Date Palm Biotechnology
Protocols Volume II

Germplasm Conservation and Molecular Breeding

Edited by

Jameel M. Al-Khayri
Department of Agricultural Biotechnology
King Faisal University
Al-Hassa, SA

S. Mohan Jain
Agricultural Sciences
University of Helsinki
Helsinki, FL

Dennis V. Johnson
Cincinnati, OH, USA

Humana Press
Biotechnology is playing an essential role in sustaining sufficient food supplies challenged by the burgeoning human population and current global environmental changes. The development of plant biotechnology has reached advanced stages of applications in several important food crops. Palm fruit crops, particularly those grown predominantly in developing countries, are attracting increasing research attention in response to greater funding allocated for plant biotechnology research in these countries. Cultivation of date palm (*Phoenix dactylifera* L.), an economically important tree species grown in the arid and semiarid regions of the Middle East and North Africa, has expanded to Australia, Southern Africa, South America, Mexico, and the southwestern USA. In addition to its high nutritive value and versatile tree by-products, dates have diverse medicinal properties due to a high content of bioactive compounds including carotenoids, polyphenols, tannins, and sterols. These compounds are known for antioxidant activity, cholesterol-lowering properties, and chemoprevention which may inhibit cancer, diabetes, and cardiovascular diseases.

Our earlier book entitled *Date Palm Biotechnology* provided a comprehensive coverage on various biotechnological aspects in relation to date palm. This book, *Date Palm Biotechnology Protocols*, is intended to supplement the previous volume and to provide precise stepwise protocols in the field of date palm biotechnology. Materials, equipment, methods, and analysis are detailed for easy adoption by novices and substantiated with relevant references for specialists desiring to indulge in new biotechnological applications.

The book consists of two volumes—Vol 1: Tissue Culture Applications and Vol 2: Germplasm Conservation and Molecular Breeding. This second volume comprises 28 chapters grouped in six parts: Part I, Germplasm In Vitro Conservation; Part II, Molecular Analysis of In Vitro Cultures; Part III, Genetic Diversity and Cultivar Identity; Part IV, Gender Identification; Part V, Genomics; and Part VI, Proteomics. Each protocol chapter starts with a brief introduction relevant to the topic methodology and then lists the necessary materials, including chemical and equipment, as well as reagent preparation, followed by detailed reproducible procedural steps supported with observational notes and illustrative photographs. The chapters in this book are authored and reviewed by prominent specialists demonstrating distinct research contributions to date palm biotechnology, invited from industry, universities, and research institutes.

This two-volume book is a valuable resource to students, researchers, scientists, commercial producers, consultants, and policymakers interested in agriculture or plant sciences particularly in date palm biotechnology. This protocol manual is highly recommended for teaching advanced level undergraduate as well as graduate courses in date palm biotechnology or other relevant courses.

We would like to express our sincere gratitude for the generosity and excellent cooperation of the chapter authors of these two volumes. In total, 125 scientists, representing 21 countries, have provided their detailed knowledge of the biotechnological protocols being

mohan.jain@helsinki.fi
employed to improve the date palm and enhance its contribution to the world’s food needs. These two scientific books represent the epitome of international cooperation, transcending the various differences, which exist in the world, and emphasizing the common solutions to feeding the burgeoning human population of the planet we all share.

Al-Hassa, SA
Helsinki, FL
Cincinnati, OH, USA

Jameel M. Al-Khayri
S. Mohan Jain
Dennis V. Johnson
## Contents

Preface ................................................................. v
Contributors .............................................................. xi

### PART I  GERMLASM IN VITRO CONSERVATION

1  Storage and Viability Assessment of Date Palm Pollen .......................... 3
   Maryam, Muhammad J. Jaskani, and Summar A. Naqvi

2  In Vitro Conservation of Date Palm Tissue Cultures ........................... 15
   Shawky A. Bekheet

3  Cryopreservation of Date Palm Pro-Embryonic Masses
   Using the D Cryo-plate Technique ............................................ 25
   Mohammad Salma and Florent Engelmann

4  In Vitro Cryopreservation of Date Palm Caulogenic Meristems .............. 39
   Lotfi Fki, Olfa Chkir, Walid Kriaa, Ameni Nasri, Emna Baklouti,
   Raja B. Masmoudi, Alain Rival, Noureddine Drira, and Bart Panis

5  In Vitro Conservation of Date Palm Shoot-Tip Explants
   and Callus Cultures Under Minimal Growth Conditions .................... 49
   Maiada M. El-Dawayati

6  In Vitro Conservation of Date Palm Somatic Embryos
   Using Growth-Retardant Conditions .......................................... 61
   Mona M. Hassan

7  Encapsulation of Date Palm Somatic Embryos: Synthetic Seeds .......... 71
   Shawky A. Bekheet

### PART II  MOLECULAR ANALYSIS OF IN VITRO CULTURES

8  Evaluation of Clonal Fidelity of Micropropagated Date Palm
   by Random Amplified Polymorphic DNA (RAPD) ............................ 81
   Arpan Modi, Bhavesh Gajera, Naraynan Subhash,
   and Nitish Kumar

9  Molecular Identification of Fungal Contamination
   in Date Palm Tissue Cultures .................................................. 91
   Mohammed H. Abass

### PART III  GENETIC DIVERSITY AND CULTIVAR IDENTITY

10 Genetic Diversity Analysis of Date Palm Using Random
    Amplified Polymorphic DNA (RAPD) and Inter-Simple
    Sequence Repeat (ISSR) ....................................................... 105
    Mahesh K. Mahatma, Vishal S. Srivashtav, and Sanjay Jha

mohan.jain@helsinki.fi
11 Date Palm Genetic Diversity Analysis Using Microsatellite Polymorphism
Hussam S.M. Khierallah, Saleh M. Bader, Alladin Hamwieh, and Michael Baum

12 Assessing Date Palm Genetic Diversity Using Different Molecular Markers
Mohamed A.M. Atia, Mahmoud M. Sakr, and Sami S. Adawy

13 Molecular Analysis of Date Palm Genetic Diversity Using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSRs)
Sherif F. El Sharabasy and Khaled A. Soliman

14 Determining Phylogenetic Relationships Among Date Palm Cultivars Using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) Markers
Nadia Haider

15 Genotyping and Molecular Identification of Date Palm Cultivars Using Inter-Simple Sequence Repeat (ISSR) Markers
Basim M. Ayesh

16 Molecular Identification of Date Palm Cultivars Using Random Amplified Polymorphic DNA (RAPD) Markers
Nasser S. Al-Khalifah and A.E. Shanavaskhan

PART IV GENDER IDENTIFICATION

17 Early Sex Identification in Date Palm by Male-Specific Sequence-Characterized Amplified Region (SCAR) Markers
Pushpa Kharb and Charu Mitra

18 Gender Identification in Date Palm Using Molecular Markers
Faisal Saeed Awan, Maryam, Muhammad J. Jaskani, and Bushra Sadia

19 Development of Sex-Specific PCR-Based Markers in Date Palm
Mohamed A.M. Atia, Mahmoud M. Sakr, Morad M. Mokhtar, and Sami S. Adawy

20 Date Palm Sex Differentiation Based on Fluorescence In Situ Hybridization (FISH)
Mohamed A.M. Atia, Sami S. Adawy, and Hanaiya A. El-Itriby

PART V GENOMICS

21 Characterization and Amplification of Gene-Based Simple Sequence Repeat (SSR) Markers in Date Palm
Tongli Zhao, Manjunath Keremane, Channapatna S. Prakash, and Guohao He

22 Mitochondrial Molecular Markers for Resistance to Bayoud Disease in Date Palm
Angad A. Saleh, Anwar H. Sharafaddin, Mahmoud H. El-Komy, Tasser E. Ibrahim, Younis K. Hamad, and Younis Y. Molan
23 Analysis of Expressed Sequence Tags (EST) in Date Palm ........................ 283
Sulieman A. Al-Faifi, Hussein M. Migdadi, Salem S. Algamdi,
Mohammad Altaf Khan, Rashid S. Al-Obeed, Megahed H. Ammar,
and Jerenj Jakse

24 Development of Genomic Simple Sequence Repeats (SSR)
by Enrichment Libraries in Date Palm ..................................... 315
Sulieman A. Al-Faifi, Hussein M. Migdadi, Salem S. Algamdi,
Mohammad Altaf Khan, Rashid S. Al-Obeed, Megahed H. Ammar,
and Jerenj Jakse

25 MicroRNA Expression in Multistage Date Fruit Development ............. 339
Wanfei Liu, Chengqi Xin, Jun Yu, and Hasan Awad Aljohi

PART VI PROTEOMICS

26 Proteome of Abiotic Stress Tolerance in Date Palm .......................... 355
Haddad A. El Rabey

27 Electrophoresis-Based Proteomics to Study Development
and Germination of Date Palm Zygotic Embryos .......................... 365
Besma Sghaier-Hammami, Noureddine Drira, Mouna Bahloul,
and Jesús V. Jorrín-Novo

28 Date Fruit Proteomics During Development and Ripening Stages .......... 381
Claudius Marondedze

Index ................................................................. 399
Contributors

MOHAMMED H. ABASS • Date Palm Research Centre, Basra University, Basra, Iraq
SAMI S. ADawy • Molecular Genetics and Genome Mapping Laboratory (MGGM), Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt
SULEIMAN A. AL-FAIFI • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia
SALEM S. ALGAMDI • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia
HASAN AWAD ALJOHI • Joint Center of Excellence for Genomics, King Abdulaziz City for Science and Technology and Chinese Academy of Sciences, Riyadh, Saudi Arabia; National Centre for Genome, Life Science & Environmental Research Institute, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia
NASSER S. AL-KHALIFAH • National Center for Agricultural Technology, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia
RASHID S. AL-OBEED • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia
MEGAHED H. AMMAR • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia
MOHAMED A.M. ATIA • Molecular Genetics and Genome Mapping Laboratory (MGGM), Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt
FAISAL SAEED AWAN • Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
BASIM M. AYESH • Department of Laboratory Medical Sciences, Alaqsa University, Gaza, Palestine
SALEH M. BADER • State Board of Agricultural Research, Ministry of Agriculture, Baghdad, Iraq
MOUNA BAILLUL • Laboratoire des Biotechnologies Végétales Appliquées à l’Amélioration des Cultures, Faculté des Sciences de Sfax, Sfax, Tunisia
EMMA BAKLOUTI • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia
MICHAEL BAUM • International Center for Agricultural Research in the Dry Areas (ICARDA), Cairo, Egypt
SHAWKY A. BEKEHT • Department of Plant Biotechnology, National Research Center, Giza, Egypt
OLFIA CHKIR • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia
NOUREDDINE DRIRA • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia
HADDAD A. EL RABEY • Bioinformatics Department, Genetic Engineering and Biotechnology Institute, Sadat City University, Sadat City, Egypt
SHERIF F. EL SHARABASY • Central Laboratory for Date Palm Research and Development, Agriculture Research Center, Giza, Egypt
MAIADA M. EL-DAWAYATI • Central Laboratory of Date Palm Research and Development, Agriculture Research Center, Giza, Egypt
Contributors

HANAIYA A. EL-ITRIBY • National Gene Bank, Agriculture Research Center, Giza, Egypt
MAHMoud H. EL-KOMY • Plant Pathology Institute, Agriculture Research Center, Giza, Egypt
FLORENT ENGELMANN • IRD, UMR DIADE, Montpellier Cedex 5, France
LOTFI FKI • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia
BHAVEsh GAJERA • Centre for Advanced Research in Plant Tissue Culture, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat, India
NADIA HAIDER • Department of Molecular Biology and Biotechnology, AECS, Damascus, Syria
YOUNIS K. HAMAD • Faculty of Agriculture, Plant Pathology Department, Alexandria University, Alexandria, Egypt
ALLADIN HAMWIEH • International Center for Agricultural Research in the Dry Areas (ICARDA), Cairo, Egypt
MONA M. HASSAN • The Central Laboratory for Date Palm Research and Development, Agricultural Research Center, Giza, Egypt
GUOHAO HE • Department of Agricultural and Environmental Science, College of Agriculture, Environment, and Nutrition Sciences, Tuskegee University, Tuskegee, AL, USA
YASSER E. IBRAHIM • Plant Pathology Institute, Agriculture Research Center, Giza, Egypt
JERENJ JAKSE • Biotechnical Faculty, Agronomy Department, University of Ljubljana, Ljubljana, Slovenia
MUHAMMAD J. JASKANI • Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
SANJAY JHA • ASPPE Shakilam Agricultural Biotech Institute, Navsari Agricultural University, Surat, Gujarat, India
JESU´S V. JORRI´N-NOVO • Agroforestry and Plant Biochemistry and Proteomics Research Group, Department of Biochemistry and Molecular Biology, University of Cordoba-CeiA3, Cordoba, Spain
MANJUNATH KEREMANE • National Clonal Germplasm Repository, USDA ARS, Riverside, CA, USA
MOHAMMAD ALTAF KHAN • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia
PUSHPA KHARB • Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University, Hisar, Haryana, India
HUSSAM S.M. KHIERALLAH • Date Palm Research Unit, College of Agriculture, University of Baghdad, Baghdad, Iraq
WALID KRIAA • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia
NITISH KUMAR • Centre of Biological Sciences (Biotechnology), School of Earth, Biological and Environmental Science, Central University of South Bihar, Patna, India
WANFEI LIU • Joint Center of Excellence for Genomics, King Abdulaziz City for Science and Technology and Chinese Academy of Sciences, Riyadh, Saudi Arabia; CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China
MAHESH K. MAHATMA • ICAR-Directorate of Groundnut Research, Junagadh, Gujarat, India

mohan.jain@helsinki.fi
Claudius Marondedze • Department of Biochemistry, Cambridge Center for Proteomics, Cambridge Systems Biology Centre, University of Cambridge, Cambridge, UK

Maryam • Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan

Raja B. Masmoudi • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia

Hussein M. Migdadi • Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia

Charu Mitra • Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University, Hisar, Haryana, India

Arpan Modi • Centre for Advanced Research in Plant Tissue Culture, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat, India

Morad M. Mokhtar • Molecular Genetics and Genome Mapping Laboratory (MGGM), Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt

Younis Y. Molan • Faculty of Agriculture, Plant Pathology Department, Alexandria University, Alexandria, Egypt

Summar A. Naqvi • Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan

Ameni Nasri • Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax, University of Sfax, Sfax, Tunisia

Bart Panis • Laboratory of Tropical Crop Improvement, Katholiekke Universiteit Leuven (K.U. Leuven), Leuven, Belgium

Channapatna S. Prakash • College of Art and Science, Tuskegee University, Tuskegee, AL, USA

Alain Rival • UMR DIADE, CiradBioS, IRD, Montpellier Cedex 5, France

Bushra Sadia • U.S.-Pakistan Center for Advanced Studies in Agriculture and Food Security (USPCAS-AFS), University of Agriculture, Faisalabad, Pakistan

Mahmoud M. Sakr • Plant Biotechnology Department, National Research Center, Dokki, Giza, Egypt

Amgad A. Saleh • Faculty of Food and Agriculture Sciences, Department of Plant Protection, King Saud University, Riyadh, Saudi Arabia

Mohammad Salma • IRD, UMR DIADE, Montpellier Cedex 5, France

Besma Sghaier-Hammami • Biotechnology Centre of Borj Cedria, Laboratory of Extremophile Plants, Hammam-Lif, Tunisia; Laboratoire des Biotechnologies Végétales Appliquées à l'Amélioration des Cultures, Faculté des Sciences de Sfax, Sfax, Tunisia; Agroforestry and Plant Biochemistry and Proteomics Research Group, Department of Biochemistry and Molecular Biology, University of Cordoba-CeiA3, Cordoba, Spain

A.E. Shanavaskhan • National Center for Agricultural Technology, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia

Anwar H. Sharafaddin • Faculty of Food and Agriculture Sciences, Department of Plant Protection, King Saud University, Riyadh, Saudi Arabia

Khaled A. Soliman • Department of Genetics, Ain Shams University, Cairo, Egypt

Vishal S. Srivastav • Department of Plant Molecular Biology and Biotechnology, Navsari Agricultural University, Navsari, Gujarat, India

Naraynan Subhash • Centre for Advanced Research in Plant Tissue Culture, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat, India

Chengqi Xin • Joint Center of Excellence for Genomics, King Abdulaziz City for Science and Technology and Chinese Academy of Sciences, Riyadh, Saudi Arabia
JUN YU • Joint Center of Excellence for Genomics, King Abdulaziz City for Science and Technology and Chinese Academy of Sciences, Riyadh, Saudi Arabia; CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China

YONGLI ZHAO • Department of Agricultural and Environmental Science, College of Agriculture, Environment, and Nutrition Sciences, Tuskegee University, Tuskegee, AL, USA
Part I

Germplasm In Vitro Conservation
Chapter 1

Storage and Viability Assessment of Date Palm Pollen

Maryam, Muhammad J. Jaskani, and Summar A. Naqvi

Abstract

Pollen storage and viability are very important for pollination, breeding, biodiversity, biotechnology, conservation, and other biological and non-biological studies of the date palm. Optimizing procedures and duration of storage are important for effective and long-term date palm pollen storage and viability. Here we describe pollen storage methods, such as room temperature (25–30 °C), refrigeration (4 °C), storage at 4 °C in desiccators, deep freezer (−20 °C), and cryopreservation (−196 °C). Based on pollen viability by staining and in vitro germination methods, cryopreservation is the best method for long-term storage without any significant effect on pollen viability (75–84%); however, the percentage of pollen viability depends on the storage period.

Key words  Acetocarmine, In vitro germination, Pollen storage, Pollen viability

1 Introduction

Pollen viability affects pollen germination and delivers the nuclei to the embryo sac for compatible fertilization [1]. Date palm is a dioecious plant, and cross-pollination is the general rule. Hence, date palm pollen storage is an expedient practice; however, storage methods and pollen viability are subjects worthy of research. Pollen can retain its viability much longer under dry conditions. Pollen is collected at anthesis and stored in stoppered bottles [2], refrigerated or at room temperature for 4 weeks without losing viability and performance [3]. Various pollen storage methods indicate that short-term storage (230 days) is the best in desiccators, whereas freeze-drying is effective for long-term storage [4]. Previously, viability of pollen grains stored at various conditions has also been examined at room temperature (25–30 °C), in a refrigerator (3–4 °C) [5], at 4 °C in desiccators (anhydrous calcium chloride) [4], deep freezer (−20 °C) [6], and cryopreservation at −196 °C [7].

Furthermore, pollen viability can be determined with Alexander’s stain, fluorescein diacetate (FDA), acetocarmine or in vitro...
pollen germination [8]. Acetocarmine is widely used to test the germination rate of stored pollen grains on germination [9]; however, viability of fresh and stored pollens (1–12 months) can also be evaluated by applying different storage methods and germination tests, as different storage conditions had no apparent effect on pollen viability as tested with acetocarmine. Different media are used to compare stored and fresh pollen germination rates and tube elongation indicating significant decrease in pollen tube length in stored pollen grains [5]. The pollen germination rate was poor (8.5%) when stored at 0 °C for 6 weeks; however, the best temperature for pollen tube elongation was 30 °C [10]. Phoenix pollen has been stored cryogenically by immersing pollen, wrapped in aluminum foil, in liquid nitrogen (−196 °C) for long-term storage [7, 11]. Pollen viability gradually decreases although its shelf life is enhanced through low-temperature storage.

Germination testing is highly reliable to determine pollen viability [12, 13]. On the other hand, pollen capacity to fertilize the ovule and set fruit is considered a measure of date fruit production [14]. In this chapter, we describe pollen storage and viability testing procedures, which are important for date palm pollination and production, due to asynchronized inflorescence emergence and anthesis between male and female date palms.

2 Materials

2.1 Plant Material
Pollen grains collected from mature spathes of date palm Halawy cv. (see Note 1).

2.2 Reagents and Supplies
1. Pollen waxing: 200 g paraffin wax, paraffin film (see Note 2).
2. Pollen germination medium: 0.42 g/L Ca(NO₃)₂·2H₂O (see Note 3), 0.2 g/L H₃BO₃, 0.1 g/L KNO₃, 0.22 g/L MgSO₄·7H₂O, 200 g/L sucrose, and 10 g/L agar in distilled water.
3. Acetocarmine staining solution (1%): 100 mL of 45% glacial acetic acid, 1 g carmine powder.

2.3 Equipment
1. Instruments: Hot air oven, magnetic stirrer, weighing balance, pH meter, autoclave, refrigerator, deep freezer, microscope, desiccator, and thermometer.
2. Glassware: Cryotubes (1 mL), glass test tubes (10 mL), beakers (50, 500 and 1000 mL), Petri plates, and measuring cylinders (50, 100, 500 and 1000 mL).
3. Supplies: Parafilm, spatula, pharmaceutical capsules (1 mL), and wax.
3 Methods

3.1 Pollen Collection

1. Select mature spathes of male date palm, which are either ready to open or already opened, and cut at the base (see Note 4).

2. Place cut spathes for 2–3 days in the dark at 25–30 °C and 30–40% relative humidity for anther dehiscence.

3. Separate pollens by shaking the inflorescence strands on a paper sheet (Fig. 1).

4. Dry pollen at room temperature and sieve (40 mesh =0.42 mm pore size) to separate petals and other inert material.

3.2 Pollen Storage

1. Encapsulate pollen for long-term storage in pharmaceutical capsules as follows:

   (a) Take 200 g paraffin wax in 250-mL beaker and heat in oven at 45 °C for 2 min.

   (b) Dip pollen-filled capsules in melted wax (35–40 °C).

   (c) After 8–10 min, transfer each waxed capsule in a Petri plate, and wrap in paraffin film.

Fig. 1 Collecting (a) and drying (b) of date palm pollen grains after dehiscence
2. Place capsules in labeled zipped polythene bags. Store bags under following storage conditions for up to 12 months (Fig. 2):
(a) Room temperature (25–30 °C).
(b) Refrigerator (4 °C).
(c) Storage at 4 °C in the desiccators (see Notes 5 and 6).
(d) Deep freezer (−20 °C).
(e) Cryopreservation (−196 °C) (see Note 7).

3. For cryopreservation, transfer the dehydrated pollen into cryotubes (0.56 g/mL), close the lid, and immerse in liquid nitrogen for 15 min.

4. Store the cryotubes in a freezer at −196 °C.

3.3 Pollen Viability Tests
3.3.1 Pollen Germination Test

1. Soak glassware in liquid detergent for 1 h and thoroughly wash with hot water. Rinse the glassware with double-distilled water, and air-dry before use (see Note 8).

2. Prepare the germination medium as follows:
(a) Weigh the chemicals for germination medium individually (for 1 L medium), and put in a 500-mL glass beaker. Add 300 mL distilled water to the beaker, and place on magnetic stirrer for dissolution of chemicals (see Note 9).
(b) Adjust the pH of medium to 5.7 prior to the addition of agar.

Fig. 2 Storage of pollen grains at different storage temperature after waxing
(c) Weigh 10 g agar, put it in 100 mL glass beaker, add 100 mL distilled water, and boil.

(d) Pour 100 mL boiled agar and 300 mL solution of salts and sucrose into a 1000 mL conical flask. Mix it vigorously; close the flask with aluminum foil, and autoclave at 121 °C for 20 min.

(e) In a laminar hood cabinet, pour the medium in sterile 50 mm diameter Petri plates and allow to solidify.

3. Sterilize camel hair brush for spreading pollen on the germination medium.

4. Thaw the frozen and cryopreserved stored pollen grains by swirling tubes in water bath, at 45 °C, until the ice melts (Fig. 3, see Note 10).

5. After thawing, dust the stored pollens with a camel hair brush in Petri plates containing pollen germination medium under a laminar airflow cabinet to minimize the risk of contamination (Fig. 4).

6. Seal the plates with paraffin film, and incubate in a growth room at 25 ± 2 °C and 16-h photoperiod of 100 μmol/m²/s.

7. Count the number of germinated and total number of pollen grains (see Note 11) in each visible field of the microscope using 200× magnification within 1–24 h (Fig. 5, see Notes 12–14).

8. Calculate pollen germination percentage by using the following formula (see Note 15).

Fig. 3 Thawing of pollen grains after storage at −20 °C
Fig. 4 Culture of pollen grains on the germination medium

Fig. 5 Viability of 12-month stored pollen grains tested by acetocarmine staining (magnification 200 ×) [NV Non-viable, V Viable]
Germination (%) = \( \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains}} \times 100 \)

9. Measure pollen tube length (see Note 16) at successive intervals under a microscope equipped with an eyepiece and stage micrometer using 200× magnification (see Note 17).

### 3.3.2 Pollen Staining Test

1. Prepare the acetocarmine solution for pollen staining as follows:
   (a) Boil 100 mL 45% glacial acetic acid in a glass beaker.
   (b) Weigh 1 g carmine powder, transfer to boiling glacial acetic acid, and boil gently for 5 min in a reflux condenser. Shake well and filter when cool.
   (c) Store the solution in a tightly closed screw-capped dark glass bottle under cool (4°C) and dry conditions (see Note 18).

2. Place one drop of 1% acetocarmine solution on a glass slide.

3. Place a drop of pollen suspension on glass slide, put a cover slip on top of it, and gently press the cover slip to remove extra stain with tissue paper/filter paper (see Note 19).

4. Examine stained and unstained pollens under the microscope at 200× magnification (Fig. 6, see Note 20).

![Fig. 6 in vitro pollen germination after 12-month storage (−20 °C) incubated at 28 °C (magnification 200×)](image)
5. Calculate stained pollen percentage by using the following formula.

\[ \text{Staining} \% = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains}} \times 100 \]

4. Notes

1. The age of the date palms in this study was about 30 years.
2. Wax the capsules, and wrap with paraffin film as a precaution to avoid possible capsule cracking that may occur due to dryness.
3. Calcium nitrate is also important for date palm pollen in vitro germination. Application of certain concentrations of calcium nitrate in culture media increased the pollen germination rates, but its higher concentration could decrease it. Pollen germination and pollen tube growth are regulated by transferring inorganic \( \text{Ca}^{2+} \) and \( \text{K}^{+} \) ions across the plasma membrane. Calcium also plays a role in determining the direction of pollen tube growth. For in vitro pollen germination and pollen tube growth, boron ion (\( \text{B}^{+} \)) is one of the essential factors.
4. Select and cut a male spathe, either already split open or about to split open, and has brown color and soft texture. To prevent wind or bees from causing loss of pollen, it is recommended that the freshly opened spathe be cut early in the morning.
5. Before placing pollen in desiccators, fill it with new silica gel for effective moisture control.
6. Desiccators are used to store dried samples in dry atmosphere by using desiccants like silica gel, whereas pollen storage in a refrigerator at low temperature slows down metabolic processes and does not lead to pollen drying.
7. Ultra-low temperature (\(-196^\circ\text{C}\)) is the best for long-term storage of date pollen without any deteriorating effect on viability.
8. Prepare all solutions using distilled water. Chemicals and reagents should be of analytical grade.
9. Gradually increase the speed of stirrer for uniform movement of magnet, otherwise it will move abruptly and the beaker may break. If chemicals do not dissolve, increase the temperature up to 40 \( ^\circ\text{C} \).
10. Do not expose the frozen pollen capsules directly to hot water, but thaw them using glass test tubes in a beaker filled with hot water. Swirl these tubes in hot water at 65 \( ^\circ\text{C} \) for 10 min. Then place the capsules on Petri plates, remove the paraffin and
wax using tissue paper, and collect the pollen on labeled Petri plates.

11. Pollen germination percentage during incubation period at 3 and 24 h ranges from 15 to 85%, respectively.

12. Pollen incubation on culture media up to 24 h ensures maximum germination.

13. A pollen grain is considered germinated when the pollen tube length is equal to or greater than the diameter of the grain.

14. Pollen culture medium can become contaminated if incubated for duration longer than 24 h. Although sterilized culture media and sterile Petri plates are used, the culture media can become contaminated when Petri plates are opened during microscopic studies.

15. Count germinated and total pollen in at least five microscopic fields, add accordingly, and then calculate the pollen germination percentage according to the given formula. For example, in the visible field of microscope, the total number of pollen are 90, out of which 75 pollen germinated, then the percent pollen germination is 83%.

16. Pollen tube length varies from 44 to 196 μm at 3 and 24 h, respectively.

17. Place the ocular micrometer inside the body tube of the microscope by unscrewing the eyepiece of the microscope. Then place the stage micrometer on the stage of the microscope, and focus the scale for calibration. The stage and ocular micrometer scales must be in the same direction. Compare and count the number of divisions of the point where both scales coincide with each other under the microscope with 40× object and 10× eyepiece magnification. Each of the 100 parts of the stage micrometer scale represents 0.01 mm, i.e., 10 μm (Fig. 7). For example, if 50 ocular micrometer scales are equal to 65 scales of stage micrometer, then

\[
\frac{50 \text{ scales of ocular micrometer}}{1 \text{ scale of ocular micrometer}} = \frac{0.65}{50} = 0.013 \text{ mm} = 13 \mu m
\]

After calibration, place the sample slide on the stage under the microscopic object for pollen tube length measurement. It is already determined that each scale of ocular micrometer is equal to 13 μm at 40× object and 10× eyepiece magnification of the microscope. The length of the pollen tube will be the number of scales multiplied by 13 μm.

18. Since the solution contains 45% glacial acetic acid, it is recommended to store the solution in a tightly closed dark screw cap glass bottle, in cool, dry, well-ventilated place away from inflammable material.
19. Place cover slip gently and press it slightly with care to avoid air bubbles.

20. The pollen grains appearing normal and stained red are considered viable, whereas weakly stained or colorless are recorded as non-viable. The viability of stained pollen varies from 65 to 87% at storage periods of 1 and 12 months, respectively.

Acknowledgment

This work was supported by the Higher Education Commission, Pakistan and Plant Tissue Culture Cell, University of Agriculture, Faisalabad 38040, Pakistan.

References


Fig. 7 Illustration of ocular and stage micrometer used for pollen tube measurement

mohan.jain@helsinki.fi
Chapter 2

In Vitro Conservation of Date Palm Tissue Cultures

Shawky A. Bekheet

Abstract

In vitro technology offers a potential solution for the conservation of date palm germplasm. Slow growth induced by low temperature allows storage from several months up to few years. Otherwise, cryopreservation is suitable for long-term in vitro conservation, at between −79 and −196 °C. This chapter describes a protocol for cold storage at 5 °C and cryopreservation of date palm tissue cultures. For cold storage, 70% of shoot buds remain healthy after storing for 12 months at 5 °C, and callus cultures remain fully viable after 12 months of storage. For cryopreservation of embryogenic cultures using dehydration by air, apparently, 20 min air drying is the best for cryopreservation. Among different types of sugars used as osmotic agents in pre-culture medium, 1 M sucrose is the best for the survival of cryopreserved cultures. However, exposure of embryogenic cultures to vitrification solution for 60 min at 0 °C gives the highest percentage of survival and conversion to plantlets.

Key words Slow growth, Low temperature, Cryopreservation, Dehydration, Vitrification

1 Introduction

In vitro conservation has been used as an alternative strategy to conserve plant genetic resources and facilitates safe international exchange of plant material. This technique requires minimal space for storing large number of germplasm collections and facilitates supply of valuable material for wild population recovery and molecular investigations and ecological studies [1]. Conservation of plant genetic resources by in vitro technology has been done by slow growth procedures and cryopreservation. In vitro slow growth is generally achieved by reducing growth rate at low temperature [2–4] and addition of osmotic agents such as sucrose, sorbitol, and mannitol to the culture medium [3, 5, 6]. The slow growth methods may be used either singly or in combination. For applications, however, in vitro storage under slow growth conditions delays the necessity for subculturing and consequently efficient utilization of labor year around [7].

Cryopreservation is the only technique that ensures the long-term conservation of plants, including nonorthodox seed species,
vegetatively propagated plants, rare and endangered species, and biotechnology products [8]. Cryopreservation is carried out in liquid nitrogen (LN) at −79 and −196 °C, by preventing ice crystal formation. The major advantage of plant material at such temperature is that both metabolic process and biological deterioration are considerably slowed or even halted. In addition, cryopreserved material remains genetically stable, thus affording an advantage over conventional conservation methods. In this context, many cryopreservation protocols use chemical solutes like dimethyl sulfoxide (DMSO), sucrose, and plant vitrification solutions (PVS) [9–11] to decrease the free water content in cells. Vitrification-based procedures are more appropriate for multicellular organs (shoot tips, embryos) which contain a variety of cell types, each with unique requirements under freeze-induced dehydration. However, classical cryopreservation techniques have been applied to undifferentiated culture systems of different plant species, such as cell suspension cultures and calli. The cryopreservation procedure has a number of steps including pre-culture in media with osmotically active compounds, treatment with cryoprotectants, cooling and storage in liquid nitrogen, thawing, post-thaw treatment, and recovery of growth. The cooling rate of explants is critical during cryopreservation as it determines the survival after exposure to LN [12].

Due the dioecious and heterozygous nature, date palm is conventionally propagated vegetatively by offshoots from mother plants. Thus, its germplasm cannot be effectively stored or handled by conventional means. Therefore, different techniques have been tried to preserve date palm cultures in vitro using slow growth [13–15]. Otherwise, cryopreservation methods have been developed for in vitro preservation [16] and improvement of biotic stress tolerance [17] in date palm. The present chapter describes a protocol for in vitro conservation of date palm tissues using cold storage and two cryopreservation techniques: dehydration and vitrification.

2 Materials

2.1 Plant Materials and Sterilization Solution

1. Explant source: Offshoots of date palm cv. Zagloul, 20–25 kg (see Fig. 1a).

2. Antioxidant solution: 100 mg/L ascorbic acid and 150 mg/L citric acid.

3. Sterilization solutions: 70% ethanol solution and 1.6% w/v sodium hypochlorite solution (30% v/v Clorox, commercial bleach).
2.2 Culture Medium

1. Basal culture medium: Stock solutions of Murashige and Skoog (MS) medium [18] (see Table 1).
2. Hormone stock solutions: 3-methyl-2-butenylaminopurine (2iP, 1 mg/mL), 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg/mL), and 1-naphthaleneacetic acid (NAA, 1 mg/mL).
3. Medium additives for each culture stage: Initiation medium (I), proliferation medium (II), callus induction medium (III), recovery and rooting (V) medium consist of MS basal medium, hormones and activated charcoal (Table 2).
4. pH adjustment solutions: 1 M KOH and HCL solutions.

2.3 Equipment

1. Microwave oven.
3. pH meter.
4. Laminar flow bench.
5. Bacti-cinerator sterilizer.
6. Incubation chamber.
7. Liquid nitrogen container.
Table 1  
Composition of Murashige and Skoog (MS) medium [18]

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration of stock solution (mg/L)</th>
<th>Final concentration in culture medium (mg/L)</th>
</tr>
</thead>
</table>
| **Major salts (stock I)**  
(20× stock) use 50 mL to prepare 1 L of medium | | |
| NH₄NO₃       | 33,000                                 | 1650                                        |
| KNO₃         | 38,000                                 | 1900                                        |
| CaCl₂·2H₂O   | 8800                                   | 440                                         |
| MgSO₄·2H₂O   | 7400                                   | 370                                         |
| KH₂PO₄       | 3400                                   | 170                                         |
| **Minor salts (stock II)**  
(200× stock) use 5 mL to prepare 1 L of medium | | |
| KI            | 166                                    | 0.83                                        |
| H₃BO₃        | 1240                                   | 6.20                                        |
| MnSO₄·2H₂O   | 4460                                   | 22.3                                        |
| ZnSO₄·7H₂O   | 1720                                   | 8.60                                        |
| Na₂MoO₄·2H₂O | 50                                     | 0.25                                        |
| CuSO₄·5H₂O   | 5                                      | 0.025                                       |
| CoCl₂·6H₂O   | 5                                      | 0.025                                       |
| **Iron stock (stock III)**  
(200× stock) use 5 mL to prepare 1 L of medium | | |
| FeSO₄·7H₂O   | 5560                                   | 27.80                                       |
| Na₂EDTA·2H₂O | 7460                                   | 37.30                                       |
| **Vitamins (stock IV)**  
(200× stock) use 5 mL to prepare 1 L of medium | | |
| Myoinositol  | 1000                                   | 20,000                                      |
| Nicotinic acid | 200                                    | 1                                           |
| Pyridoxine-HCl | 200                                    | 1                                           |
| Thiamine-HCl  | 2000                                   | 10                                          |
| Glycine       | 400                                    | 2                                           |
| Biotin        | 200                                    | 1                                           |
| **Carbon source** | | |
| Sucrose       | –                                      | 30 g/L                                      |
| **Gelling agent** | | |
| Agar          | –                                      | 7 g/L                                       |
Table 2
Growth regulators and activated charcoal additives supplemented to the MS medium

<table>
<thead>
<tr>
<th>Culture stage</th>
<th>2,4-D (mg/L)</th>
<th>2iP (mg/L)</th>
<th>NAA (mg/L)</th>
<th>AC (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation medium (I)</td>
<td>–</td>
<td>2</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Proliferation medium (II)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Callus induction medium (III)</td>
<td>10</td>
<td>3</td>
<td>–</td>
<td>1.5</td>
</tr>
<tr>
<td>Recovery medium (IV)</td>
<td>3</td>
<td>5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rooting medium (V)</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

1. Prepare antioxidant solution by mixing 100 mg ascorbic acid and 150 mg citric acid and dissolving them in suitable volume of distilled water using magnetic stirring plate and then raise the volume to 1 L.

2. Prepare stock solutions of 2,4-D (1 mg/mL) by dissolving 100 mg in 2 mL 0.5 M NaOH and NAA by dissolving 100 mg in 2 mL 0.5 M NaOH and 100 mg 2iP in 2 mL 0.1 N HCl, and raise each volume to 100 mL with distilled water. Store at 4°C.

3. Prepare initiation medium (I): Mix MS medium stock solutions and additives (Table 1) including 2 mg/L 2iP and 1 mg/L NAA (Table 2).

4. Prepare proliferation medium (II): Mix MS medium stock solutions and additives (Table 1) devoid of plant growth hormones (Table 2).

5. Prepare callus induction medium (III): Mix MS medium stock solutions and additives (Table 1) including 10 mg/L 2,4-D and 3 mg/L 2iP (Table 2).

6. Prepare recovery medium (IV): MS medium (Table 1) containing 5 mg/L 2,4-D and 3 mg/L 2iP (Table 2).

7. Prepare rooting medium (V): MS medium (Table 1) containing 1 mg/L NAA and 2 g/L charcoal (Table 2).

8. Adjust the pH of the media to 5.8 with 1 N HCl or KOH.
Table 3
Components of verification solution

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>22</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>15</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>15</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>7</td>
</tr>
</tbody>
</table>

9. Add 0.7% (w/v) agar and dissolve in a microwave oven for 10 min; dispense into the glass jars and close them with plastic caps.

10. Sterilize the media by autoclaving at 121 °C and 1.2 kg/cm² for 20 min.

11. Store the autoclaved media at room temperature in the dark for up to 4 weeks.

12. Prepare vitrification solution (Table 3) (see Notes 1 and 2).

13. Prepare 1 M sucrose and 0.5 M sorbitol solutions by dissolving 85.5 g sucrose in 250 mL distilled water and 22.7 g sorbitol in 250 mL distilled water.

3.2 Preparation and Sterilization of Plant Material

1. Detach offshoots from adult female plant.

2. Remove leaves from offshoots until reaching tender portion using saw and knives (see Note 3).

3. Excise shoot tips when they are approximately 3–5 cm in length (Fig. 1b).

4. Keep the shoot tips in the antioxidant solution (Fig. 1c).

5. Disinfect the shoot tips with ethanol solution for 30 s and then by sodium hypochlorite solution for 20 min (see Note 4).

6. Wash the explant three times with sterile distilled water.

7. Remove external leaves until shoot tip reaches 1.5 cm.

3.3 Culturing and Incubation of Explants

1. Excise shoot tips with part of sub-meristematic tissue.

2. Place the explant on initiation medium (I) (Table 2).

3. Incubate the cultures in darkness at 24 ± 2 °C for 6 weeks.

4. Transfer the explants onto a fresh initiation medium three times at 5-week intervals to obtain embryonic cultures.

5. Use the embryonic cultures for in vitro cryopreservation.

6. For shoot bud differentiation, transfer the cultures into proliferation medium (II) (Fig. 1d).
7. For callus formation, transfer the cultures into callus induction medium (III) (Fig. 1e).
8. Incubate the cultures (shoot buds and callus) at 25 ± 2 °C under 16 h photoperiod of 45 μmol/m²/s for 6 weeks.

3.4 Pretreatment and Storage in Liquid Nitrogen (LN)

3.4.1 Dehydration by Air Drying

1. Place small pieces (1 cm³ size) of embryonic cultures on dry sterile filter paper in the laminar airflow cabinet for 20 min.
2. Transfer the cultures to sterile 2-ml cryovials for the storage in LN.

3.4.2 Osmotic Dehydration

1. Transfer embryonic culture into MS medium (Table 1) containing 1 M sucrose for 1 week.
2. Transfer cultures to sterile cryovials without nutrient medium for storage in LN.

3.4.3 Dehydration by Vitrification

1. Transfer embryonic culture into MS liquid medium (Table 1) containing 0.5 M sorbitol for 48 h.
2. Keep the cultures into the vitrification solution (Table 3) for 1 h at 0 °C in darkness (see Notes 5 and 6).
3. Transfer the cultures into sterile cryovials for the storage in LN.
4. Keep the cryovials containing all pretreated date palm cultures at 0 °C for 2 h, and then plunge them directly into LN container (Fig. 2a) for 48 h (see Note 7).

3.5 Rewarming and Recovery

1. Gently, remove the cryovials from the LN (Fig. 2b) and then put them in water bath at 37 °C (see Note 8).
2. Transfer the cultures on the recovery medium (IV) and inoculate at 25 ± 2 °C under 16-h photoperiod of 45 μmol/m²/s.
3. Monitor regeneration and shoot formation for 4–6 weeks after inoculation.

3.6 In Vitro Rooting and Acclimatization

1. Transfer the regenerated plantlets (5 cm length) on rooting medium (V) and inoculate at 25 ± 2 °C under light conditions of 16-h photoperiod 45 μmol/m²/s for 6 weeks.
2. Wash the rooted plantlets (Fig. 2c) with tap water and disinfect by soaking in Benlate solution (1 g/l) for 20 min.
3. Transplant into plastic pots containing peat moss and vermiculite (1:1).
4. Cover the pots with clear polyethylene bags (Fig. 2d) for 4 weeks of transplanting, and then gradually remove the covers.
3.7 Storage at Low Temperature

1. Transfer shoot bud cultures into jars containing 25 mL proliferation medium (II).

2. Transfer callus inoculum into jars containing 25 mL callus induction medium (III).

3. Incubate both types of cultures in incubator chamber at 5 °C in the dark for 12 months (Fig. 3a, b).

4. Check for contamination, necrosis, browning, and senescence, and discard as necessary (see Note 9).

5. Evaluate the survival and viability at 3-month interval by counting the number of shoots and callus vigor as indication for viability (see Note 10).

---

Fig. 2 (a) Plunging the cryovials into LN container, (b) cryovials containing embryonic cultures after exposing to LN, (c) rooted plantlets including MS medium plus 1 mg/L NAA and 2 g/L charcoal, (d) acclimatization of plantlets to free-living conditions
Notes

1. Vitrification solution should be freshly prepared before use.
2. Filter sterilize vitrification solution to avoid degradation by heating.
3. Cut and remove the white leaf layers one by one from the outside without damaging the next internal one while removing the leaves from offshoots.
4. Use only freshly made sodium hypochlorite solution for surface sterilization of explants to avoid dilution of the active agent.
5. Observe the exposure time carefully because overexposure to vitrification solution is toxic for the cultures.
6. Make sure the cultures are in contact with the vitrification solution and shake the cryovials gently if necessary.
7. Storage of date palm embryonic cultures on LN for 6 months does not show any significant decrease of survival rate as compared with after 60 min exposure which reach 70% of survival.
8. Thawing should be performed quickly at 37 °C to avoid ice formation during rewarming.

Fig. 3 (a) Shoot bud, (b) callus cultures of date palm stored for 12 months at 5 °C in the dark
9. It is recommended to evaluate genetic stability of cultures, stored for more than 12 months to avoid somaclonal variation.

10. It is recommended to modify protocol for each genotype to be used because of genotypic effect in vitro response.

References


mohan.jain@helsinki.fi
Chapter 3

Cryopreservation of Date Palm Pro-Embryonic Masses Using the D Cryo-plate Technique

Mohammad Salma and Florent Engelmann

Abstract

In this chapter, we describe a cryopreservation (liquid nitrogen, $-196 \, ^\circ C$) protocol developed for long-term storage of date palm pro-embryonic masses (PEMs), which uses the recently established D cryo-plate technique. Clumps of PEMs (3–5 mm in size) were dissected from PEM cultures and placed on pretreatment medium containing 171 g/L sucrose for 3 days. Clumps were placed in the wells of aluminum cryo-plates in which they were made to adhere using droplets of 3% calcium alginate. PEMs were treated for 20 min with a loading solution containing 184 g/L glycerol and 136.8 g/L sucrose. They were then dehydrated for 90–120 min in the air current of a laminar airflow cabinet and immersed directly in liquid nitrogen. For rewarming, the cryo-plates holding the PEMs were immersed for 15 min in an unloading solution containing 410.4 g/L sucrose. The PEMs were then detached from the cryo-plates, placed for 3 days in the dark on posttreatment medium containing 102.6 g/L sucrose, and transferred on recovery medium under light conditions. Using this protocol, 74.6 and 95.8% recovery were achieved with the PEMs of the two cultivars tested, Sukkari and Sultany.

Key words Date palm, Pro-embryonic masses, Cryopreservation, D cryo-plate

1 Introduction

Cryopreservation (liquid nitrogen [LN], $-196 \, ^\circ C$) is currently the only technique available to ensure the safe and cost-effective long-term conservation of nonorthodox seed species, of vegetatively propagated plant species, and of biotechnology products such as calli, cell suspension cultures, somatic embryos, multiple bud cultures, and shoot tips of in vitro cultured plants [1]. Cryopreservation protocols are now available for several hundred of plant species from temperate and tropical origin [1–3]. There are two main types of cryopreservation techniques: two-step controlled cooling protocols and vitrification-based protocols. In the latter category, the droplet-vitrification (DV) technique has been developed by Panis et al. in 2005 [4]. The achievement of very high cooling and warming rates is one of the keys to the high efficiency.
of DV, in which explants are in direct contact with LN during cooling and with the unloading solution during warming [3]. The DV technique has been successfully applied to a range of different plant species [5]. Very recently, a further improvement to DV has been made with the development of the cryo-plate technique [6, 7]. The main advantage of this technique lies with the facilitated manipulation of explants, which adhere to the cryo-plate.

The traditional method for propagation of date palm uses offshoots from mother-palms. However, offshoots are produced only during the juvenile phase of palm life and in very low numbers (10–30 offshoots/palm), thereby limiting multiplication potential [8, 9]. In vitro culture protocols based on organogenesis [10] and somatic embryogenesis [11] have been developed to ensure the large-scale propagation of elite varieties. All these cultures, including embryogenic calluses, somatic embryos, and multiple bud cultures, are cryostored for the long-term conservation of genetic resources and to facilitate management of large-scale production.

So far, work on date palm cryopreservation has not been done extensively. However, two-step controlled cooling protocols have been developed for callus cultures [12], somatic embryos [13], and apices [14, 15]. Vitrification-based protocols have been developed for somatic embryos [16], undifferentiated tissues [17], pro-embryonic masses (PEMs) [18], and cell suspensions [19]. In 2014 [20], we used the D cryo-plate protocol for the cryopreservation of PEMs, reaching 74.6 and 95.8% recovery with the two cultivars tested, Sukkari and Sultany. In this chapter, we describe the D cryo-plate protocol employed for the cryopreservation of date palm PEMs.

## Materials

### 2.1 Plant Material

PEMs of two date palm commercial cultivars Sukkari and Sultany, diameter 3–5 mm, produced from juvenile leaves of offshoots [18]. PEMs consist of aggregates of approximately 15 pro-embryos, of a size of approximately 500 μm each (Fig. 1).

### 2.2 Tissue Culture Media and Solutions

1. Basal culture medium: Murashige and Skoog’s (MS) [21] basal salt prepared mixture, MS vitamin mixture, and other additives are listed in Table 1.
2. Hormone stock solution: 2,4-D (30 mg/100 mL).
3. Glutamine stock solution: 2 g/100 mL.
4. Adenine stock solution: 0.6 g/100 mL.
5. MS vitamin mixture stock solution: 10.31 g/100 mL (see Note 1).
6. PEM proliferation medium: basal culture medium supplemented with 51.3 g/L sucrose, 0.3 mg/L 2,4-D, and 8 g/L agar (see Note 1).

7. Somatic embryo (SE) regeneration medium: basal culture medium with 51.3 g/L sucrose, 8 g/L agar, devoid of hormones.

8. pH adjustment solutions: 1 N NaOH and 1 N HCl.

2.3 Cryopreservation Media and Solutions (Table 2)

1. Pretreatment medium: basal culture medium with 171 g/L sucrose and 8 g/L agar.

2. Alginate medium: deionized water with 51.3 g/L sucrose and 30 g/L sodium alginate (medium viscosity, 250 cps).

3. Polymerization medium: basal culture medium with 51.3 g/L sucrose and 11 g/L CaCl₂.

4. Loading solution: basal culture medium with 184 g/L glycerol and 136.8 g/L sucrose.

5. Unloading solution: basal culture medium with 410.4 g/L sucrose.

6. Posttreatment medium: basal culture medium with 102 g/L sucrose, 8 g/L agar, and 0.3 mg/L 2,4-D.

7. PEM proliferation medium: basal culture medium with 51.3 g/L sucrose, 8 g/L agar, and 0.3 mg/L 2,4-D.
2.4 Equipment

1. Instruments: magnetic stirrer cum hotplate, media dispenser, analytical balance (0.1 mg–few grams), top pan balance (few hundreds of grams), gas stove, pH meter, set of micropipettes (10–5000 μL), autoclave, refrigerator, laminar air flow cabinet, and bead sterilizer. Dewar vessel low form (approx. 1 L volume), polyethylene Dewar vessel (2–4 L), aluminum cryo-plates (7 × 37 × 0.5 mm with 10 wells of 1.5 Ø and 0.75 mm depth (Fig. 2)), aluminum cryocanes, forceps and tweezers of

<table>
<thead>
<tr>
<th>Macromolecules mg/L</th>
<th>Micromolecules mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃ 1900</td>
<td>MnSO₄ 4H₂O 22.3</td>
</tr>
<tr>
<td>NH₄NO₃ 1650</td>
<td>ZnSO₄ 7H₂O 8.6</td>
</tr>
<tr>
<td>CaCl₂ 4H₂O 441.1</td>
<td>H₃BO₃ 6.2</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O 369.8</td>
<td>KI 0.83</td>
</tr>
<tr>
<td>KH₂PO₄ 204.1</td>
<td>CuSO₄ 5H₂O 0.025</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄ 2H₂O 0.25</td>
</tr>
<tr>
<td></td>
<td>CoCl₂ 6H₂O 0.025</td>
</tr>
<tr>
<td></td>
<td>FeSO₄ 7H₂O 27.85</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA 2H₂O 37.25</td>
</tr>
<tr>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td>Inositol 100</td>
<td></td>
</tr>
<tr>
<td>Thiamine 100</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid 500</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCl 500</td>
<td></td>
</tr>
<tr>
<td>Additives</td>
<td></td>
</tr>
<tr>
<td>Adenine 15</td>
<td></td>
</tr>
<tr>
<td>Glutamine 100</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Composition of MS medium [21] and additives used as the basal medium in this protocol

mohan.jain@helsinki.fi
### Table 2
Composition of cryopreservation media and solutions used for various stages

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Alginate medium</th>
<th>Polymerization</th>
<th>Loading</th>
<th>Unloading</th>
<th>Posttreatment</th>
<th>PEM proliferation</th>
<th>SE regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS basal mixture</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
<td>4.3 g/L</td>
</tr>
<tr>
<td>MS vitamin solution(^a)</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
<td>1 mL/L</td>
</tr>
<tr>
<td>Adenine solution(^b)</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
<td>2.5 mL/L</td>
</tr>
<tr>
<td>Glutamine solution(^c)</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
<td>5 mL/L</td>
</tr>
<tr>
<td>Sucrose</td>
<td>171 g/L</td>
<td>51.3 g/L</td>
<td>51.3 g/L</td>
<td>136.8 g/L</td>
<td>410.4 g/L</td>
<td>102.6 g/L</td>
<td>51.3 g/L</td>
</tr>
<tr>
<td>Agar</td>
<td>8 g/L</td>
<td>8 g/L</td>
<td>8 g/L</td>
<td>8 g/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>184 g/L</td>
</tr>
<tr>
<td>2,4-D solution(^d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 mL</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 g/L</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11 g/L</td>
</tr>
</tbody>
</table>

\(^a\): MS vitamin mixture solution at 10.31 g/100 mL  
\(^b\): adenine solution at 0.6 g/100 mL  
\(^c\): glutamine solution at 2 g/100 mL  
\(^d\): 2,4-D solution at 30 mg/100 mL
different lengths with fine and medium tips and lancets with N°11 blades, liquid nitrogen (LN) transport tank, and LN storage tank (Fig. 3).

2. Glassware/plastic ware: reagent bottles, conical flasks (100, 250, and 500 mL capacity), beakers (various capacities), measuring cylinders (various capacities), culture tubes (25 x 150 mm) and racks, plastic caps, glass rods, and sterile plastic Petri dishes (60 and 100 mm Ø). Whatman filter paper Ø 90, 100 mm Ø glass Petri dishes, sterile 2 mL polypropylene cryotubes with internal thread, cryostorage, and box for 2 mL cryotubes.

3 Methods

3.1 Tissue Culture Medium Preparation

1. Prepare 1 L of PEM proliferation medium and somatic embryo regeneration medium each by adding 4.3 g MS [21] prepared basal salt mixture powder, 1 mL MS vitamin solution, 2.5 adenine solution, 5 mL glutamine solution (see Note 1), and 51.3 g sucrose.

2. For the PEM proliferation medium, add 1 mL 2,4-D stock solution for a final concentration of 0.3 mg/L; whereas, for SE regeneration, the medium is devoid of hormones.

3. Make up the volume to 1 L with deionized water.
4. Stir the medium to achieve dissolution of all chemicals and adjust the pH to 5.75 using NaOH.
5. Progressively add 8 g agar and stir to homogenize it in the medium and heat until agar is dissolved.
6. Distribute the medium in test tubes using a media dispenser (20 mL/test tube) and autoclave at 121 °C for 20 min.
7. After autoclaving, let the medium cool at room temperature and store the test tubes in the refrigerator until use.

Fig. 3 View of a liquid nitrogen storage tank
3.2 Tissue Culture Procedures

1. Subculture PEMs every 4–6 weeks on PEM proliferation medium.
2. Take PEMs for cryopreservation use, 1 week after the last subculture.
3. For plantlet production, transfer the PEMs to SE regeneration medium.
4. Maintain cultures in a growth chamber at 27°C under a 12 h light/12 h dark photoperiod of 50 μmol/m²/s.

3.3 Cryopreservation Media Preparation

3.3.1 Pretreatment Medium

Same as steps 1–5 in Subheadings 3.1 for proliferation medium but add 171 g sucrose. Then:

1. Transfer the medium in a 2 L screw-top glass flask and autoclave at 121°C for 20 min.
2. After autoclaving, under the laminar airflow, distribute the medium in sterile Ø 60 mm plastic Petri dishes (25 mL/Petri dish) (see Note 2).
3. Seal the Petri dishes with cling film, wrap them in cling film by packs of 10, and store them in the dark at 4°C until use.

3.3.2 Alginate Medium

1. Prepare 1 L alginate medium by adding 51.3 g sucrose in a graduated cylinder and make up the volume to 1 L with deionized water.
2. Stir the medium to achieve dissolution of sucrose, and adjust the pH to 5.75 using NaOH.
3. Add progressively 30 g sodium alginate (medium viscosity, 250 cps) and stir to homogenize it in the medium (see Note 3).
4. Transfer the medium in a 2 L screw-top glass flask and autoclave at 121°C for 20 min.
5. Store in the dark at room temperature.

3.3.3 Polymerization Medium

1. To prepare 1 L polymerization medium, add 4.3 g MS basal salt mixture (see Note 1), 1 mL MS vitamin solution, 2.5 mL adenine solution, 5 mL glutamine solution, 51.3 g sucrose, and 11 g CaCl₂ in a graduated cylinder, and make up the volume to 1 L with deionized water.
2. Stir the medium to achieve dissolution of all chemicals, and adjust the pH to 5.75 using NaOH.
3. Transfer the medium in a 2 L screw-top glass flask and autoclave at 121°C for 20 min.
4. Store in the dark at room temperature.
3.3.4 Loading Solution

The loading solution used is that developed by Matsumoto et al. [22]:

1. To prepare 1 L loading solution, add 4.3 g MS basal salt mixture (see Note 1), 1 mL MS vitamin solution, 2.5 mL adenine solution, 5 mL glutamine solution, 136.8 g sucrose, and 184 g glycerol in a graduated cylinder, and make up the volume to 1 L with deionized water.

2. Stir the medium to achieve dissolution of all chemicals (see Note 4), and adjust the pH to 5.75 using NaOH.

3. Under the laminar flow, filter-sterilize the loading solution using a disposable filtering unit (pore 0.2 μm), and store it in the dark at 4 °C until use.

3.3.5 Unloading Solution

Same as Subheading 3.3.4 Loading Solution but add 205.2 g sucrose and do not add glycerol. Then:

1. Transfer the medium in a 2 L screw-top glass flask and autoclave at 121 °C for 20 min.

2. Store in the dark at room temperature.

3.3.6 Posttreatment Medium

Same as Subheading 3.3.1 Pretreatment Medium but add 102.6 g sucrose.

3.3.7 PEM Proliferation Medium

Same as steps 1–5 in Subheadings 3.1, then:

1. Transfer the medium in a 2 L screw-top glass flask and autoclave at 121 °C for 20 min.

2. After autoclaving, under the laminar airflow, distribute the medium in sterile Ø 60 mm plastic Petri dishes (25 mL/Petri dish) (see Note 2).

3. Seal the Petri dishes with cling film, wrap them in cling film by packs of 10, and store them in the dark at 4 °C until use.

3.4 Preparation of Items for Cryopreservation Using the D Cryo-Plate

3.4.1 Cryo-Plates

1. For sterilization, place cryo-plates in glass Petri dishes (10–20 cryo-plates per dish, depending on the experiment), wrap them individually in aluminum foil, and autoclave them at 121 °C for 20 min. A schematic representation of the D cryo-plate protocol is shown in Fig. 4.

2. Store at room temperature.

3.4.2 Filter Paper

1. Place precut circles of Whatman paper Ø 90 mm in 100 mm glass Petri dishes (10 circles/dish), wrap them in aluminum foil and autoclave at 121 °C for 20 min (see Note 5).

2. Store at room temperature.
3.4.3 Pipette Tips and Automatic Pipettes

1. Autoclave sterile pipette tips (0.2–10 μL and 1–200 μL) in racks at 121 °C for 20 min and get adapted automatic pipettes.
2. Store the racks at room temperature.

3.5 Cryopreservation Protocol

3.5.1 Dissection and Pretreatment of PEMs

1. Place large clumps of PEM cultures in a Petri dish under a stereomicroscope. Isolate clumps of PEMs, 3–5 mm in size, consisting of approximately 15 pro-embryos, 500 μm in size (measured using a graded scale placed under the stereomicroscope) (Fig. 1).
2. Transfer ten clumps of PEMs per Petri dish containing pretreatment medium.
3. Seal the Petri dishes with cling film and place them for 3 days in a growth chamber at 27 ± 2 °C under a 12 h light/12 h dark photoperiod and a light intensity of 50 μmol/m²/s.

3.5.2 Encapsulation of PEMs

1. Place cryo-plates in empty Petri dishes (2–3 cryo-plates/Ø 60 mm dish).
2. Pour 5 mL sodium alginate medium in a beaker.
3. Pour 5 mL polymerization medium in a beaker.
4. Place two filter papers in a Ø 100 mm Petri dish.
5. In each well of the cryo-plates, add a 1–2 μL alginate medium drop with an automatic pipette with a 10 μL tip.
6. Carefully place a PEM in each well of the cryo-plate, and then add another 1–2 μL alginate medium drop on top of the PEM.

Fig. 4 Schematic representation of the D cryo-plate protocol developed by Salma et al. [20] for cryopreservation of date palm PEMs
7. Gently pour polymerization medium on each cryo-plate until all PEMs are well covered. Wait for 15 min to allow polymerization of the sodium alginate in calcium alginate.

8. After 15 min, remove the polymerization medium with a pipette, and blot dry the cryo-plates on the filter paper.

3.5.3 Loading and Dehydration of PEMs

1. Fill Ø 60 mm Petri dishes with loading solution.
2. Place two filter papers in a Ø 100 mm Petri dish.
3. Immerse 2–3 cryo-plates with PEM/dish in loading solution for 20 min.
4. Retrieve the cryo-plates from the loading solution and blot them dry on a filter paper. Transfer them in empty Petri dishes, and allow PEMs to dehydrate under the laminar airflow for 90–120 min (RT: 23 °C; RH: 40–50%).

3.5.4 Immersion and Storage of PEMs in Liquid Nitrogen

1. Place the low form Dewar vessel (or the Styrofoam box (see Note 6)) under the laminar airflow cabinet and clean it (inside and outside) with 95° ethanol.
2. Place inside the Dewar vessel a cryostorage box, which has been cleaned with 95° ethanol.
3. Fill a Ø 60 mm Petri dish with unloading solution.
4. Insert open cryotubes (see Note 7) in their slots in the cryobox (one cryotube/cryo-plate).
5. A few min before the end of the PEM dehydration period, fill the cryotubes with LN, and fill the Dewar vessel with LN up to 2–3 mm from the top of the cryotubes.
6. Take the cryo-plates with forceps and immerse them rapidly in the cryotubes (one cryo-plate/cryotube). If necessary, add additional LN to fill up cryotubes up to the top. When performing experiments, PEMs are rewarmed after a few minutes of LN storage to evaluate recovery.
7. For long-term storage, close cryotubes with screw caps and transfer them in LN storage tanks, either in cryoboxes inserted in cryoracks or clipped to cryocanes inserted in cryocans (see Note 8).

3.5.5 Rewarming

For rewarming, cryo-plates are retrieved from the cryotubes and immersed in unloading solution for 15 min at RT.

3.5.6 Posttreatment and Recovery

1. After rewarming, blot dry the cryo-plates on a filter paper. With the tip of a lancet blade, gently detach PEMs from the cryo-plates, remove the excessive unloading solution with a filter paper, and transfer the PEMs in Petri dishes containing posttreatment medium.
2. Seal Petri dishes with cling film and maintain at 27 ± 2 °C in the dark for 3 days, then transfer on recovery medium to light conditions, 12 h photoperiod, light intensity 50 μmol/m²/s for further regrowth.

3. After 1–2 subcultures on proliferation medium, recovering PEMs are transferred on regeneration medium for plantlet production.

4 Notes

1. Stock solutions of MS macroelements, microelements, and vitamins can also be prepared and used as an alternative to ready-to-use powder (see e.g., [23] for preparation of stock solutions).

2. Glass Petri dishes can also be used in case disposable ones are not available. They are wrapped in aluminum foil and autoclaved at 121 °C for 20 min.

3. Gently heating the medium helps in dissolving the alginate. Do not add MS mixture or MS macroelements in the presence of calcium, otherwise sodium alginate will react to form calcium alginate.

4. Gently heating the medium helps dissolving the chemicals.

5. Alternatively, circles can be cut manually in large sheets of Whatman paper.

6. Alternatively, Styrofoam boxes (e.g., ice cream boxes) of a similar volume can be efficiently used.

7. When performing experiments, cryotubes can be reused several times after cleaning and autoclaving at 121 °C for 20 min in an airtight container. It is worth mentioning that currently the only manufacturer of the cryo-plates is Taiyo Nissan Co Ltd., Japan.

8. For security reasons, equally split cryostored material in two replicates, and store in separate LN containers, ideally in two different locations. LN storage containers are equipped with racks and cryoboxes containing the cryotubes or with cans containing cryocanes holding the cryotubes. Their volume and cryotube storage capacity vary depending on the number of samples to be stored.

Acknowledgments

The date palm PEMs used to establish the D cryo-plate protocol were kindly provided by Dr. Lotfi Fki, Plant Biotechnology Laboratory, Faculty of Sciences, Sfax, Tunisia.
References

Chapter 4

In Vitro Cryopreservation of Date Palm Caulogenic Meristems

Lotfi Fki, Olfa Chkir, Walid Kriaa, Ameni Nasri, Emna Baklouti, Raja B. Masmoudi, Alain Rival, Noureddine Drira, and Bart Panis

Abstract

Cryopreservation is the technology of choice not only for plant genetic resource preservation but also for virus eradication and for the efficient management of large-scale micropropagation. In this chapter, we describe three cryopreservation protocols (standard vitrification, droplet vitrification, and encapsulation vitrification) for date palm highly proliferating meristems that are initiated from vitro-cultures using plant growth regulator-free MS medium. The positive impact of sucrose preculture and cold hardening treatments on survival rates is significant. Regeneration rates obtained with standard vitrification, encapsulation-vitrification, and droplet-vitrification protocols can reach 30, 40, and 70%, respectively. All regenerated plants from non-cryopreserved or cryopreserved explants don’t show morphological variation by maintaining genetic integrity without adverse effect of cryogenic treatment. Cryopreservation of date palm vitro-cultures enables commercial tissue culture laboratories to move to large-scale propagation from cryopreserved cell lines producing true-to-type plants after clonal field-testing trials. When comparing the cost of cryostorage and in-field conservation of date palm cultivars, tissue cryopreservation is the most cost-effective. Moreover, many of the risks linked to field conservation like erosion due to climatic, edaphic, and phytopathologic constraints are circumvented.

Key words  Cryopreservation, Caulogenic meristems, Encapsulation, Vitrification

1 Introduction

Biotechnology has been used to propagate, improve, and preserve plant genetic resources [1–4]. In date palm (Phoenix dactylifera L.), biotechnological techniques have already been employed for in vitro propagation [5, 6]. However, very few commercial tissue culture laboratories are now able to provide suitable quantities of date palm vitro-plants because it is recalcitrant to in vitro regeneration. In fact, date palm tissue cultures grow very slowly: the initiation phase may require more than 24 months, especially when low amounts of plant growth regulators are used to avoid potential somaclonal variation [7]. The presence of uncontrollable
endophytic bacteria is another serious constraint hampering the large-scale micropropagation of date palm [8].

Cryopreservation refers to the long-term storage of living tissues at an ultra-low temperature (−196 °C) so that it can be revived without loss of regeneration capacity and genetic fidelity. Various technical approaches exist in order to establish cryopreservation techniques. Some are based on a slow freezing and others are based on a rapid-freezing process known as vitrification which enables hydrated living cells to be cooled to cryogenic temperatures without ice formation [1, 2]. All living cells suffer from severe osmotic stress and/or ice crystal damage during the freezing and thawing processes. The most effective ways to minimize such lethal effects are: (a) to use cryoprotective compounds in the culture medium prior to material freezing and (b) to control the transient cooling and warming rates during preservation. Cryopreservation circumvents problems related to the regular re-initiation of in vitro cultures and to the long-term maintenance of proliferating cultures that include risks of contamination, somaclonal variation, or loss of regeneration competency. Several studies have been published on date palm cryopreservation although research is still needed before routine use [9–11]. The successful cryopreservation of proliferating tissue can be of great interest for the development of commercial large-scale micropropagation strategies. Indeed, the availability as a safe backup of cryopreserved germplasm enables the sequential rejuvenation of cultures under production, thus circumventing unwanted drifts linked to long-term proliferation such as hormone habituation, loss of regeneration capacity, or somaclonal variation.

This chapter describes three cryopreservation protocols for date palm highly proliferating caulogenic meristems: standard vitrification, droplet-vitrification, and encapsulation-vitrification protocols.

2 Materials

2.1 Plant Material

Adventitious caulogenic meristems (dedifferentiating cell aggregates) of date palm Deglet Noor cv. from in vitro cultures at an exponential proliferation phase, produced in RITA bioreactors (Fig. 1; see Note 1).

2.2 Culture Medium and Cryopreservation Solutions

1. Basal culture medium: Murashige and Skoog (MS) medium [12] (Table 1).
2. Preculture medium (PM): Hormone-free MS medium containing 0.52 M (180 g/L) sucrose and 8 g/L agar.
3. Bioreactor medium (BM): Hormone-free liquid MS medium containing 0.2 M (70 g/L) sucrose.
4. Loading solution (LS): Hormone-free liquid MS medium containing 2 M (145.36 mL/L) glycerol and 0.4 M (136.9 g/L) sucrose.

5. Vitrification solution (VS2) [13]: Hormone-free liquid MS medium containing 3.26 M (236.93 mL/L) glycerol, 2.42 M (136.66 mL/L) ethylene glycol, 1.9 M (135.09 mL/L) dimethyl sulfoxide (DMSO), and 0.4 M (136.9 g/L) sucrose.

6. Recovery solution (RS): Hormone-free liquid MS medium containing 1.2 M (410.7 g/L) sucrose and 22 μM (0.1 mg/L) 2,4-dichlorophenoxyacetic acid (2,4-D).

7. Posttreatment medium (PTM): Hormone-free liquid MS medium containing 0.52 M (180 g/L) sucrose.

8. Regeneration medium (RM): MS medium supplemented with 0.14 M (50 g/L) sucrose, 22 μM (0.1 mg/L) 2,4-D and 8 g/L agar.

9. Elongation medium (EM): Hormone-free liquid MS medium supplemented with 0.14 M (50 g/L) sucrose.

10. Rooting medium (RoM): Liquid MS medium supplemented with 0.14 M (50 g/L) sucrose and 4 mg/L Indole-3-butyric acid (IBA).

11. Solutions to adjust pH: 0.5 N NaOH and 0.5 N HCl.

---

Fig. 1 (a) Caulogenic cultures growing in a RITA bioreactor, scale bar: 0.5 cm, (b) histological section showing the explants (caulogenic meristems) used for cryopreservation, scale bar: 0.5 mm

mohan.jain@helsinki.fi
2.3 Reagents

1. Liquid nitrogen.
2. Fixative solution (FS): Chromic acid 5 g/L, glacial acetic acid 50 mL/L formaldehyde 150 mL/L, and ethanol 50 mL/L.
3. Hematoxylin Solution (HS): 100 mg/L hematoxylin in ethanol.
4. Staining solution (SS): 100 mL/L hematoxylin and 100 mL/L glycerol.

<table>
<thead>
<tr>
<th>Medium composition</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major inorganic nutrients</strong></td>
<td></td>
</tr>
<tr>
<td>Mg SO$_4$·7H$_2$O</td>
<td>370</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>440</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
</tr>
<tr>
<td><strong>Minor inorganic nutrients</strong></td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$·4H$_2$O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>8.6</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Iron source</strong></td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>27.84</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>37.24</td>
</tr>
<tr>
<td><strong>Vitamins and amino acids</strong></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
</tr>
<tr>
<td>Glutamine</td>
<td>200</td>
</tr>
</tbody>
</table>
2.4 Equipment  
1. Horizontal laminar flow hood.  
2. RITA Bioreactors.  
3. Aluminum foil.  
4. Micropipette.  
5. Cryotubes.  
7. Rotary microtome.  
8. Autoclave.

3 Methods

3.1 Culture Media Preparation  
1. Refer to Subheading 2.2 and Table 1 for the composition of culture media, mix components, and adjust the pH to 5.8.  
2. Sterilize all media for 15 min using a standard autoclave.

3.2 Caulogenic Cultures Maintenance  
1. For caulogenic vitro-cultures multiplication, use RITA bioreactors for the temporary immersion of cultures in liquid medium. The RITA vessel is made of two compartments: the explants are cultivated in the upper compartment, and the lower one holds the liquid medium (Fig. 1a).  
2. Cultivate 6-bud clusters per bioreactor using 200 mL BM. The immersion cycle is 15 min every 24 h, and the culture medium is renewed once every 4 months (see Note 2).  
3. Incubate cultures in a growth chamber set at 28 °C and 16-h photoperiod (30 μmol/m²/s).

3.3 Histology  
1. Put the explant in the fixative solution for 24 h.  
2. Dehydrate gradually the explant with ethanol solutions (50, 60, 70, 80, 90, and 100%) for 1 h each.  
3. Embed the explant in paraffin.  
4. Perform serial sections (10 μm) with a rotary microtome.  
5. Mount the sections on glass slides and stain with SS solution for 4 h.

3.4 Cryopreservation Solutions Preparation  
1. Refer to Subheading 2.2 for the composition of cryopreservation solutions, mix components of each solution, and adjust the pH to 5.8.  
2. Sterilize all solutions using 0.22 μm membrane filters, and keep them at −20 °C.
3.5 Cryopreservation Protocols

1. Excise the caulogenic meristems from tissue showing bud initiation (Fig. 1b; see Note 3).

2. Culture tissue-bearing caulogenic meristems on preculture medium (PM) or incubate them at 4 °C (cold hardening treatment) for 2 days (see Notes 3 and 4).

3.5.1 Standard Vitrification Protocol

1. Transfer the caulogenic meristems (explants) to a Petri dish containing 15 mL loading solution (LS), and incubate at room temperature for 20 min (Fig. 2).

---

**Fig. 2** Description of the cryopreservation protocols. LS loading solution; VS vitrification solution; RS recovery solution

mohan.jain@helsinki.fi
2. Replace the loading solution by ice-cooled VS2 solution.
3. Put the explants in a Petri dish containing VS2 solution for 30 min at 0 °C.
4. Transfer the explants into 2 mL cryotubes containing 0.5 mL VS2, and then plunge them into liquid nitrogen.
5. After 1 h in liquid nitrogen storage, thaw the cryotubes in a water bath at 40 °C for 2 min.
6. Put the explants in a Petri dish containing RS at room temperature for 15 min.
7. Place the explants onto two sterile filter papers on top of PTM.
8. Incubate the explants in the dark at 28 °C.
9. After 2 days, transfer the explants onto RM.

3.5.2 Droplet-Vitrification Protocol

1. Transfer explants (tissue-bearing caulogenic meristems) to 15 mL loading solution (LS) for 20 min.
2. Replace the loading solution by ice-cooled VS2 solution.
3. Place the explants in a Petri dish containing VS2 solution for 30 min at 0 °C.
4. Transfer the explants to a droplet of VS2 on a strip of aluminum foil (3/7 mm), and then plunge them into liquid nitrogen.
5. For permanent cryostorage, transfer frozen foil strips to 2 mL cryotubes filled with liquid nitrogen.
6. After 1 h in LN storage, treat in a Petri dish the explants by RS at room temperature for 15 min.
7. Place the explants onto two sterile filter papers on top of PTM.
8. Incubate the explants in the dark at 28 °C.
9. After 2 days, transfer the explants onto RM (see Note 5).

3.5.3 Encapsulation-Vitrification Protocol

1. Put the explants bearing caulogenic meristems into previously autoclaved 3% (w/v) sodium alginate, dissolved in MS medium amended with 0.2 M (68.4 g/L) sucrose without CaCl2. Suck them up with a micropipette, and drop them gently into 75 mM CaCl2·2 H2O in MS medium supplemented with 0.2 M (68.4 g/L) sucrose (see Note 6).
2. Transfer the encapsulated plant tissue into the loading solution for 20 min (Fig. 2).
3. Replace the loading solution by ice-cooled VS2 solution.
4. Hold the encapsulated explants with VS2 solution for 60 min at 0 °C.
5. Transfer the encapsulated explants into 2 mL cryotubes containing 0.5 mL VS2, and then plunge them into liquid nitrogen (LN).

6. After 1 h in LN, thaw the cryotubes containing the alginate beads in a water bath at 40 °C for 2 min.

7. Put the beads in a Petri dish containing RS at 25 °C for 15 min.

8. Place the alginate beads onto two sterile filter papers on top of PTM.

9. Incubate the beads in the dark at 28 °C.

10. After 2 days, transfer the explants onto RM to get buds (Fig. 3a; see Note 7).

### 3.6 Plant Regeneration Protocol

1. To generate multiple bud clusters, subculture the obtained buds onto RM medium once every 2 months for many times.

2. For shoot elongation, transfer bud clusters onto a thin film of liquid RM medium once every 2 months for many times (Fig. 3b).

3. For shoots rooting, transfer shootlets (5–10 cm height) onto RoM medium.

### 4 Notes

1. A caulogenic meristem is a tissue that can produce an adventitious bud. It is important to point out that this protocol is also effective for two other tested date palm cultivars, Barhee and Khenaizi.

2. Cultivation of date palm shoots in RITA vessels proliferates efficiently by using liquid medium and the control of the gaseous environment of in vitro cultures [14].

3. In this protocol, only small explants (<2 mm) are able to tolerate cryo-treatments [15].

4. The beneficial effect of both sucrose preculture and cold hardening on post-thaw regeneration is clearly demonstrated [16, 17]. Sucrose treatments increase the osmolarity of intracellular solutes, thus reducing the detrimental effect of VS2 and the formation of intracellular ice upon subsequent immersion of the explants in liquid nitrogen. Both sucrose and cold acclimation treatments increase proline content in date palm tissue.

5. The droplet vitrification is the most efficient strategy for the cryo-banking of date palm tissue. The highest regeneration rates obtained with the standard vitrification, encapsulation-vitrification, and droplet-vitrification protocols are 30, 40, and 70%, respectively [1].
6. Alginate is nontoxic to date palm meristems and protects efficiently against cryodamages [1].
7. We have to wait for 3–5 months to produce buds from survived meristems [18].

Fig. 3 Regrowth (see arrows) of date palm caulogenic meristems after cryopreservation using encapsulation-vitrification protocol. (a) 2 months after thawing, (b) 6 months after thawing, scale bar: 0.5 cm
Acknowledgments

This work was supported financially by the Ministry of Higher Education and Scientific Research in Tunisia, the International Atomic Energy Agency (IAEA), the Arab League Educational, Cultural and Scientific Organization (ALECSO), the European Cooperation in Science and Technology (COST), the Swiss National Science Foundation (SNSF), and the Technical Centre of Dates in Tunisia (TCDT).

References


mohan.jain@helsinki.fi
Chapter 5

In Vitro Conservation of Date Palm Shoot-Tip Explants and Callus Cultures Under Minimal Growth Conditions

Maiada M. El-Dawayati

Abstract

Date palm fruit production has great economic significance for many countries. There is a fundamental necessity to conserve valuable date palm germplasm, but there are various problems with in vivo and ex situ conservation. In vitro storage has several advantages over conventional germplasm conservation methods. The in vitro technique offers a developed method of slow-growth storage, which is considered as an alternate solution for short- and medium-term storage of date palm germplasm under controlled conditions. Minimal growth conditions for germplasm conservation are generally achieved by reducing growth rate through modification of environmental growing conditions and culture, by using low temperatures, and the addition of growth retardants and osmotic agents. This chapter describes a protocol for short-term in vitro conservation of date palm shoot-tip and callus cultures under slow-growth storage conditions, using sucrose as an osmotic agent and abscisic acid (ABA) as a growth retardant at 15 °C for 12 months.

Key words Conservation, Germplasm, Growth retardants, In vitro, Low temperature, Osmotic regulators, Slow-growth storage

1 Introduction

Date palm (Phoenix dactylifera L.) is one of the oldest cultivated crops and an important multipurpose tree. It is one of the most economically important fruit trees in the desert areas of the Middle East and North Africa [1]. Significant progress has been made to improve plant regeneration by using tissue culture means through organogenesis and somatic embryogenesis [2–6]. Date palm germplasm cannot be conserved by storage, or handled easily, by conventional means [7]. The maintenance of a germplasm collection by in vitro techniques could reduce many problems of traditional storage methods [8]. Conservation of plant germplasm by in vitro techniques has been done using slow-growth procedures or cryopreservation [9]. Date palm germplasm can be preserved in vitro as cultured material in the form of shoot tips, callus cultures, somatic embryos, and shoot clusters [10–12].
Slow growth can be achieved by modifying the environmental conditions and the culture medium through several methods [8, 9, 13]. Storage under low temperature is a major tissue technique used for date palm conservation. Bekheet [10] described preservation of Egyptian date palm tissue cultures (shoot buds and callus cultures) at 5 °C in the dark. El-Dawayati [14] conserved date palm shoot-tip explants at 5 °C and 15 °C temperatures for 12 months. A high survival rate of 91.85% was achieved at 15 °C, while at 5 °C survival was only 45.17%.

It has been demonstrated that medium supplemented with sucrose or sorbitol at 0.3 M seems to be the most effective in producing the highest significant survival percentages of date palm shoot-tip and callus explants, compared with mannitol addition [14–17]. Regarding reduced growth conditions for date palm germplasm by using abscisic acid (ABA) as a growth retardant, El-Dawayati [14] found that increasing ABA concentrations in the presence of sucrose, sorbitol, or mannitol, added to conservation medium, reduced growth significantly and extended the subculture interval to 12 months for date palm shoot-tip explants.

This chapter describes a protocol for short-term in vitro conservation of date palm shoot-tip and callus cultures under slow-growth storage condition, using sucrose as an osmotic agent and abscisic acid (ABA) as a growth retardant, at 15 °C for 12 months, to achieve successful germplasm storage and the best recovery of conserved tissue.

2 Materials

2.1 Plant Materials and Sterilization

1. Explant source: Shoot tips of date palm offshoots 3–5 years and 5–7 kg of Gondola cv.

2. Clorox disinfectant solution: 1.6% (w/v) sodium hypochlorite solution (10% v/v Clorox, commercial bleach) containing 3 drops of Tween 20 per 100 mL.

3. Mercury disinfectant solution: Mercuric chloride (HgCl2) (0.1 mg/L).

4. Antioxidant solution: Ascorbic acid (150 mg/L) and citric acid (100 mg/L).

2.2 Basal Culture Medium and Hormone Stock Solutions

1. Basal culture medium: MS medium salts and vitamins [18] (Table 1).

2. Hormone stock solutions (1 mg/mL each): 2,4-dichlorophenoxyacetic acid (2,4-D), 2-isopentenyladenine (2iP), benzyladenine (BA), naphthaleneacetic acid (NAA), and abscisic acid (ABA).
<table>
<thead>
<tr>
<th>Medium composition</th>
<th>Stock concentration (mg/L)</th>
<th>Final concentration in culture medium (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock I: Major inorganic nutrients (20× stock) use 50 mL to prepare 1 L of medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>33,000</td>
<td>1650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>38,000</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>8800</td>
<td>440</td>
</tr>
<tr>
<td>MgSO₄·2H₂O</td>
<td>7400</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3400</td>
<td>170</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>3400</td>
<td>170</td>
</tr>
<tr>
<td><strong>Stock II: Minor inorganic nutrients (200× stock) use 5 mL to prepare 1 L of medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>166</td>
<td>0.83</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1240</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO₄·2H₂O</td>
<td>4460</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1720</td>
<td>8.6</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Stock III: Iron source (200× stock) use 5 mL to prepare 1 L of medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>5560</td>
<td>27.8</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>7460</td>
<td>37.3</td>
</tr>
<tr>
<td><strong>Stock IV: Vitamins (200× stock) use 5 mL to prepare 1 L of medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>25,000</td>
<td>125</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Glycine</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>Biotin</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>–</td>
<td>200</td>
</tr>
<tr>
<td>Citric acid</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>
3. Basal MS medium (Table 1) containing additives listed in Table 2 according to tissue and stage of the conservation process, including shoot-tip explant conservation of medium (SC), shoot-tip explant recovery medium (SR), callus conservation medium (CC), and callus recovery medium (CR).

4. Basal MS medium (Table 1) containing additives listed in Table 3 according to the culture stages, including shoot-tip establishment and callus proliferation medium (E), somatic embryo induction medium (SEI), somatic embryo germination medium (SEG), and plantlet rooting medium (PR).

### 2.3 Equipment

1. Instruments: Incubator chamber, laminar airflow hood, and autoclave.
2. Glassware: Measuring cylinder, conical flask, pipettes, beakers, and culture jars (150 mL).
3. Tools: Scalpel, forceps, and spatula.
3 Methods

3.1 Explant Preparation

1. Separate young offshoots, 2–3 years old and 5–7 kg, from the mother palm, Gondola cv.

2. Remove all older leaves of offshoot and then transfer to the laboratory. Clean leaf fibers from the base, and remove the leaf sheaths one by one (see Note 1).

3. Wash excised apical meristems, 3–4 cm in width and 6–8 cm in length, under running tap water with detergent for 5–10 min.

4. Dip apical meristems in disinfectant solution 1 for 5 min.

5. Soak apical meristems in sterile aqueous solution of antioxidant solution for 30 min, and then rinse once with sterile distilled water (see Note 2).

6. Surface sterilize the apical meristems with disinfectant solution 2 for 10 min, and thoroughly rinse with sterilized distilled water and again soak them for another 50 min, and thoroughly rinse three times with sterilized distilled water.

7. Remove the first young leaf with a sterile scalpel from apical meristem explant, and then divide into four equal longitudinal sections for use as the explants.

3.2 Preparation of Culture Medium

1. Prepare stock solutions of plant growth regulators by dissolving 2,4-D, NAA, and ABA in 95% ethanol or 1 N NaOH and 2iP and BA using 1 N HCl; make up the required volume by adding double-distilled water. Store in the refrigerator at 4 °C for up to 1 month.

2. Mix MS salt solutions (Table 1) and other components according to the culture stage and preserved tissue (Tables 2 and 3).

3. Adjust pH to 5.7 ± 0.1 with 0.1 N NaOH and 0.1 N HCl before adding agar.

4. Make up the volume of prepared media with distilled water and then add agar.

5. Heat the medium until the agar is fully dissolved.

6. Distribute media in small culture jars, 30 mL per jar, for tissue culture stages, and 50 mL per jar for conservation procedures (see Note 3).

7. Cap culture jars with polypropylene lids and autoclave for 20 min at 121 °C and 1.2 kg/cm² pressure.

3.3 Establishing Shoot-Tip Explants

1. Culture sterilized shoot-tip explants on nutrient medium for the establishment of explants (E medium, Table 3).

2. Incubate the cultures for 10 days in the dark at 27 ± 1 °C in order to eliminate fungal- or bacterial-contaminated cultures.
3.4 Conservation Conditions for Shoot-Tip Explants

1. Collect contamination-free healthy shoot-tip explants and culture on conservation medium (SC medium, Table 2) (Fig. 1a; see Notes 4–6).

2. Incubate the shoot-tip explants jars in an incubator at 15 °C in complete darkness for 12 months.

3.5 Recovery of Conserved Shoot-Tip Explants

1. Transfer culture jars to room temperature in the dark for 3 days to acclimatize the explants to the new growth conditions.

2. Transfer the shoot-tip explants onto the recovery medium (SR medium, Table 2), and incubate under complete darkness at room temperature. Recover all good, healthy surviving explants after 4–6 weeks (Fig. 1b).

3. Transfer the recovered shoot-tip explants at 6-week intervals for 6–8 months until full formation of callus clumps (Fig. 1c) using the same fresh culture medium.

4. Transfer to a culture medium with reduced 2,4-D concentration from 10 mg/L to 3 mg/L to induce embryonic callus formation.

Fig. 1 (a) Uncontaminated shoot-tip sections cultured on prepared conservation medium for shoot tips, incubated at 15 °C for 12 months; (b) surviving shoot-tip explants after conservation period for 12 month under minimal growth conditions at 15 °C, exhibiting good and healthy appearance after 4–6 weeks cultured on recovery medium; (c) formation of callus clumps on culture nutrient media for recovery at normal condition of growth, for conserved shoot-tip explants after 12 month of conservation period under minimal growth conditions at 15 °C; (d) germinated embryos and development stages received from conserved shoot tips under minimal growth conditions for 12 months at 15 °C healthy and fully rooted plantlets after conservation process.
5. Collect embryonic callus and culture on SEI medium (Table 3). Maintain for 12 weeks by subculturing at 6-week intervals to induce somatic embryo differentiation and maturation.

6. Transfer fully differentiated somatic embryos to SEG medium (Table 3), and incubate at 27 ± 1 °C under 16-h photoperiods of cool-white florescent light (40 μmol m²/s).

7. Transfer germinated somatic embryos to rooting medium (PR medium, Table 3) to achieve healthy and fully rooted plantlets after the conservation process (Fig. 1d).

3.6 Callus Culture Establishment
1. Culture shoot-tip explants on E medium (Table 3) for 8–10 months and subculture at 6 weeks intervals until callus formation.

2. Incubate cultures at 27 ± 1 °C under complete darkness. Friable embryogenic callus is used for the conservation process.

3.7 Conservation Conditions for Callus
1. Collect embryogenic callus and culture on callus conservation medium (CC medium, Table 2).

2. Incubate the callus cultures at low temperature (15 °C) in complete darkness (see Notes 7).

3.8 Recovery of Conserved Callus
1. Transfer callus cultures to 27 ± 1 °C and keep for 3 days to acclimatize the cultures to the new growth conditions (Fig. 2a).

2. Transfer callus to recovery nutrient medium for conserved callus (CR medium, Table 2) and incubate under complete darkness at 27 ± 1 °C.

3. Identify the surviving explants, which exhibit a good healthy appearance after 4–6 weeks.

4. Culture embryogenic callus on SEI medium (Table 3), and subculture twice at 6-week intervals to induce somatic embryo differentiation and maturation (Fig. 2b) (see Notes 8 and 9).

5. Transfer fully developed somatic embryos to SEG medium (Table 3), and incubate at 27 ± 1 °C and 16-h photoperiod of cool-white florescent light (40 μmol/m²/s) to resume their germination.

6. Transfer geminated somatic embryos to rooting medium (PR medium, Table 3) to achieve healthy and fully rooted plantlets after the conservation processing. (Fig. 2c).
4 Notes

1. While removing shoot tips, care must be taken to prevent any shock or damage to the core soft tissues.

2. In this step antioxidant solution is used to reduce further formation of phenolic compounds which cause browning and reduce viability of cultured explants.

3. Increase the amount of nutrient medium inside culture jars, which are used in the conservation process to provide sufficient amount of nutrient medium to the conserved tissue throughout the conservation period.

Fig. 2 (a) Conserved callus explants after 12 months of conservation period at 15 °C, (b) differentiated somatic embryos on recovery medium received from conserved embryonic callus explants under minimal growth conditions for 12 months at 15 °C, (c) healthy and fully rooted plantlets after conservation process obtained from conserved embryonic callus explants under minimal growth conditions for 12 months at 15 °C
4. Important consideration should be given while choosing conserved explants, namely, (a) the organ that serves as a tissue source and (b) the physiological and ontogenic stage of the organ.

5. Sucrose, sorbitol, and mannitol as osmotic regulators act to retard growth by causing osmotic stress to the explants to be conserved. Carbohydrates added to the culture medium reduce hydric potential and restrict the water availability to the conserved explants. Sucrose is the most suitable energy source and osmotic agent added to conservation medium for date palm callus and shoots-tip explants. It gives the highest survival rates and effective regeneration after the conservation period. Shoot-tip explants conserved on a medium supplemented with sorbitol are stimulated rapidly to form direct somatic embryos when returned to normal growth conditions for regeneration. However, the addition of mannitol to the conservation media reduces the survival percentage and weak recovery after the conservation period. Mannitol is metabolically inactive sugar alcohol and results in poor results. It is one of the compatible solutes that can decrease plant water potential (drying) by osmotic adjustment. A large amount of mannitol in the cell can increase drastically the osmotic pressure of the medium. A high osmotic potential in the external medium can induce protoplast water loss, causing plasmolysis and eventually permanent cell damage.

6. Conservation of explants, besides low temperature and osmotic adjustment, is also routinely used with ABA. Application of exogenous ABA improves in vitro conservation and the adaptive response of plant cells and tissue to various environmental stresses.

7. Optimal conditions for date palm cold storage are unsatisfactory by decreasing the temperature to 5 °C. In vitro preservation of different explants of date palm such as shoot tip, callus, somatic embryos, and shoot clusters under slow-growth condition at 15 °C as a low temperature for conservation gave satisfying results for survival and recovery of conserved explants, especially conserved callus explants. Although conserved explants survive at 5 °C after the conservation period, recovery potential under normal growth conditions is low. Recovery of conserved callus explants at 5 °C shows poor regeneration rate, high hyperhydricity, and high propensity for abnormal somatic embryo formation. The main point is how can conserved explants react and retain their regeneration capacity. Preservation protocols should reflect maximum survival rates and recovery potential after the conservation period. Genotypic differences in cold hardness should be taken into account before planning storage procedures.
8. The conversion from friable callus to embryonic callus occurs in the darkness. Once the nodular callus appears, add ABA to the culture nutrient media for 2–3 subcultures to achieve proper maturation of differentiated somatic embryos from embryonic callus. The addition of ABA is critical for enhanced production of somatic embryos. The favorable effect of ABA on somatic embryogenesis may be due to increased production of proteins, triglycerides, and lipid; also, it improves sucrose uptake and starch synthesis, as reported in several studies.

9. Conserved callus explants often faced with hyperhydricity problem after the conservation period. This can be eliminated in different ways during the recovery stage as follows: (a) subculture callus for 2–3 times at every 8 weeks interval on nutrient medium with NO₃⁻ as the sole N source for MS salts; (b) callus explants can be cultured for 1 month on MS nutrient medium containing 20 g/L polyethylene glycol for 1 month to induce dehydration; and (c) culture callus for 1 month on MS nutrient medium supplemented with 0.2 mg/L AgNO₃ to induce dehydration for callus tissue.

Acknowledgment

The author wishes to acknowledge the researcher Mr. Mahmoud Hamza for the vital help to achieve this work.

References

2. El-Dawayati MM (2000) Physiological studies on date palm micropropagation. M.Sc. Thesis, Department of Pomology, Faculty of Agriculture, Cairo University, Egypt
11. Hassan M (2002) In vitro studies on somatic embryogenesis conservation of date palm. Ph. D. Thesis Department of Pomology, Faculty of Agriculture, Cairo University, Egypt
12. El-Dawayati MM (2008) Using tissue culture technology to storage some plant tissues of
date palm. Ph.D. Thesis, Department of Pomology, Faculty of Agriculture, Cairo University, Egypt


Chapter 6

In Vitro Conservation of Date Palm Somatic Embryos Using Growth-Retardant Conditions

Mona M. Hassan

Abstract

In vitro conservation is carried out to maintain disease-free genetic materials, in a small area, protected against pests, insects, soil problems (alkaline, acidic, excess salinity, lack of organic matter, too dry, or too wet), climatic changes, and high-multiplication potential. A requirement of successful in vitro conservation is that the plants can be regenerated into complete plants rapidly when desired. The current work describes in vitro propagation and conservation techniques employing slow-growth conditions of date palm somatic embryo cultures. Clusters of somatic embryos resulting from an indirect micropropagation protocol are conserved in MS culture medium supplemented with an osmotic agent (sucrose at 90 g/L) combined with a growth-retardant hormone (abscisic acid) at 2 mg/L incubated at low temperature (18°C) and low light intensity (10 μmol/m²/s). The survival and plant recovery rates are recorded after 10 months. Date palm somatic embryo clusters can be conserved for up to 10 months without subculture with high survival rates. Successfully conserved somatic embryos multiply and germinate to regenerate plants with well-developed shoots and roots, which survive acclimatization and field transfer.

Key words Abscisic acid (ABA), Osmotic agent, Conservation, Somatic embryos, Tissue culture

1 Introduction

Date palm (*Phoenix dactylifera* L.) is a monocotyledonous dioecious tree species of major economic importance in the arid regions of the Middle East and North Africa [1]. The potential of date palm conservation appears to be most important in high-production countries, which hold significant amounts of genetic diversity that can be utilized for genetic improvement and development of crop cultivars for domestic and foreign markets [2]. Fruit tree germplasm is conserved mainly in the form of field gene banks, which require a considerable amount of labor, expense, and land. Moreover, field germplasm collections are exposed to natural disasters and are subject to attack by pests and pathogens [3]. In addition, conservation of genetic resources of date palm seeds is not an ideal method because they are heterogeneous and do not reproduce...
true-to-type plants. Tissue culture and cryopreservation techniques may prove the most effective conservation approach as the genetic erosion of conserved cultures is minimized [4, 5]. The success of date palm propagation using different in vitro techniques enables the large-scale multiplication of desired genotypes [6] and facilitates preserving in vitro cultures with extended subculture intervals thus reducing costs associated with frequent subcultures [7].

The maintenance of large collections of micropropagated materials under optimum growth condition, which involves subculturing at regular intervals, exposes the cultures to the risk of contamination and genetic variation [8]. Several approaches have been found to reduce the growth rate and extend subculture intervals by incubation at reduced temperature and low light intensity [9–13], changing the osmotic potential of conservation medium either by the addition of sugar alcohols (mannitol or sorbitol) or increasing the sugar concentration. In addition, decreasing the supply of inorganic nutrients [14, 15] or adding growth retardants to culture medium [16] has also been used. Minimal-growth storage is a very simple technique that allows storage of plants in vitro for periods ranging from 6–60 months, depending on the species. These stored plants can be micropropagated rapidly when desired [17]. This chapter describes a simple and effective protocol to conserve date palm somatic embryos using a combination of sucrose, as an osmotic agent, and abscisic acid, as a growth-retardant hormone, and incubation at a low temperature and low light intensity.

2 Materials

2.1 Plant Material and Disinfection

1. Healthy offshoots of 5–7 kg in weight and 50–70 cm in length from date palm cv. Sewy.
2. Ethanol, 70%.
3. Clorox disinfection solution: 50% (v/v) Clorox commercial bleach (2.6% w/v, sodium hypochlorite NaOCl) containing 0.1% Tween 20.
4. Mercuric chloride (HgCl2) solution: 1 g/L.
5. Filter sterilized antioxidant solution: 100 mg/L citric acid and 150 mg/L ascorbic acid.

2.2 Culture Medium

1. Basal culture medium: Stock solutions of Murashige and Skoog (MS) medium [18] (Table 1).
2. Plant growth regulators stock solutions (1 mg/mL each): 2,4-Dichlorophenoxyacetic acid (2,4-D), 2-isopentenyladenine (2iP), abscisic acid (ABA), ancymidol, benzyladenine (BA), kinetin, naphthaleneacetic acid (NAA), and paclobutrazol (PBZ).
Medium additives for various culture stages: The strength of basal culture medium and additives (sucrose, hormones, agar, activated charcoal, and polyethylene glycol) for each culture stage are shown in Table 2. They include callus initiation medium (M1), embryogenic callus formation medium (M2), somatic embryo multiplication medium (M3), rooting medium (M4), and pre-acclimatization medium (M5).

### Table 1
Modifications of the MS basal medium [18] composition for various culture stages of date palm micropropagation

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Callus initiation, mg/L</th>
<th>Embryogenic callus formation, mg/L</th>
<th>Somatic embryo multiplication, mg/L</th>
<th>Rooting medium, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroelements I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>850</td>
<td>850</td>
<td>850</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>1900</td>
<td>950</td>
<td>950</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>440</td>
<td>440</td>
<td>220</td>
<td>220</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370</td>
<td>370</td>
<td>185</td>
<td>185</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>170</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td><strong>Minor elements II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄ × 7H₂O</td>
<td>27.8</td>
<td>27.8</td>
<td>27.8</td>
<td>27.8</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.3</td>
<td>37.3</td>
<td>37.3</td>
<td>37.3</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>22.3</td>
<td>22.3</td>
<td>22.3</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄·4H₂O</td>
<td>8.6</td>
<td>8.6</td>
<td>8.6</td>
<td>8.6</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>CuSO₄ × 5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Organic constituents II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>L glutamine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>Adenine sulfate</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 2
Different culture stages and their corresponding additives supplemented to the basal MS medium composition shown in Table 1

<table>
<thead>
<tr>
<th>Culture stage</th>
<th>MS medium strength</th>
<th>Growth regulators (mg/L)</th>
<th>AC (g/L)</th>
<th>Sucrose (g/L)</th>
<th>Agar (g/L)</th>
<th>PEG (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2,4-D 2iP  BA  Kin  NAA  ABA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus initiation (M1)</td>
<td>Full</td>
<td>10 3 – – – –</td>
<td>1.5</td>
<td>30</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Embryogenic callus formation (M2)</td>
<td>Full except NH₄NO₃ at ½</td>
<td>5 3 – – – –</td>
<td>1.5</td>
<td>45</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Somatic embryo multiplication (M3)</td>
<td>Half major and full minor elements</td>
<td>– – 0.5 0.5 – –</td>
<td>1</td>
<td>45</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Rooting medium (M4)</td>
<td>Half major and full minor elements</td>
<td>– – – – 1 –</td>
<td>1</td>
<td>30</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Pre-acclimatization medium (M5)</td>
<td>Half</td>
<td>– – – – 0.1 – –</td>
<td>15</td>
<td>–</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Somatic embryo conservation medium (CM)</td>
<td>Full</td>
<td>– – 0.5 0.5 – 2 –</td>
<td>90</td>
<td>6</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

4. Conservation medium (CM): MS basal medium supplemented with 0.5 mg/L BA, 0.5 mg/L kinetin, 2 mg/L ABA, 90 g/L sucrose, and 6 g/L agar (Table 2).

2.3 Equipment
1. Instruments: Laminar airflow hood, culture incubator, autoclave, stirring hot plate and magnetic stirrers, pH meter.
2. Tools: Scalpel handle, stainless steel sterile surgical blades, and forceps.
3. Glassware and culture vessels: Beakers (1 L), test tubes (2.5 × 15 cm), test tubes (2.5 × 25 cm), small jars (200 mL), and large jars (375 mL).

3 Methods
3.1 Establishment of In Vitro Culture
3.1.1 Explants Preparation and Sterilization
1. Select healthy date palm offshoots and carefully detach them from mother plants (Fig. 1a). Transfer selected offshoots to the laboratory after detaching.
2. Remove adventitious roots, older leaves, and other external tissue (Fig. 1b). Continue to remove leaves from offshoots until the appearance of white soft leaves (Fig. 1c).
3. Place shoot tip (surrounded by 4–6 leaf primordia) in a 1 L beaker and wash thoroughly with running tap water for 1 h.

4. In the laminar airflow hood, place the explants in 70% ethanol for 1 min (Fig. 1d).

5. Decant the ethanol and place in the Clorox disinfection solution for 25 min (see Note 1).

6. Wash the explants three times with sterile distilled water and remove 1–2 leaves under aseptic condition (Fig. 1e).

Fig. 1 Stages of date palm micropropagation and callus formation: (a) selection of offshoots, (b) preparation of offshoot, (c) shoot-tip explant, (d) sterilization of shoot tip, (e) removal of 1–2 leaves, (f) shoot-tip sections, (g) shoot-tip section in test tube, (h) swelling of explant, (i) callus cultures induced from shoot tip cultured for 9 months on callus induction medium.

mohan.jain@helsinki.fi
7. Immerse explants in 1 g/L mercuric chloride solution for 5 min and wash explants with sterilized distilled water three times.

8. Immerse the shoot tip in antioxidant solution for 30 min (see Note 2).

9. Slice the shoot tip longitudinally into four sections (see Note 3, Fig. 1f).

### 3.2 Initiation of Callus Cultures

1. Inoculate slices of sterilized shoot-tip explants in test tubes (2.5 × 15 cm) containing 15 mL callus initiation medium (M1, Table 2). Incubate test tubes in the dark, at 27 ± 1 °C for 6-weeks (Fig. 1g).

2. Transfer shoot-tip explants to fresh medium dispensed in small jars (200 mL) containing 35 mL, at 6-week intervals, five times to produce white soft callus (see Note 4, Fig. 1h, i).

3. Culture 0.5–1.0 g fragile callus in small glass jars (200 mL) containing 35 mL embryogenic callus medium (M2, Table 2) for 3 months, at 6-week intervals to form embryogenic callus (see Note 5, Fig. 2a).

### 3.3 Improvement of Date Palm Somatic Embryo Clusters

1. Transfer embryogenic callus to growth regulators-free medium (see Note 6).

2. Incubate cultures at 27 ± 2 °C in complete darkness for 3 months, and subculture at 4-week intervals to produce somatic embryogenesis.

3. During the aforementioned period, two types of embryos form, individual well-developed somatic embryos and secondary somatic embryos (see Note 7).

4. Culture clusters of somatic embryos into somatic embryo multiplication medium (M3, Table 2) [11].

5. Keep cultures at 27 ± 2 °C under 16-h photoperiod of 10 μmol/m²/s, and subculture five times at 3-week intervals to obtain sufficient stock of somatic embryo clusters (Fig. 2b).

### 3.4 Somatic Embryo Conservation

1. Collect clusters of somatic embryos, each consisting of 5–8 somatic embryos from the multiplication stage.

2. Inoculate collected somatic embryo clusters in conservation medium (CM) in 200-mL glass jars containing 50 mL medium (Table 2) (see Note 8).

3. Incubate culture jars for 1 week under 27 °C and 16-h photoperiod of 20 μmol/m²/s light intensity provided by cool white fluorescent lamps (see Note 9).

4. Transfer all culture jars after 1 week to incubators set at 18 °C. Expose all cultured jars to low light intensity (10 μmol/m²/s) during conservation.
3.5 Determination of Viability After Conservation

1. Transfer somatic embryo clusters to recovery multiplication medium (M3, Table 2) in order to assess their viability after conservation period.

2. Keep somatic embryos in recovery multiplication medium growing at standard growth conditions for 1–2 months.

3. Record survival percentages as recovery rate defined as germination of embryos and production of secondary somatic embryos (see Notes 10–16).

4. After determination of plant recovery, transfer clusters of germinated somatic embryos (shoots) into larger jars (375 mL) containing rooting medium (M4, Table 2), light intensity 40 μmol/m²/s, and subculture two times at 1-month intervals, to form roots (Fig. 2d).

5. Culture rooted shoots in test tubes 2.5 × 25 cm containing 1/2 MS liquid culture medium (M5, Table 2), and grow for 1 month at 50 μmol/m²/s light intensity (Fig. 2e).

6. Transfer rooted plantlets to the greenhouse for further growth and record survival (Fig. 2f).

Fig. 2 Stages of date palm embryo formation and subsequent growth after preservation: (a) embryogenic callus culture 3 months in reduced 2,4-D medium (M2), (b) clusters of somatic embryos in multiplication medium (M3), (c) somatic embryo recovery in multiplication medium, (d) rooted shoots in solid rooting medium (M4), (e) rooted shoots in liquid pre-acclimatization medium (M5), (f) regenerated plants established in the greenhouse after 6 months.
4 Notes

1. Sterilization must be carried out with continuous shaking of the explants for uniform exposure to the sterile solution.

2. To avoid culture browning, it is more effective to use filter sterilized antioxidant before culturing rather than before sterilization.

3. Quick dipping of shoot-tip slices in a diluted disinfection solution (5% Clorox solution or 0.1 g/L mercuric chloride) immediately before placing on the medium is effective in reducing contamination percentage. Normally this mild treatment does not negatively affect explant responses; however, shoot-tip explants smaller than 0.5 cm in length occasionally deteriorate, and they can be excluded.

4. When browning exudation from the explants is very high, remove brown tissue from the base, and reduce the subculture interval to 3–4 weeks, instead of 5–6 weeks, especially in the first and second subculture in order to accelerate the explant response.

5. Reduce 2,4-D concentration, use half NH₄NO₃ strength, and increase sucrose concentration up to 45 g/L for reducing hyperhydricity of callus cultures and increasing its ability to induce globular embryos. Also, it is important to discard all hyperhydric tissues.

6. The addition of ancymidol, paclobutrazol, or ABA at 0.25–0.5 mg/L is beneficial to enhance somatic embryo formation.

7. Clusters of somatic embryos are highly suitable for in vitro conservation rather than single embryos due to better survival rate in conservation and increased regeneration rate after recovery.

8. The clusters of somatic embryos should be immersed deeply in the medium in order to protect them from dehydration and increased conservation period.

9. It is important to incubate culture for 1 week before conservation to assure these cultures are free of contamination.

10. The survival of date palm somatic embryo cultures at cool temperature (18 °C) is enhanced by addition of ABA and high sucrose concentration to the medium, and the period of conservation can be extended to at least 18 months with higher survival rates. However increasing conservation period beyond 10 months reduces survival rates gradually. The combination of treatments preferred will vary with cultivars.
11. Explant necrosis is common following conservation treatments; however, these explants should be subcultured twice in the growth room under standard conditions.

12. Normally conserved clusters are fully viable after 10 months.

13. High sucrose concentration induces proline accumulation and reduces moisture content, which are indicators of water stress.

14. Conservation of cultures beyond 4 months causes the loss of somatic embryo viability on culture medium containing 30 g/L sucrose and devoid of ABA.

15. Avoid using sucrose concentration higher than 90 g/L in the presence of ABA. Under such conditions, the degree of browning of conserved clusters increases (which is a normal reaction of living tissues to stress) as well as reduction in survival rate, attributed to high stress [11].

16. Conserved explants exhibited higher multiplication rate after recovery, especially after third subculture under normal conditions [19] (Fig. 2c).

References

1. Al-Khayri JM (2002) Growth, proline accumulation, and ion content in NaCl-stressed callus cultures of date palm (*Phoenix dactylifera* L.) In Vitro Cell Dev Biol Plant 38:79–82


11. Hassan MM (2002) In vitro studies on somatic embryogenesis conservation of date palm. Ph. D. Thesis, Faculty of Agriculture, Cairo University, Cairo, Egypt


mohan.jain@helsinki.fi


Encapsulation of Date Palm Somatic Embryos: Synthetic Seeds

Shawky A. Bekheet

Abstract

Synthetic seed or encapsulated somatic embryos may be used for propagation, storage, and exchange of plant germplasm and have many diverse applications in date palm cultivation. They have advantages over conventional use of offshoot material for germplasm propagation, maintenance, exchange, and transportation. This chapter describes a protocol for date palm synthetic seed production by encapsulation of somatic embryos with sodium alginate. Among three concentrations used, 3% sodium alginate followed by dropping into 2.5% calcium chloride ($\text{CaCl}_2$) solution shows the best concentration of gel matrix for both maintenance and recovery. In addition, storage of the encapsulated date palm somatic embryos at 5 °C improves the survival and conversion into plantlets; otherwise, 20 g/L sucrose in the culture medium enhances conversion of the recovered somatic embryos to plantlets. This protocol is promising for in vitro conservation and international exchange of date palm germplasm.

Key words  Encapsulation, Sodium alginate, Somatic embryos

1 Introduction

Synthetic seeds are basically defined as encapsulated somatic embryos which functionally mimic zygotic seeds and can germinate to grow as seedlings under sterile conditions; moreover, encapsulated buds or any other form of meristems can regenerate plants [1]. However, the encapsulation technique has the potential of easy handling, exchange of germplasm, and efficient in vitro short- or long-term storage [2]. Alginate-encapsulated germplasm can also be stored at room temperature for several years and are suitable for the conservation under ultralow-temperature conditions. Significant progress has been made to make synthetic seeds by using different explants. Unipolar propagules such as micro-bulbs, rhizomes, protocorm [3–5], nodal cuttings [6], and shoot buds [7, 8] besides bipolar somatic embryos [9, 10] have been subjected to encapsulation for storage, easy handling, transport, prevention of dehydration and mechanical damage, delivery, and...
establishment under in vitro and/or ex vitro conditions. In this respect, establishment of synthetic seeds using somatic embryos has multiple advantages over other plant tissue cultures, including ease of handling, higher scale-up potential, low cost, and the direct development to complete plantlets [11].

Several studies have been carried out to develop a synthetic seed protocol for date palm. Ibrahim et al. [12] described a method for date palm artificial seeds through coating the somatic embryos with sodium alginate. The frequency of conversion to plantlets was affected by sodium alginate concentration, gel matrix composition, and duration of exposure to calcium chloride used for capsule hardening. A successful system for preservation of date palm germplasm via artificial seeds was recognized by Bekheet et al. [13]. Somatic embryos proliferated in vitro from shoot-tip cultures were encapsulated in sodium alginate capsules and stored for several months and then regenerated to plantlets. Recently, Fki et al. [14] reported applicable methods for somatic embryogenesis and synthetic seeds of date palm. This chapter presents a protocol for the preparation of artificial seeds of date palm by encapsulating somatic embryos within sodium alginate capsules.

2 Materials

2.1 Plant Materials and Disinfection Solution

2. Antioxidant solution: 100 mg/L ascorbic acid and 150 mg/L citric acid.
3. Disinfection solutions: 70% ethanol solution and sodium hypochlorite solution (1.6% w/v; 30% v/v Clorox, commercial bleach).

2.2 Culture Medium

1. Basal culture medium: Stock solutions of Murashige and Skoog (MS) medium [15] (Table 1).
2. Hormone stock solutions (1 mg/mL each): 3-methyl-2-butenylamino-purine (2iP) and 2,4-dichloro-phenoxycetic acid (2,4-D).
3. Hormone solvent solutions: 0.5 N NaOH and 0.1 N HCl.
4. pH adjustment solutions: 0.1 N and 1 N each of KOH and HCL solutions.
5. Medium additives: Sucrose, hormones, and agar according to the culture stages as shown in Table 2 including initiation medium (I), embryo induction medium (II), and recovery medium (III).
Table 1
Murashige and Skoog (MS) basal medium composition [15]

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration of stock solution (mg/L)</th>
<th>Final concentration in culture medium (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Major salts (20×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use 50 mL to prepare 1 L of medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>33,000</td>
<td>1650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>38,000</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>8800</td>
<td>440</td>
</tr>
<tr>
<td>MgSO₄·2H₂O</td>
<td>7400</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3400</td>
<td>170</td>
</tr>
<tr>
<td>**Minor salts (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use 5 mL to prepare 1 L of medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>166</td>
<td>0.83</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1240</td>
<td>6.20</td>
</tr>
<tr>
<td>MnSO₄·2H₂O</td>
<td>4460</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1720</td>
<td>8.60</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td>**Iron (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use 5 mL to prepare 1 L of medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>5560</td>
<td>27.80</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>7460</td>
<td>37.30</td>
</tr>
<tr>
<td>**Vitamins (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use 5 mL to prepare 1 L of medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>1000</td>
<td>20,000</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>2000</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>Biotin</td>
<td>200</td>
<td>1</td>
</tr>
</tbody>
</table>
2.3 Encapsulation

1. Sodium alginate.
2. Calcium chloride (CaCl₂).
3. Antibiotic mixture [16]: Rifampicin, cefotaxime, and tetracycline HCl.
4. Pure sucrose.

2.4 Equipment

1. Microwave oven.
3. pH meter.
4. Laminar airflow hood.
5. Bacti-Cinerator sterilizer.

3 Methods

3.1 Preparation of Stock Solutions and Culture Media

1. Prepare antioxidant solution: Add 100 mg ascorbic acid and 150 mg citric acid in suitable volume of distilled water, and dissolve them by stirring using a magnetic stirring plate and then raise the volume to 1 L.
2. Prepare hormone stock solutions (1 mg/mL each): Dissolve 100 mg 2,4-D in 2 mL 0.5 N NaOH and 100 mg 2iP in 2 mL 0.1 N HCl; raise volume to 100 mL with distilled water of each growth hormone. Store at 4 °C.
3. Prepare initiation medium (I): Mix MS medium stock solutions and additives (Table 1) including 10 mg/L 2,4-D and 3 mg/L 2iP (Table 2).
4. Prepare somatic embryo induction medium (II): Mix MS medium stock solutions and additives (Table 1) devoid of plant growth hormones (Table 2).
5. Prepare recovery medium (III): Mix MS medium stock solutions and additives (Table 1) without adding any hormones (Table 2).
6. Adjust the pH of the media to 5.8 with HCl and KOH.

<table>
<thead>
<tr>
<th>Culture stage</th>
<th>2,4-D (mg/L)</th>
<th>2iP (mg/L)</th>
<th>Sucrose (g/L)</th>
<th>Agar (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation medium (I)</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>Embryo induction medium (II)</td>
<td>–</td>
<td>–</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>Recovery medium (III)</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

1. Shawky A. Bekheet

mohan.jain@helsinki.fi
7. Add agar 0.7% (w/v) in the media and heat to dissolve in a microwave oven for 10 min.
8. Dispense the media into 350 mL glass jars and close the jars with plastic caps.
9. Sterilize the media by autoclaving for 20 min at 121 °C and 1.2 kg cm⁻².
10. Store the autoclaved media at room temperature in the dark, maximum for 4 weeks.

3.2 Explant Preparation

1. Remove woody tissues and sheathing leaf bases. Excise external leaves until shoot tip is 3 cm long.
2. Take the shoot apices with small part of sub-meristematic tissues and keep them in the antioxidant solution (see Subheading 2.1).
3. Disinfect the shoot tips with sterilization solution (see Subheading 2.1) under air laminar cabinet. Use ethanol for 30 s followed by immersion in Clorox for 20 min and wash the explants three times with sterile distilled water.
4. Trim external leaves until shoot tip is 1.5 cm long and cut into 8–12 small pieces to serve as explants.

3.3 Regeneration of Somatic Embryos

1. Place shoot-tip explants on the culture initiation medium (I) (Tables 1 and 2).
2. Incubate cultures in the dark at 25 °C for 6 weeks.
3. Transfer explants three times onto the fresh culture medium (I) (Tables 1 and 2) with similar composition, at 5-week intervals.
4. Transfer the cultures to MS hormone-free medium (II) (Table 2) and incubate in 16-h photoperiod of 45 μmol/m²/s and 25 ± 2 °C for somatic embryo formation (Fig. 1a).

3.4 Encapsulation of Somatic Embryos

1. Prepare sodium alginate solution (3%): Dissolve 3 g sodium alginate in 100 mL distilled water. Sterilize solution by autoclaving for 20 min at 121 °C and 1.2 kg cm⁻².
2. Prepare calcium chloride solution (2.5%): Dissolve 2.5 g calcium chloride in 100 mL distilled water. Sterilize solution by autoclaving for 20 min at 121 °C and 1.2 Kg cm⁻².
3. Suspend the somatic embryos in 3% sodium alginate and mix them well with the gel (see Note 1).
4. Add and mix gently the filter-sterilized antibiotic mixture (60 mg rifampicin, 250 mg cefotaxime, and 25 mg tetracycline HCl) dissolved in 5 mL dimethyl sulfate.
5. Gently, pick up the alginate-coated somatic embryos with forceps and immerse in the 2.5% calcium chloride solution for...
30 min to form beads with each bead containing one embryo (see Notes 2 and 3, Fig. 1b) and then place them on filter paper.

6. Leave the coated somatic embryos to air-dry for 10 min under the laminar flow hood.

7. Transfer somatic embryos in beads to 2 mL sterile Eppendorf tubes (see Note 4) and store them at 5 °C in the dark up to 12 months.

### 3.5 Recovery

1. Wash beads with sterilized distilled water to get rid of gel around encapsulated somatic embryos in order to increase and accelerate somatic embryo sprouting on recovery medium.

2. Culture the somatic embryos on recovery MS medium devoid of hormones (Table 2, medium III).
3. Incubate the cultures at 25 ± 2 °C and 16-h photoperiod of 45 μmol/m²/s light intensity.

4. Monitor the survival and somatic embryo germination (see Note 5) for 5 weeks after inoculation (Fig. 1c).

5. Subculture the germinated somatic embryos on medium III containing 40 g/L sucrose for further full plantlet development (see Notes 6–8, Fig. 1d).

4 Notes

1. Use late cotyledonary somatic embryos for producing date palm synthetic seed because of their high capability for plantlet development.

2. Encapsulation procedure must be done under aseptic conditions.

3. The texture of the beads depends on the concentration of sodium alginate and calcium chloride solutions and duration of completion and recovery. Lower concentrations of sodium alginate and calcium chloride resulted in soft beads that are too delicate to handle, while higher concentrations of both resulted in hard beads that cause considerable delay in the embryo germination.

4. Position the somatic embryos in the center of the capsule to ensure better protection during handling.

5. Some beads do not produce healthy plantlets after removing gels from encapsulated embryos and recovery. This decline in the recovery response attributed to the inhibition of tissue respiration by the alginate matrix or a loss of moisture due to the partial desiccation during storage.

6. Addition of nutrients to the alginate beads may be useful for viability and germination of somatic embryos.

7. Post-thawing culture condition such as low light intensity is useful to enhance organized growth.

8. Optimized micropropagation or plant regeneration systems would be required prior to synthetic seed development.

References


Part II

Molecular Analysis of In Vitro Cultures
Evaluation of Clonal Fidelity of Micropropagated Date Palm by Random Amplified Polymorphic DNA (RAPD)

Arpan Modi, Bhavesh Gajera, Naraynan Subhash, and Nitish Kumar

Abstract

Date palm is a fruit-bearing tree commonly found in arid and semiarid regions. It is a dioecious plant, producing fruit on female plants and a limited number of basal offshoots for propagation. To produce large numbers of uniform plantlets, tissue culture techniques are required. It is highly advisable to detect genetic variation that may occur through micropropagation techniques as it may lead to phenotypic alterations. Random amplified polymorphic DNA (RAPD) is a simple and PCR-based molecular marker technique which can be employed to check the somaclonal variation. Screening of markers requires repeated confirmation of the pattern obtained in individual samples.

Key words Clonal fidelity, RAPD, Phoenix dactylifera, Somaclonal variation

1 Introduction

Date palm (Phoenix dactylifera L.) (2n = 36), a member of Arecales family, is dioecious and has slow plant growth rate leading to a late reproduction phase. It is a perennial and monocotyledonous fruit-bearing tree. Date palms are cultivated under extremely harsh climatic conditions in arid and semiarid regions within economically and environmentally sustainable agricultural systems. Date fruits as well as other parts of the tree generate foreign exchange [1]. The tree is generally cultivated through offshoots to maintain the genetic integrity and to avoid the genetic variability produced from seed propagation; however, dates produce a low number of offshoots. Micropropagation as a technique is being widely used worldwide to produce true-to-type plants. Propagation of date palm through in vitro techniques has been reported by several researchers using different explants sources as well as regeneration pathways [2–6]. Micropropagation techniques are often vulnerable to somaclonal variation and do not maintain genetic fidelity of micropropagated plants [7]. The most likely reasons for somaclonal variation are DNA methylation, chromosomal rearrangements, and
point mutation [8]. The molecular marker-based approach is most suitable, among other biochemical and physiological markers, for the detection of somaclonal variation. Among the various DNA-based markers, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have been used to screen for genetic stability/instability in tissue culture raised plants. However, these methods become unaffordable with the use of expensive enzymes and radioactive labeling. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers require only small amounts of DNA per sample, without involving radioactive labels, and are simple and rapid. RAPD has proven to be quite efficient in detecting genetic variations, even in closely related organisms such as two near-isogenic lines (NIL) [9–13]. This chapter describes the evaluation of various RAPD markers to detect somaclonal variation among regenerated in vitro date palm plantlets.

2 Materials

2.1 Micropropagated Date Palm Plants

1. Explant material: Offshoots of cv. Barhee, collected from the Kutch region, Gujarat state, India.

2. Basal culture medium: Murashige and Skoog (MS) [14] medium stock solutions (MS stocks I, II, III, and IV) (Table 1). Store in a freezer or cold room at 4 °C (see Note 1).

3. Surface sterilization: 0.1% mercuric chloride (see Note 2).

4. Plant growth regulator stock solutions: Micropropagation—stock solutions of 200 μM 6-benzylaminopurine (BAP), 200 μM indole-3-acetic acid (IAA), 4000 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 200 μM indole-3-butyric acid (IBA). Store hormonal stock solutions at 4 °C.

5. Sucrose (2%) and charcoal (0.5%).

2.2 DNA Extraction

1. CTAB extraction buffer: Stock solutions of 1 M Tris-HCl (pH 8.0), 5 M sodium chloride, 0.5 M EDTA (ethylenediaminetetraacetic acid) and store them at room temperature (see Note 3).

2. Chloroform/isoamyl alcohol solution in a ratio of 24:1 and stored at 4 °C.

3. Isopropanol stored at 4 °C having one tenth volume of 3 M sodium acetate.

4. 70% ethanol stored at 4 °C.

5. TE buffer: 1 M Tris–HCl (pH 8.0), 0.5 M EDTA (see Note 4).
2.3 Polymerase Chain Reaction

1. Master Mix (2×): Taq DNA polymerase, buffer components, magnesium chloride, and dNTPs and store at –20 °C.
2. Primer stock solution: 100 pmol and store at –20 °C (see Note 5).

2.4 Agarose Gel Electrophoresis

1. Agarose gel (1.3%) contains 0.1 μg/mL ethidium bromide (final concentration) in Tris borate EDTA buffer (pH 8.8) (see Note 6).

3 Method

3.1 Surface Sterilization

1. From the offshoots, thoroughly wash growing shoot tips and primordial leaf with double distilled water.
2. Establish aseptic cultures by treating these explants with 0.1% HgCl₂ for 12 min.
3. Rinse explants three times with sterile distilled water.

Table 1
Chemical composition of MS medium

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Stock concentration (mg/L)</th>
<th>Volume per liter (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major inorganic nutrients (20×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>33,000</td>
<td>50</td>
</tr>
<tr>
<td>KNO₃</td>
<td>38,000</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>8800</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·2H₂O</td>
<td>7400</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3400</td>
<td></td>
</tr>
<tr>
<td>Minor inorganic nutrients (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>166</td>
<td>5</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1240</td>
<td></td>
</tr>
<tr>
<td>MnSO₄·2H₂O</td>
<td>4460</td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1720</td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Iron source (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>5560</td>
<td>5</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>7460</td>
<td></td>
</tr>
<tr>
<td>Organic supplements (200×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>20,000</td>
<td>5</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Carbon source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>As per the experiment</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Callus Induction, Somatic Embryo Induction, and Embryo Germination

1. Place the explant on MS medium containing 2% sucrose, 33.75 μM BAP, 42.5 μM IAA, and 0.5% charcoal.

2. Transfer the induced callus on MS medium supplemented with 100 μM 2,4-D and 0.5% charcoal to induce somatic embryos.

3. Place the somatic embryos on MS medium amended with 11.2 μM IAA, 25 μM IBA, and 0.5% charcoal.

4. Randomly select 10% TC raised plants for the evaluation of clonal fidelity from regenerated tissue cultured plant (Fig. 1).

Fig. 1 Micropropagation of date palm through somatic embryogenesis. (a) Embryogenic callus, (b) development of somatic embryo, (c) germination of somatic embryo, (d) regenerated plantlets, (e) hardened plants in the nursery (Source: [16])

mohan.jain@helsinki.fi
3.3 DNA Extraction

DNA is extracted according to the widely used Doyle and Doyle method [15] with minor modifications as follows:

1. Take 200 mg leaf sample and crush in liquid nitrogen using mortar and pestle.
2. Take the sample powder in 2 mL autoclaved vial and add 1 mL pre-warmed extraction buffer in the sample.
3. Keep the sample at 65 °C for 60–90 min with occasional mixing.
4. Centrifuge the sample at 1957 × g for 5 min at 4 °C and take aqueous phase.
5. Add equal volume of chloroform/isoamyl alcohol (24:1) to the sample (800 μL approx.).
6. Centrifuge the sample at 7826 × g for 8 min at 4 °C and take 600 μL aqueous phase from it.
7. Transfer the aqueous phase in 2 mL autoclaved vial and add equal volume of chloroform/isoamyl alcohol (24:1) to it.
8. Centrifuge the sample at 7826 × g for 8 min at 4 °C and take 500 μL aqueous phase from it.
9. Transfer the aqueous phase in 1.5 mL autoclaved vial and add 1.0 mL absolute alcohol (100% alcohol) containing 10% 3 M sodium acetate to it.
10. Keep the sample at −20 °C for 3 h.
11. Centrifuge the sample at 11269 × g for 15 min at 4 °C.
12. Wash the pellet with 80% ethanol and centrifuge at 5009 × g for 10 min at 4 °C.
13. Air-dry the pellet and resuspend in 100 μL of 0.3× TE buffer.
14. Quantify the DNA and use 20 ng/μL for PCR.

3.4 RAPD Primer Screening and Polymerase Chain Reaction

1. Primer screening is done by taking Operon RAPD primer, and out of 160 primers, 27 primers (OPA-02, OPA-03, OPA-05, OPA-10, OPA-15, OPA-18, OPB-04, OPB-07, OPB-10, OPB-17, OPC-05, OPC-08, OPC-11, OPD-02, OPD-18, OPD-20, OPE-01, OPE-15, OPE-18, OPF-10, OPF-12, OPG-02, OPG-03, OPG-18, OPH-19, OPH-04, and OPH-13) were selected which produced sharp, clearly distinguishable, and more numbers of bands (Table 2). DNA fingerprinting pattern of D series primers is shown in Fig. 2.
2. Primer screening is a selection procedure of commercially available primer for its suitability to discriminate DNA fingerprinting pattern of two or more individuals. For this procedure, all the primers to be tested are employed to obtain banding pattern of any one sample of the population. For the clonal fidelity testing, DNA of the mother plant is taken, and PCR reaction is performed with different primers in 25 μL mixture containing...
5 μL DNA (100 ng), 12.5 mL Master Mix, and 1 μL 10 pmol 10-mer oligodeoxynucleotide primer. The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles comprising denaturation at 94 °C for 1 min, annealing at 38 °C for 1 min and extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA-02</td>
<td>TGCCGAGCTG</td>
</tr>
<tr>
<td>2</td>
<td>OPA-03</td>
<td>AGTCAGCCAC</td>
</tr>
<tr>
<td>3</td>
<td>OPA-05</td>
<td>AGGGGTCTTTG</td>
</tr>
<tr>
<td>4</td>
<td>OPA-10</td>
<td>GTGATCGCAG</td>
</tr>
<tr>
<td>5</td>
<td>OPA-15</td>
<td>TTCCGAACCC</td>
</tr>
<tr>
<td>6</td>
<td>OPA-18</td>
<td>AGGTGACCGT</td>
</tr>
<tr>
<td>7</td>
<td>OPB-04</td>
<td>GGACTGGAAGT</td>
</tr>
<tr>
<td>8</td>
<td>OPB-07</td>
<td>GGTGACGCAG</td>
</tr>
<tr>
<td>9</td>
<td>OPB-10</td>
<td>CTGCTGGGAC</td>
</tr>
<tr>
<td>10</td>
<td>OPB-17</td>
<td>AGGGAACGAG</td>
</tr>
<tr>
<td>11</td>
<td>OPC-05</td>
<td>GATGACCGCC</td>
</tr>
<tr>
<td>12</td>
<td>OPC-08</td>
<td>TGGACCCTTG</td>
</tr>
<tr>
<td>13</td>
<td>OPC-11</td>
<td>AAAGCTGCAG</td>
</tr>
<tr>
<td>14</td>
<td>OPD-02</td>
<td>GGAACCCACC</td>
</tr>
<tr>
<td>15</td>
<td>OPD-18</td>
<td>GAGAGCCAAC</td>
</tr>
<tr>
<td>16</td>
<td>OPD-20</td>
<td>ACCCGGTCAC</td>
</tr>
<tr>
<td>17</td>
<td>OPE-01</td>
<td>CCCAAGGTCC</td>
</tr>
<tr>
<td>18</td>
<td>OPE-15</td>
<td>ACGCACAACC</td>
</tr>
<tr>
<td>19</td>
<td>OPE-18</td>
<td>GGACTGCAGA</td>
</tr>
<tr>
<td>20</td>
<td>OPF-10</td>
<td>GGAAGCTTGAG</td>
</tr>
<tr>
<td>21</td>
<td>OPF-12</td>
<td>ACGGTACCCG</td>
</tr>
<tr>
<td>22</td>
<td>OPG-02</td>
<td>GGCACTGAGG</td>
</tr>
<tr>
<td>23</td>
<td>OPG-03</td>
<td>GAGGCTGCAA</td>
</tr>
<tr>
<td>24</td>
<td>OPG-18</td>
<td>GGCTCATGGG</td>
</tr>
<tr>
<td>25</td>
<td>OPG-19</td>
<td>GTCAGGGCCA</td>
</tr>
<tr>
<td>26</td>
<td>OPH-04</td>
<td>GGAAGTGGCC</td>
</tr>
<tr>
<td>27</td>
<td>OPH-13</td>
<td>GACGCCACAC</td>
</tr>
</tbody>
</table>
3. The PCR products are visualized on 1.3% agarose gel prepared by taking 1.3 g agarose in 100 mL running buffer used in electrophoresis containing 1 μL 10 mg/mL solution of ethidium bromide. Samples are loaded in wells by mixing them with gel loading dye containing both loading and tracking dyes. After the run, the gel is then visualized in a gel documentation system.

4. Run all the DNA samples using screened primers in PCR.

5. Run PCR products on agarose gel electrophoresis and check for polymorphisms (Fig. 3).

4 Notes

1. Prepare stock solutions of Murashige and Skoog medium and store at 4 °C (Table 1). After dissolving all the stock solutions in enough deionized water, make it up to 1 L, adjust the pH to 5.8 (add 2 g/L Gelrite or 8 g/L agar for solid medium), and autoclave for 25 min at 120 °C.
2. HgCl\textsubscript{2} is very hard to dissolve in water. Prepare 0.1% HgCl\textsubscript{2} solution by dissolving in sterilized distilled water prior to surface sterilization.

3. CTAB extraction buffer: Composed of 10 mL 1 M Tris–HCl (pH 8), 28 mL 5 M NaCl, 4 mL 0.5 M EDTA, 2 g CTAB, 0.8 g polyvinylpyrrolidone (PVP), and 1 mL β-mercaptoethanol. Dissolve properly and make the final volume to 100 mL with Molecular Grade Water. CTAB is dissolved in the buffer at 65 °C.

4. TE buffer (1×): Consists of 1 mL 1 M Tris–HCl (pH 8), 0.2 mL 0.5 M EDTA, and 98.5 mL distilled water for a final volume of 100 mL.

5. Operon primers for RAPD series are supplied in lyophilized powder. The working concentration is diluted to 10 pmol per μL using distilled water. Primers are prepared by dissolving in 0.3× TE buffer under sterile conditions. Dilute the stock solution ten times before use for polymerase chain reaction.

6. Tris borate EDTA buffer (10×): TBE buffer is prepared by taking 108 g Tris and 55 g boric acid in 800 mL distilled water and kept on stirrer for complete dissolution. To the solution, add 40 mL 0.5 M Na\textsubscript{2}EDTA, adjust to pH 8, and raise the final volume to 1 L with distilled water. From this stock solution, 1× working stock is prepared by ten times’ dilutions.

Fig. 3 Clonal fidelity testing of micropropagated plants with D-18 primer (5’ GAGAGCCAAC 3’) of operon RAPD series. A represents mother plant and samples B–O represent 14 tissue culture raised clones. L represents 100-base-pair DNA ladder.
Acknowledgment

This work was carried out with the funding given by the Rashtriya Krishi Vikas Yojna scheme, and we are highly obliged to Dr. Ranbir Singh Fougat, Head, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, India, for providing laboratory as well as hardening facilities.

References


mohan.jain@helsinki.fi
Chapter 9

Molecular Identification of Fungal Contamination in Date Palm Tissue Cultures

Mohammed H. Abass

Abstract

Fungal contamination of in vitro cultures of date palm (Phoenix dactylifera L.) is the major constraint to their initiation and maintenance. Different molecular approaches have been applied successfully to analyze both inter- and intraspecific variation among fungal species as well as determine their identity. This chapter describes step-by-step procedures of molecular identification of fungal contaminants by internal transcribed spacer (ITS) products of the most common fungal contaminants of date palm tissue culture. To begin with, samples of genera Alternaria, Aspergillus, Cladosporium, Epicoccum, and Penicillium were collected to isolate each fungal genus and extraction of genomic DNA. Polymerase chain reactions were accomplished by ITS primers (ITS1 and ITS4) for each fungal contaminant as well as for sequencing. Subsequently, they are analyzed by Basic Local Alignment Search Tool (BLAST) search of ITS sequence to reveal the identity of each individual fungal contaminant species. The molecular identification herein is a rapid and reliable procedure to identify date palm fungal contaminants which is very important in their control and treatment.

Key words BLAST, Fungal contamination, ITS, Molecular identification, Tissue cultures, Sequencing

1 Introduction

Date palm (Phoenix dactylifera L.) is one of the most important cultivated species of the Arecaceae family, grown mainly for its fruit; date fruit is highly nutritious and very rich in sugar, minerals, and vitamins. Additionally, all other parts of date palm tree have high socioeconomic importance in industrial and traditional applications [1]. Different procedures have been used to propagate date palm including seed propagation, offshoot propagation, and, most importantly, micropropagation [2, 3].

Micropropagation faces numerous challenges, such as microbial contamination which is rapidly becoming one of the most challengeable constraints [4, 5]. Fungal contamination of date palm cultured tissues is a major problem and causes degradation, browning, and death of infected tissue [6, 7]. Several studies
revealed that the highest fungal contamination of date palm cultures occurred during the initial stages of micropropagation [6, 7]. For fungal identification, the morphological characterization is mainly depending on macro- and microscopic features including colony morphology, size and shape of conidia, and presence of setae and teleomorph. Morphological identification is not reliable because of fungal morphology instability, which is significantly influenced by environmental factors and cultivation media as well as overlapping of morphological features among closely related taxa [8, 9]. Herein, molecular marker-assisted identification of fungal species is reliable to analyze genetic variation among isolated fungi [10–12].

This chapter describes a step-by-step protocol for the isolation and incubation of predominant fungal contamination of in vitro date palm cultures at the initial stages of development: DNA extraction and PCR amplification by using ITS primers (ITS1 and ITS4) and sequence analysis for fungal identification.

2 Materials

2.1 Plant Materials and Sterilization

1. Explant source: Date palm cv. Halawy offshoots, 10–15 kg, detached from the adult female trees (see Subheading 3).
2. Antioxidant solution: 100 mg/L ascorbic acid and 150 mg/L citric acid.
3. Sterilization solutions: 70% ethanol, 20% commercial bleach (sodium hypochlorite solution) containing 2–3 drops of Tween 20 per 100 mL solution.

2.2 Callus Initiation Medium (CIM)

1. Basal culture medium: Murashige and Skoog medium [13] (Table 1; see Note 1).
2. Hormone stock solutions: 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg/mL), 2-isopentyladenine (2iP, 1 mg/mL).
3. Culture initiation medium: MS medium (Table 1) containing 50 mg/L 2,4-D and 3 mg/L 2iP, 8 g/L agar, and 1.5 g/L activated charcoal (AC).

2.3 Fungal Growth Media and Antibiotics

1. Potato dextrose agar (PDA) medium: 200 g fresh potato, 20 g dextrose, 20 g agar, and 1000 mL water.
2. Potato carrot agar (PCA) medium: 200 g potato, 20 g fresh carrot, 20 g agar, and 1000 mL water.
3. Antibiotic: Chloramphenicol, 150 mg/L.
Table 1
MS basal medium components, plant hormones, additives, and activated charcoal for date palm micropropagation at initial stage

<table>
<thead>
<tr>
<th>Medium components</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrient</strong></td>
<td></td>
</tr>
<tr>
<td>Potassium nitrate (KNO₃)</td>
<td>1900</td>
</tr>
<tr>
<td>Ammonium nitrate (NH₄NO₃)</td>
<td>1650</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄·2H₂O)</td>
<td>370</td>
</tr>
<tr>
<td>Potassium phosphate (KH₂PO₄)</td>
<td>170</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂·2H₂O)</td>
<td>440</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate NaH₂PO₄·H₂O</td>
<td>170</td>
</tr>
<tr>
<td><strong>Micronutrient</strong></td>
<td></td>
</tr>
<tr>
<td>Boric acid (H₃BO₃)</td>
<td>6.2</td>
</tr>
<tr>
<td>Potassium iodide (KI)</td>
<td>0.83</td>
</tr>
<tr>
<td>Manganese sulfate (MnSO₄·2H₂O)</td>
<td>22.3</td>
</tr>
<tr>
<td>Zinc sulfate (ZnSO₄·7H₂O)</td>
<td>8.6</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Cupric sulfate (CuSO₄·5H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Cobalt chloride (CoCl₂·6H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Sodium molybdate (Na₂EDTA·2H₂O)</td>
<td>37.3</td>
</tr>
<tr>
<td>Ferrous sulfate (FeSO₄·7H₂O)</td>
<td>27.8</td>
</tr>
<tr>
<td><strong>Vitamins and amino acids</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Biotin</td>
<td>1</td>
</tr>
<tr>
<td><strong>Charcoal, sucrose, and agar</strong></td>
<td></td>
</tr>
<tr>
<td>Activated</td>
<td>1500</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30,000</td>
</tr>
<tr>
<td>Agar</td>
<td>8000</td>
</tr>
<tr>
<td><strong>Plant growth regulators</strong></td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>50</td>
</tr>
<tr>
<td>2iP</td>
<td>3</td>
</tr>
</tbody>
</table>
2.4 Fungal DNA Extraction

1. Extraction buffer: 600 μL 1% hexadecyltrimethylammonium bromide, 0.7 M NaCl, 50 mM Tris–HCl (pH 8), 10 mM EDTA, 1% 2-mercaptoethanol.
2. Chloroform/isoamyl alcohol (24:1 v/v) (see Note 2).
3. TE buffer: 10 mM Tris–HCl (pH 8), 1 mM EDTA (see Note 3).
4. Liquid nitrogen.

2.5 Agarose Gel Analyses

1. 0.5× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0, H2O up to 1 L (see Note 4).
2. Ethidium bromide solution: 0.5 μg/mL.
3. Loading buffer: 60% (v/v) glycerol, 60 mM EDTA, 0.09% (w/v) xylene cyanol, 0.09% (w/v) bromophenol blue.

2.6 PCR

1. 10× Polymerase buffer: 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3.
2. 8 μL dNTPs: 200 μM of each deoxynucleotide.
3. 1 μL DNA polymerase: Taq (see Note 5).
4. 1 μL forward and reverse primer (see Note 6).

3 Methods

3.1 Explant Preparation

1. Detach 10–15 kg date palm offshoots from adult female trees of Halawy cultivar. Remove the outer and internal leaves gradually (Fig. 1a, b; see Note 7).
2. Immerse the 6-cm-long shoot-tip tissue immediately in antioxidant solution to prevent browning (Fig. 1c).
3. Sterilize the explants in 70% ethanol for 1 min and in disinfectant solution for 20 min. Rinse the explants three times in sterile distilled water (Fig. 1d).
4. Remove more of the tip-surrounding tissue until the shoot tip is about 1 cm in length. Excise the tip and cut into four segments for culturing.

3.2 Callus Initiation

1. Culture shoot-tip explants on the callus initiation medium (Table 1).
2. Incubate cultures in the dark at 27 ± 2 °C.
3. Subculture on a fresh medium at 4-week intervals for 12 weeks until callus initiation.

3.3 Fungal Isolation

1. Collect contaminated date palm tissue cultures (Fig. 2).
2. Isolate a fragment of mycelia growth on a PDA medium supplemented with 150 mg/L chloramphenicol (see Note 8).
3. Incubate at 30 ± 2 °C for 7 days.
Fig. 1 Date palm offshoot dissecting. (a, b) Date palm shoot tip before excision. (c) Shoot tip after excision. (d) Shoot tips
4. Record the morphological characters of each individual contaminant.
5. Separate each fungal contaminant and identify the genus according to morphological and microscopic features.

3.4 DNA Extraction

1. Grow each fungal contaminant by a single spore on PCA medium supplemented with 150 mg/L chloramphenicol.
2. Incubate at 30 ± 2 °C for 7 days.
3. Collect mycelial and conidial growth, and place in a mortar and pestle.
4. Grind 10 g mycelial and conidial growth in liquid nitrogen to a fine powder.
5. Gently transfer 1–2 g ground tissue powder into a 1.5 mL Eppendorf tube.
6. Add 600 μL freshly prepared extraction buffer, and add the 2-mercaptoethanol to extraction buffer immediately prior to use.
7. Heat aliquot in water bath (60 °C) for 30 min.
8. Add equal volume of chloroform/isoamyl alcohol mixture.
9. Centrifuge for 5 min, 18,928 × g.
10. Transfer the aqueous phase into a new tube containing an equal volume of isopropanol.
12. Remove the supernatant and resuspend the DNA pellet in 100 μL TE buffer (see Note 9).
13. Quantify the DNA by either spectrophotometer or gel electrophoresis (see Notes 10 and 11).
3.5 PCR Amplification

1. Use 0.2 mL polypropylene tubes.
2. Add 4 ng of each DNA template.
3. Add 5 μL 10× polymerase buffer.
4. Add 8 μL dNTPs.
5. Add 1 μL DNA polymerase.
6. Add 1 μL of forward and reverse ITS primers each (see Note 6 for primer sequence).
7. Raise the volume to 50 μL by adding double-distilled sterilized water.
8. PCR conditions of ITS primers: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min, with 35 cycles.

3.6 DNA Gel Electrophoresis

1. Prepare 2% w/v gel by mixing 1 g agarose gel with 50 mL 0.5× TBE buffer (see Note 12).
2. Heat in a microwave for 1 min to dissolve the agarose.
3. Leave on bench for 5 min to cool slightly.
4. Add 1 μL ethidium bromide and mix well (see Note 13).
5. Pour the gel slowly into a gel tank (avoid bubbling), and insert comb.
6. Leave the cast for 30 min.
7. Use 0.5× TBE as a running buffer, and ensure to cover the gel surface with TBE buffer.
8. Preparation of DNA samples:
   (a) Transfer 20 μL of each PCR product into a new microfuge tube.
   (b) Add 2 μL 10× loading buffer and mix well.
   (c) Load the first well with 10 μL DNA ladder.
   (d) Load 20 μL of the PCR product of each sample into each well.
   (e) Close the tank; switch on the power source to 100 V.
   (f) After 30 min, stop the run when the loading dye (bromophenol blue) reaches ¾ of the gel length.
   (g) Switch off the power source; check the gel under UV light in a dark room.

3.7 DNA Sequencing

1. Check DNA quantity after PCR purification (see Note 14).
2. Use PCR primers as sequencing primers.
3. The concentration of DNA PCR product should not exceed 10 ng/μL.
4. Set PCR conditions as instructed in Subheading 3.5 for each specific primer.

5. Perform gel electrophoresis (2%) as explained in Subheading 2.4.

3.8 Analysis of DNA Sequencing Results

1. Use Chromas software to get DNA sequence of each fungal contaminant (Fig. 2) (see Note 15).

2. Use BLAST search to match fungal identities (www.ncbi.nlm.nih.gov). Use FASTA form to insert the DNA sequence (Fig. 3; see Note 16). Use BLAST search to match fungal identities (Fig. 4) (www.ncbi.nlm.nih.gov).

4 Notes

1. All solutions and buffers should be prepared using double-distilled sterile H₂O. Prepare and store all solutions and buffers at room temperature unless otherwise indicated.

2. Use freshly prepared mixture of chloroform/isoamyl alcohol.

3. Use freshly prepared mixture of TE buffer.

4. Prepare a stock of 10X TBE buffer and store at room temperature.

5. Different types of DNA polymerase are commercially available, with different proofreading and fidelity features, such as Taq, Pfu, and Pwo.
6. Select primers herein to amplify ITS of rDNA regions; the ITS1 sequence is 5'-TCCGTAGGTGAACCTGCGG-3', which hybridizes at the end of 18S rDNA, and ITS4 5'-TCCTCCGCTTATTGATATGC-3', which hybridizes at the beginning of 28S rDNA [15].

7. All of the procedures should be performed at room temperature unless otherwise specified.

8. Chloramphenicol antibiotic has excellent thermostability (5 h at 100 °C), which is suitable to add in the culture medium before autoclaving. Always check the thermostability of antibiotics before adding to the culture medium and avoid autoclaving thermolabile antibiotics.

9. Mix the TE buffer well with DNA pellets and leave it on the bench for 5 min at room temperature.
10. Several procedures for DNA quantification could be followed. Spectrophotometry is traditionally used by measuring the absorbance at 260 nm. Spectrophotometry is simple to apply with little sample-to-sample variation.

11. Gel electrophoresis could be applied as an extra step subsequent to DNA quantification to ensure DNA integrity before using in the PCR.

12. Mostly agarose concentration is used between 0.5 and 2%; for large DNA products, (5–10 kbp) use 0.5%, whereas for small-size products (0.2–1 kbp), use 2%. Our PCR product is approximately 300–650 bp.

13. Ethidium bromide is widely known as a mutagenic agent; therefore, an extreme care should be considered when handling this material. Dispose of used tips in ethidium bromide waste container.

14. A cleanliness of PCR products (purification) should be followed before DNA sequencing; the DNA products should be free from DNA polymerase, unincorporated dNTPs, salt, primers, and impurities. Many commercial kits could be used for purification of PCR products.

15. Chromas program is suitable for DNA sequencing without alignment capabilities.

16. Basic Local Alignment Sequence Tool (BLAST) (www.ncbi.nlm.nih.gov): It enables to search any selected sequence database either proteins or DNA nucleotide sequences and compare a query sequence with database library of known sequences. Herein, the nucleotide sequences (ITS) of each individual fungal contaminant genera are introduced to a BLAST search, and results revealed the identity at species level according to the database library of known sequences (fungal species, ITS fragment size, and accession number illustrated in Table 2).

<table>
<thead>
<tr>
<th>Fungal contaminant genera</th>
<th>Amplified products/ bp with ITS primers</th>
<th>Identity of fungal contaminant according to BLAST search</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria</td>
<td>305</td>
<td>A. alternata</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>531</td>
<td>A. niger</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>512</td>
<td>C. cladosporioides</td>
</tr>
<tr>
<td>Epicoccum</td>
<td>440</td>
<td>E. nigrum</td>
</tr>
<tr>
<td>Penicillium</td>
<td>560</td>
<td>P. expansum</td>
</tr>
</tbody>
</table>

Table 2
Molecular identification of fungal contaminants associated with date palm tissue culture
References

Part III

Genetic Diversity and Cultivar Identity
Chapter 10

Genetic Diversity Analysis of Date Palm Using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR)

Mahesh K. Mahatma, Vishal S. Srivashtav, and Sanjay Jha

Abstract

Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are PCR-based molecular techniques used for evaluation and characterization of date palm to find the best genotype and male/female identification at an early stage. Genetic fingerprinting using molecular markers is an important tool for the analysis of genetic diversity and cultivar identification. Here, we present an improved DNA extraction protocol using leaf tissue, based on the standard cetyltrimethyl ammonium bromide (CTAB) protocol, which yields large amounts of high-quality amplifiable DNA. RAPD and ISSR markers reveal sufficient genetic diversity as well as give some unique markers in some genotypes with a maximum number of bands.

Key words Biotechnology, CTAB, DNA isolation, Molecular marker, RAPD, ISSR

1 Introduction

DNA fingerprinting in plants is primarily used for identification of genetic diversity, protection of biodiversity or germplasm conservation, and identifying markers associated with specific traits [1]. Random amplified polymorphic DNA (RAPD), a PCR-based molecular marker, demonstrated by utilizing single short (usually ten bases) oligonucleotide primers of arbitrary sequence for the amplification of segments, distributed randomly throughout the genome. The result is the generation of amplification products (amplicons) that represent some multiple anonymous sites, which are characteristic of the studied genome [2]. The RAPD technique has been used for cultivar genotyping [3, 4], estimation of the genetic distance for studying genetic diversity [2, 5], a trait important for parent selection associated with genetic mapping, and marker-assisted selection in breeding programs [6]. However, RAPD shows a low rate of polymorphism [7]. Moreover, it is
widely criticized for the reproducibility, the structure of the primers, the dominance of markers, and the independence of loci. Inter-simple sequence repeats (ISSRs) involve amplification of DNA segments at an amplifiable distance between two identical microsatellite repeat regions oriented in the opposite direction [2]. The technique uses microsatellites as primers in a single primer PCR simple sequence repeats of different sizes. The ISSR targets multiple genomic loci and usually yields dominant markers [8]. Moreover, they are more reproducible than the RAPD technique [9]. However, in some cases ISSRs also enabled distinction between homozygotes and heterozygotes and thus are considered as codominant markers [10–13]. The ISSR technique combines most of the benefits of amplified fragment length polymorphism (AFLP) and microsatellite analysis with the universality of RAPD. However, ISSR markers have drawbacks such as sensitivity to the variation of the amount of template DNA as well as the annealing temperature, which must be carefully developed [14]. RAPD and ISSR are powerful techniques, which can be used to identify and determine plant genomes or to estimate the phylogenetic relationship among genotypes of date palm [15, 16]. This chapter describes a procedure of DNA extraction from date palm leaves and detailed protocols for RAPD and ISSR marker analysis for the determination of genetic diversity.

2 Materials

2.1 Plant Material

Fresh juvenile leaf samples of date palm genotypes comprising four females, cvs. Early maturing, Ghanshyam, late maturing, and seasonal (also known as timely maturing), and four male plants (Male-1, Male-2, Male-3, and Male-4) from the Kutch Region, India.

2.2 Isolation of Total Genomic DNA

1. DNA extraction buffer: 100 mM Tris–HCl (5 mL 1 M Tris–HCl, pH 8), 0.04 M ethylenediaminetetraacetic acid (EDTA) (4 mL 0.5 M EDTA, pH 8), 1.5 M NaCl (15 mL 1.5 M NaCl), 1% β-mercaptoethanol (500 μL β-mercaptoethanol), 2% CTAB (1 g CTAB), and 1% PVP (0.5 g PVP). Make up to 50 mL with sterile deionized water (see Note 1).
2. Chloroform/isoamyl alcohol (24:1): 96 mL chloroform and 4 mL isoamyl alcohol.
3. Ice-cold isopropanol.
4. 70% ethanol: 70 mL absolute ethanol and 30 mL H2O.
5. RNase A: 50 μg/mL.
2.3 Gel Electrophoresis

1. Stock solution (5×) TBE in 1 L H₂O: 54 g Tris base, 27.5 g boric acid, and 20 mL EDTA (0.5 M, pH 8) for running and preparation of gel. The 0.5× working solution is 45 mM Tris-borate and 1 mM EDTA.

2. Agarose gel (0.8%) in 0.5× TBE buffer: 0.8 g agarose powder in 100 mL 0.5× TBE buffer.

3. Ethidium bromide (10 mg/mL): 1 g ethidium bromide in 100 mL H₂O (see Note 2).

4. Bromophenol blue solution BPB (6×): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 30% (v/v) glycerol in H₂O.

2.4 RAPD and ISSR PCR Analysis

1. Template DNA: Plasmid DNA, genomic DNA.

2. Primers: Random decamers for RAPD and forward and reverse PCR primers (only for ISSR).

3. 25 mM MgCl₂.

4. dNTPs mixture: 2.5 mM dATP, dCTP, dGTP, and dTTP.

5. PCR buffer (10×): 500 mM KCl, 100 mM Tris–HCl (pH 8.3).


7. Agarose gel: 1.5% gel prepare in 0.5× TBE running buffer.

3 Methods

3.1 Genomic DNA Isolation Using 2% CTAB Method

1. Grind 500 mg of date palm leaves of each tested tree into a fine powder in liquid nitrogen using autoclaved prechilled mortar and pestle (see Note 3).

2. Transfer the powdered sample to a test tube and suspend in 2.5 mL preheated extraction buffer. Incubate test tubes at 60 °C for 1 h with intermittent shaking and swirling every 15 min.

3. Add equal volume of chloroform/isoamyl alcohol (24:1 v/v) to the suspension and mix the content by inversion to form an emulsion. Centrifuge the mixture at 12,000 × g for 10 min at room temperature to separate the phases.

4. Transfer the supernatant containing upper phase to a new sterile tube (avoid shearing) and repeat step 3 (see Note 4).

5. Precipitate the aqueous solution with 0.6 volume of ice-cold isopropanol in tubes and incubate at −20 °C overnight (see Note 5).

6. Centrifuge the precipitated sample at 10,000 rpm for 25 min at 4 °C (see Note 6).
7. Carefully decant the supernatant without disturbing the pellet (see Note 7), and wash the pellet twice with 70% ethanol, and then centrifuge at 10,000–15,000 × g for 5–15 min at 4 °C (see Note 8).

8. Air-dry the DNA pellet at the room temperature (see Note 9) and resuspend the DNA pellet in 1 mL TE or double-distilled water. Store samples at 4 °C until use; if DNA will not be used for a long time, store at −20 °C (see Note 10).

3.2 RAPD and ISSR PCR Programming

1. For RAPD, program the thermal cycler (Eppendorf thermocycler with 4 °C/s ramp rate) to denature for 1 min at 94 °C, and anneal for 1 min at 35 °C (see Note 11).

2. Allow primer extension for 2 min at 72 °C for the first 5 cycles. Then, program for 40 cycles of 1 min denaturation at 94 °C, 1 min of annealing at 38 °C (see Note 11), and 2 min primer extension at 72 °C.

3. Set the final extension period for 8 min at 72 °C. In ISSR annealing temperature varies according to the length of primers and sequence.

3.3 Setting Up the PCR for RAPD and ISSR Analysis and Gel Electrophoresis

1. Prepare the PCR master mix in 0.2 mL thin-walled reaction tubes containing approximately 50 ng P. dactylifera genomic DNA (see Note 12), 5 μL 10× PCR standard buffer, 200 μM dNTPs, 5 μL 25 mM MgCl2 (see Note 13), 1 μM primer, and 1.0 U Taq polymerase Genei, (see Note 14), and make up to 50 μL final reaction volume with double-distilled deionized sterile water.

2. Mix these reagents well by vortexing and then overlay with 40 μL mineral oil to prevent evaporation and internal condensation. Spin for 10 s to make interface between the aqueous and the mineral oil layer.

3. Preparation of the agarose gel: Separate the amplified products in 1.5% agarose gel because the molecular weight of the resultant amplicons is in the range of 0.25–2 kb. Dissolve agarose in the same electrophoresis running buffer, 0.5× TBE. Include 0.5 μg/mL ethidium bromide during the preparation of the gel.

4. Sample preparation for gel loading: Mix well the retrieved aqueous amplification mixture (10 μL) with the bromophenol blue loading buffer (1 μL) and load into wells in an agarose gel together with 100–500 bp DNA size marker (see Note 15).

5. Apply samples in gel loading buffer to the wells of the gel (see Note 16).

6. Connect the electrodes so that the DNA will migrate toward the anode (positive electrode) (see Note 17).
7. Turn on the power supply and run the gel at 1–10 V/cm until the dyes have migrated to the end of the gel. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required (see Notes 18–20).

8. After completion of electrophoresis, view and capture the image of amplified product of RAPD and ISSR with gel documentation system by switching on UV lamp (Fig. 1).

4 Notes

1. Prepare all solutions using ultrapure deionized water, to attain a sensitivity of 18 MΩ cm at 25 °C and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Carefully follow all waste disposal regulations when disposing of waste materials.

2. Ethidium bromide is a mutagen and must be handled carefully. Wrap the ethidium bromide container in aluminum foil or transfer to a dark bottle and store at room temperature.

Fig. 1 RAPD and ISSR fingerprinting of eight date palm genotypes with primer OPE-17 (5' CTAAGGCGG 3') for RAPD (a) and HB-10 (5' (GA)6CC 3') for ISSR (b) obtained after electrophoretic separation on 1.5% agarose gel. M is the DNA marker, lanes 1–4 are female (cvs. F1 (Ghanshyam), F2 (early maturing), F3 (late maturing), and F4 (seasonal)), and lanes 5–8 are the males from different locations of Kutch, India (M1, M2, M3 and M4). Note: M1, M2, and M4 are from Bhuj, while M3 is from Mandvi, Kutch, India.
3. Transfer the tissue powder and liquid nitrogen to 20 mL tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Proceed immediately to the DNA preparation protocol.

4. Repetition of the chloroform treatment produces cleaner DNA, but lower yield.

5. Use all solutions at room temperature to minimize coprecipitation of salt. Do not use polycarbonate tubes for precipitation as polycarbonate is not resistant to isopropanol.

6. Centrifugation should be carried out at 4 °C to prevent overheating of the sample.

7. Care should be taken when removing the supernatant as pellets from isopropanol precipitation are more loosely attached to the side of the tube. Carefully tip the tube with the pellet on the upper side to avoid dislodging the pellet.

8. Centrifuge the tube in the same orientation as previously done to recover the DNA into a compact pellet.

9. Do not overdry the pellet and resuspend in 100 μL TE buffer.

10. DNA that is frozen and thawed repeatedly will begin to break after each freezing session, so freeze DNA only for long-term storage and preferably after all testing is finished.

11. Annealing temperature: This is an important parameter that needs optimization in RAPD, and ISSR depends on primer length and sequence. The melting temperature (Tm) of a primer is proportional to its length and the G + C content. Tm for primer template can be determined using the following formula:

\[ Tm = \left[ \frac{\text{(number of A + T)}}{2} \times 2^\circ C + \frac{\text{(number of G + C)}}{2} \times 4^\circ C \right] \]

Generally, in RAPD, the first few cycles are performed at a low annealing temperature (5 °C below the calculated Tm), and subsequent cycles are performed at 38 °C, while ISSR cycles are performed with 45 °C annealing temperature for HB-10 primer (sequence 5'-3' (GA)_6 CC). Annealing temperature of ISSR primers depend on the nucleotides sequence of the primer.

12. Template: It is well known that the purity of the template is critical for producing reproducible fingerprinting patterns; thus, DNA devoid of proteins and RNA must be used at all times. For instance, impurities, such as phenol remnants in DNA, can be removed by repeated washing in 70% ethanol. Also, DNA isolation methods that lead to the degradation of DNA and inhibit the activity of DNA polymerase should be avoided.
13. Ionic composition: The concentration of ionic components is critical for RAPD and ISSR. Magnesium is important because different thermostable polymerases have different affinities for magnesium. Generally, the higher the concentration of magnesium ions, the lower the specificity, and vice versa. Our results show reproducible fingerprints with 1.5 mM magnesium for *P. dactylifera*.

14. DNA polymerase: The activity of polymerases is highly variable [13], and, therefore, subtle differences in the specificity of these enzymes can influence the fingerprint profiles and the multiplex ratio [14, 17]. The polymerase activity is regulated to a great degree by the buffer components.

15. Add color to the samples through the use of dyes such as bromophenol blue, which increases the density of the samples facilitating loading into the well. Allow tracking of the electrophoresis due to co-migration of the dyes with DNA fragments of a specific size. The bromophenol blue solution should be stored in at 4°C.

16. Prior to sample loading, rinse wells with the electrophoresis buffer. Make sure that the entire gel is submerged in the running buffer.

17. Electrophoresis apparatus should always be covered to protect against electric shocks.

18. Avoid use of very high voltages which can cause trailing and smearing of DNA bands in the gel, particularly with high-molecular-weight DNA.

19. Monitor the temperature of the buffer periodically during the run. Adjust the temperature when buffer becomes too hot by reducing the voltage.

20. Melting of an agarose gel during electrophoresis is a sign that the voltage is too high and that the buffer may have been incorrectly prepared or has become exhausted during the run.

**References**


mohan.jain@helsinki.fi
Date Palm Genetic Diversity Analysis Using Microsatellite Polymorphism

Hussam S.M. Khierallah, Saleh M. Bader, Alladin Hamwieh, and Michael Baum

Abstract

Date palm (Phoenix dactylifera L.) is considered one of the great socioeconomic resources in the Middle East and the Arab regions. The tree has been and still is at the center of the comprehensive agricultural development. The number of known date palm cultivars, distributed worldwide, is approximately 3000. The success of genetic diversity conservation or any breeding program depends on an understanding of the amount and distribution of the genetic variation already in existence in the genetic pool. Development of suitable DNA molecular markers for this tree may allow researchers to estimate genetic diversity, which will ultimately lead to the genetic conservation of date palm. Simple sequence repeats (SSRs) are DNA strands, consisting of tandemly repeated mono-, di-, tri-, tetra-, or penta-nucleotide units that are arranged throughout the genomes of most eukaryotic species. Microsatellite markers, developed from genomic libraries, belong to either the transcribed region or the non-transcribed region of the genome, and there is rarely available information on their functions. Microsatellite sequences are especially suited to distinguish closely related genotypes due to a high degree of variability making them ideally suitable in population studies and the identification of closely related cultivars. This chapter focuses on the methods employed to characterize date palm genotypes using SSR markers.

Key words Molecular marker, Molecular characterization, Simple sequence repeats (SSRs)

1 Introduction

Date palm (Phoenix dactylifera L.) \((2n = 2x = 36)\) is one of the world’s oldest cultivated fruit tree. There are about 3000 date palm cultivars worldwide. The accurate identification of cultivars for breeding purposes and to assess the accurate genetic variability within the germplasm are key pillars of any successful improvement program. In date palm, traditional breeding objectives are focused on yield enhancement, disease and pest resistance, tolerance of saline water, saline soil, and drought resistance. Accordingly, the study of genetic variability and population structure of date palm will be helpful in (a) collection of genetically diverse date
palm cultivars in newly established orchards in order to withstand various biotic and abiotic stresses, (b) selection of segregating progenies with high genetic variability, and (c) selection of genetic variability for molecular mapping [1]. Morphological traits have been used to describe such genetic variation in date palm cultivars, e.g., fruits [2], which are influenced by the environment [3, 4]. In addition, biochemical studies, including peroxidases, have been used to characterize date palms in Morocco and Tunisia [5], which do not reflect precise occurrence of polymorphisms.

DNA marker analysis in date palm is at an early stage. Microsatellite markers were used for investigation of genetic diversity in date palm [6]. Reports were encountered on utilizing microsatellite markers characterizing cultivars grown in various date-producing countries including Tunisia [7], Oman [8], Sudan [9], and Qatar [10]. Researchers have developed numerous microsatellite markers [11] and simple sequence repeat (SSR) markers [12] in date palm by mining genome sequencing data for this vital crop. We have investigated the genetic diversity in 30 date palm cultivars in Iraq representing 24 female and 6 male cultivars [13]. Genetic diversity and population structure of native and introduced date palm germplasm in the United Arab Emirates [14] and recently in date palm accessions extending from Mauritania to Pakistan using a set of SSR markers and plastid minisatellites was assessed [15].

This protocol describes the procedures of utilizing microsatellite to examine the genetic diversity and genetic relationships among date palm cultivars using SSR markers.

2 Materials

2.1 Plant Materials

Leaves (unexpanded young and white in color) from 30 well-defined reference date palm cultivars (Table 1).

2.2 Buffers and Reagents

1. Extraction buffer (2×): 2% Cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.1 M Tris–HCl pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2% β-mercaptoethanol (see Note 1).
3. Isopropanol.
4. Ethanol, 70%.
5. Washing buffer: 7.5 M ammonium acetate and 95% ethanol.
6. Tris-EDTA (TE) buffer: 10 mM Tris–HCl pH 8 and 1 mM EDTA.
7. Liquid nitrogen.
Table 1
Names, sex, and sources of collection of 30 date palm cultivars grown in Iraq

<table>
<thead>
<tr>
<th>No.</th>
<th>Source and cultivars</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Al-Mahaweel Date Palm Station</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Usta Umran</td>
<td>Female</td>
</tr>
<tr>
<td>2</td>
<td>Tabarzal</td>
<td>Female</td>
</tr>
<tr>
<td>3</td>
<td>Um Al-Dihen</td>
<td>Female</td>
</tr>
<tr>
<td>4</td>
<td>Guntar</td>
<td>Female</td>
</tr>
<tr>
<td>5</td>
<td>Kustawy</td>
<td>Female</td>
</tr>
<tr>
<td>6</td>
<td>Bream</td>
<td>Female</td>
</tr>
<tr>
<td>7</td>
<td>Ashrasi</td>
<td>Female</td>
</tr>
<tr>
<td>8</td>
<td>Maktoum Asfar</td>
<td>Female</td>
</tr>
<tr>
<td>9</td>
<td>Buliani</td>
<td>Female</td>
</tr>
<tr>
<td>10</td>
<td>Leelwi</td>
<td>Female</td>
</tr>
<tr>
<td>11</td>
<td>Shwethi Ahmer</td>
<td>Female</td>
</tr>
<tr>
<td>12</td>
<td>Jamal Al-Dean</td>
<td>Female</td>
</tr>
<tr>
<td>13</td>
<td>Qul Husaini</td>
<td>Female</td>
</tr>
<tr>
<td>14</td>
<td>Halawy</td>
<td>Female</td>
</tr>
<tr>
<td>15</td>
<td>Qitaz</td>
<td>Female</td>
</tr>
<tr>
<td>16</td>
<td>Chipchab</td>
<td>Female</td>
</tr>
<tr>
<td>17</td>
<td>Zahidi</td>
<td>Female</td>
</tr>
<tr>
<td>18</td>
<td>Shwethi Asfar</td>
<td>Female</td>
</tr>
<tr>
<td>19</td>
<td>Khadrawy</td>
<td>Female</td>
</tr>
<tr>
<td>20</td>
<td>Baw Adem</td>
<td>Female</td>
</tr>
<tr>
<td>21</td>
<td>Deari</td>
<td>Female</td>
</tr>
<tr>
<td>22</td>
<td>Barhee</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>Al-Za’afarania Date Palm Station</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Meer Haj</td>
<td>Female</td>
</tr>
<tr>
<td>24</td>
<td>Um Al-Blaliz</td>
<td>Female</td>
</tr>
<tr>
<td>25</td>
<td>Ghanami Akhder</td>
<td>Male</td>
</tr>
<tr>
<td>26</td>
<td>Ghanami Ahmer</td>
<td>Male</td>
</tr>
<tr>
<td>27</td>
<td>Khekri</td>
<td>Male</td>
</tr>
<tr>
<td>28</td>
<td>Smeasmi</td>
<td>Male</td>
</tr>
<tr>
<td>29</td>
<td>Ghulami</td>
<td>Male</td>
</tr>
<tr>
<td>30</td>
<td>Greatli</td>
<td>Male</td>
</tr>
</tbody>
</table>
2.3 Agarose Gel Electrophoresis

1. Tris-borate-EDTA (TBE) buffer (10×): 0.89 M Tris-base, 0.88 M boric acid, and 0.5 M EDTA (pH 8).
2. Loading buffer: 0.25 g bromophenol blue dye and 30 mL glycerol.
3. Undigested lambda DNA ladder: 50 ng DNA.

2.4 Microsatellite Amplification

1. A total of 33 date palm-specific primer pairs as indicated in Table 2 (see Note 2).
2. PCR mixture (20 μL): 13.9 μL H2O, 50 ng total cellular DNA (2 μL) as template, 1 μL PCR buffer, 1 μL 0.2 mM dNTP PCR mix, 0.625 U Taq DNA polymerase (0.1 μL), and 1 μL 0.2 mM of each primer using forward primer end labeled (6FAM, NED, or TET).
3. For capillary electrophoresis final analyses, 2 μL amplified DNA (diluted to 1/10) and 8 μL (Rox) DNA standard size.

2.5 Equipment

1. Instruments: Mastercycler thermocycler, pH meter, weighing balances, rotary shaker, hot plate, magnetic stirrer, autoclave, water bath shaker, high-quality water purification system, cool centrifuge, micro centrifuge, NanoDrop spectrophotometer, microwave oven, horizontal gel electrophoresis unit, UV transilluminator, gel documentation system, different volume micropipettes, vortex mixer, and ABI 3100 DNA analyzer (Applied Biosystems).
2. Tools: Pestles, mortars, aluminum foil, Eppendorf tubes, different size micropipette tips and racks.
3. Glassware: 1 L conical flasks, 500 mL beakers, and 10 mL graduated cylinders.

3 Methods

3.1 DNA Extraction

Extract total cellular DNA according to Benito et al. [16] with some modifications as follow:

1. Prepare CTAB solution by mixing 2% CTAB, 1.4 M NaCl, 0.1 M Tris–HCl pH 8, 20 mM EDTA, and 0.2% β-mercaptoethanol. The solution should be sterilized in an autoclave and stored at room temperature.
2. Prepare Tris-EDTA (TE) buffer by dissolving 0.1211 g Tris-base (10 mM) and 0.0372 g Na2EDTA (1 mM) in deionized water, adjust pH to 8, and complete volume with deionized water to 100 mL, sterilize by autoclaving and store at 4 °C.
3. Collect healthy young unexpanded leaf samples (white in leaf color) from date palm trees, and wrap the leaves in aluminum foil and in cool icebox while transferring to the laboratory.
<table>
<thead>
<tr>
<th>No.</th>
<th>Primer name</th>
<th>Motif repeat</th>
<th>Primer sequences (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mPdClR010</td>
<td>(GA)22</td>
<td>ACCCCGGAGCTGAGGTG</td>
<td>[6]</td>
</tr>
<tr>
<td>2</td>
<td>mPdClR016</td>
<td>(GA)14</td>
<td>AGCGGGGAAATGAAAAGGTAT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>mPdClR025</td>
<td>(GA)22</td>
<td>GCACAGAGGCTCTCTTTGAG</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>mPdClR032</td>
<td>(GA)19</td>
<td>CAAACTTTGCCGGTAG</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>mPdClR035</td>
<td>(GA)15</td>
<td>ACAACCGGGGATGGATTAC</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>mPdClR050</td>
<td>(GA)15</td>
<td>CTGCCATTCTCTCTGAC</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>mPdClR057</td>
<td>(GA)20</td>
<td>AAGACACGGCCCTTCCTCTTAT</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>mPdClR070</td>
<td>(GA)17</td>
<td>CAAGACCAAGGCTAAC</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>mPdClR078</td>
<td>(GA)13</td>
<td>TGGATTTCTATCTGAG</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>mPdClR085</td>
<td>(GA)29</td>
<td>GAGAGAGGGCGGATGTTATT</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>mPdClR090</td>
<td>(GA)26</td>
<td>GCAGTCAGGCTCCCTCTA</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>mPdClR093</td>
<td>(GA)17</td>
<td>CCAATTTATCATTCCTCTCTTG</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>PDCAT4</td>
<td>(CA)8(TT)(CA)</td>
<td>TAACGAGTCCACACAC</td>
<td>[11]</td>
</tr>
<tr>
<td>14</td>
<td>PDCAT5</td>
<td>(AG)16</td>
<td>GCCCCGGCTCTGGGATTAGAG</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>PDCAT6</td>
<td>(CA)14(GA)23</td>
<td>AATAGCCGGACACAGCCA</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>PDCAT11</td>
<td>(TC)7(TC)20</td>
<td>TTAGTGAAGCTCCCAAGGCTCT</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>PDCAT12</td>
<td>(TC)19</td>
<td>CATCGTTGATCTCTAAGACCTCT</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>PDCAT14</td>
<td>(TC)19(TC)16</td>
<td>TGCTGCAATCTAAGTGCAAG</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>PDCAT15</td>
<td>(GA)13-(GA)</td>
<td>ACAGAGAGGTCGTCTCTCTTCTG</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>PDCAT17</td>
<td>(GA)21</td>
<td>CAGCGGGAGGGTGGGCCTCG</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>PDCAT18</td>
<td>(CT)13G(CT)8CG</td>
<td>CTTACGTCAGAATCGAAGCA</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>PDCAT21</td>
<td>(GA)5(T)(GA)2TA</td>
<td>GTTGTATAGTGATTTT</td>
<td></td>
</tr>
</tbody>
</table>

Genetic Diversity Analysis Using Microsatellite Polymorphism
mohan.jain@helsinki.fi
4. Grind 0.1 g of leaves to a fine powder using liquid nitrogen (see Note 3).

5. Add 5 mL hot CTAB extraction buffer, mix well, and incubate for 60 min at 60 °C in a water bath. Swirl the sample tube gently every 15 min during this incubation period.

6. Cool the samples to room temperature and extract the resulting cell lyses by adding an equal volume of chloroform solution.

7. Centrifuge the cell lysate at 4000 × g and 20 °C for 15 min.

8. Transfer the aqueous phase into another tube and add 1.5 mL isopropanol.

9. Collect the precipitate by centrifugation at 10,000 × g and 20 °C for 10 min.

10. Wash the DNA pellets with 1 mL washing buffer. Repeat washing three times.

11. Dry the DNA pellets and dissolve overnight at 4 °C in 1 mL TE buffer.

3.2 Estimation of the DNA Concentration
Determine the DNA sample concentration and its purity with a NanoDrop spectrophotometer to measure the optical density (OD) at 260 and 280 nm wavelengths (see Note 4).

3.3 Microsatellite Amplification

1. Prepare the PCR mixture (20 μL) by mixing 13.9 μL H₂O, 50 ng total cellular DNA (2 μL) as template, 1 μL PCR buffer, 1 μL 0.2 mM of dNTP PCR mix, 0.1 μL (0.625 U) Taq DNA polymerase, and 1 μL 0.2 mM of each primer using forward primer end labeled (6FAM, NED, or TET).

2. Perform PCR under the following cycle conditions: A denaturation step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 1 min at 52 °C, and 1 min at 72 °C and a final extension step at 72 °C for 7 min.

3. At the testing stage, separate the amplification products by electrophoresis according to their molecular weight in 1.4% agarose gels. Prepare gel by dissolving 1.4 g agarose in 100 mL 1 × TBE buffer. Heat the agarose solution in microwave oven until boiling to completely dissolve the agarose. Put the comb in the tray of the electrophoresis unit. Cool the gel solution to 55 °C and gently pour the gel into the unit [17].

4. Prepare 10 × Tris-borate-EDTA (TBE) buffer by dissolving 108 g Tris-base (0.89 M), 55 g boric acid (0.88 M), and 40 mL 0.5 M EDTA (20 mM) (pH 8) in an appropriate amount of deionized water, adjust pH to 7.8, and complete to 1 L with deionized water. Sterilize the solution in an autoclave and store at room temperature [17].
5. Prepare loading buffer by dissolving 0.25 g bromophenol blue dye in 50 mL deionized water, add 30 mL glycerol, and complete volume to 100 mL with deionized water [18].

6. Mix 8 μL from PCR product with 2 μL loading buffer and load it in the hole of the gel and migrate at 100 volts for 2.5 h.

7. Stain the gels with ethidium bromide for the detection of DNA bands [18]. Prepare ethidium bromide dye (10 mg/mL) by dissolving 1 g ethidium bromide in 100 mL sterile deionized water, and keep the bottle in the dark [17] (see Note 5).

8. Visualize the DNA profiles on UV transilluminator and document by using a gel documentation system (see Notes 6 and 7).

9. For final analyses, load 2 μL amplified DNA (diluted to 1/10) and 8 μL (Rox) DNA standard size into 96-well plates.

### 3.4 SSR Genotyping

1. Perform allele size scoring by gene mapper software (GeneMapper® Software version 3.7, Applied Biosystems, Carlsbad, California).

2. Estimate major allele frequency, heterozygosity, gene diversity, and polymorphism information content (PIC) using a software package (PowerMarker version 1.31) [19] (Table 3) (see Note 8).

3. Draw the phylogenetic diagram by PAST software version 1.91 [20] on the basis of the Hamming similarity index with 100 bootstraps (Fig. 1, see Notes 9 and 10).

4. Conduct principal coordinate analysis (PCA) according to Euclidean similarity index using the PAST software (Fig. 2) (see Note 11).

### 4 Notes

1. Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2. Of the 33 primers used, 16 were developed by Billotte et al. [6] and 17 developed by Akkak et al. [11].

3. It is better to chill the mortar, pestle, and spatula to 4 °C when grinding samples.

4. Also use the NanoDrop spectrophotometer to estimate the DNA purity ratio according to this formula: DNA purity ratio = OD 260/OD 280. This ratio is used to detect nucleic acid contamination in protein preparations.
5. Since ethidium bromide is a powerful mutagen, wear plastic gloves and a mask during weighing and through all steps of handling.

6. According to the primer test stage, choose only primers which give good amplification and polymorphic banding pattern for further final analysis.

Table 3
Major allele frequency, number of genotypes that showed polymorphic bands, number of alleles generated, heterozygosity, gene diversity, and polymorphism information content (PIC) estimated by 22 SSR markers of 30 Iraqi date palm cultivars (see Notes 7 and 8)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Major allele frequency</th>
<th>Genotypes (no.)</th>
<th>Alleles (no.)</th>
<th>Heterozygosity</th>
<th>Gene diversity</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPdClR010</td>
<td>0.204</td>
<td>19</td>
<td>14</td>
<td>0.852</td>
<td>0.890</td>
<td>0.880</td>
</tr>
<tr>
<td>mPdClR016</td>
<td>0.500</td>
<td>8</td>
<td>5</td>
<td>0.529</td>
<td>0.654</td>
<td>0.602</td>
</tr>
<tr>
<td>mPdClR025</td>
<td>0.224</td>
<td>15</td>
<td>8</td>
<td>0.690</td>
<td>0.822</td>
<td>0.798</td>
</tr>
<tr>
<td>mPdClR032</td>
<td>0.333</td>
<td>13</td>
<td>5</td>
<td>0.815</td>
<td>0.772</td>
<td>0.736</td>
</tr>
<tr>
<td>mPdClR035</td>
<td>0.481</td>
<td>10</td>
<td>7</td>
<td>0.519</td>
<td>0.695</td>
<td>0.659</td>
</tr>
<tr>
<td>mPdClR050</td>
<td>0.370</td>
<td>17</td>
<td>12</td>
<td>0.926</td>
<td>0.813</td>
<td>0.797</td>
</tr>
<tr>
<td>mPdClR057</td>
<td>0.909</td>
<td>3</td>
<td>3</td>
<td>0.136</td>
<td>0.168</td>
<td>0.160</td>
</tr>
<tr>
<td>PdClR070</td>
<td>0.533</td>
<td>3</td>
<td>3</td>
<td>0.000</td>
<td>0.604</td>
<td>0.536</td>
</tr>
<tr>
<td>mPdClR078</td>
<td>0.320</td>
<td>16</td>
<td>15</td>
<td>0.760</td>
<td>0.832</td>
<td>0.816</td>
</tr>
<tr>
<td>mPdClR085</td>
<td>0.313</td>
<td>8</td>
<td>7</td>
<td>0.250</td>
<td>0.779</td>
<td>0.746</td>
</tr>
<tr>
<td>mPdClR090</td>
<td>0.391</td>
<td>10</td>
<td>9</td>
<td>0.261</td>
<td>0.781</td>
<td>0.757</td>
</tr>
<tr>
<td>mPdClR093</td>
<td>0.741</td>
<td>6</td>
<td>6</td>
<td>0.296</td>
<td>0.434</td>
<td>0.415</td>
</tr>
<tr>
<td>PDCAT4</td>
<td>0.407</td>
<td>13</td>
<td>7</td>
<td>0.556</td>
<td>0.738</td>
<td>0.701</td>
</tr>
<tr>
<td>PDCAT5</td>
<td>0.276</td>
<td>13</td>
<td>7</td>
<td>0.862</td>
<td>0.816</td>
<td>0.791</td>
</tr>
<tr>
<td>PDCAT6</td>
<td>0.231</td>
<td>21</td>
<td>21</td>
<td>0.654</td>
<td>0.903</td>
<td>0.897</td>
</tr>
<tr>
<td>PDCAT11</td>
<td>0.600</td>
<td>7</td>
<td>7</td>
<td>0.100</td>
<td>0.598</td>
<td>0.566</td>
</tr>
<tr>
<td>PDCAT12</td>
<td>0.850</td>
<td>5</td>
<td>4</td>
<td>0.100</td>
<td>0.269</td>
<td>0.256</td>
</tr>
<tr>
<td>PDCAT14</td>
<td>0.220</td>
<td>19</td>
<td>10</td>
<td>0.960</td>
<td>0.865</td>
<td>0.850</td>
</tr>
<tr>
<td>PDCAT15</td>
<td>0.440</td>
<td>8</td>
<td>6</td>
<td>0.480</td>
<td>0.732</td>
<td>0.699</td>
</tr>
<tr>
<td>PDCAT17</td>
<td>0.385</td>
<td>11</td>
<td>9</td>
<td>0.500</td>
<td>0.774</td>
<td>0.746</td>
</tr>
<tr>
<td>PDCAT18</td>
<td>0.140</td>
<td>19</td>
<td>19</td>
<td>0.560</td>
<td>0.923</td>
<td>0.918</td>
</tr>
<tr>
<td>PDCAT21</td>
<td>0.731</td>
<td>6</td>
<td>4</td>
<td>0.269</td>
<td>0.431</td>
<td>0.394</td>
</tr>
<tr>
<td>Mean</td>
<td>0.436</td>
<td>11.364</td>
<td>8.545</td>
<td>0.503</td>
<td>0.695</td>
<td>0.669</td>
</tr>
</tbody>
</table>

mohan.jain@helsinki.fi
7. Test 16 primer pairs of Billotte et al. [6] and 17 developed by Akkak et al. [11] to generate expected SSR banding patterns in Iraqi date palms. A total of 22 primers show polymorphic bands among the 30 cvs. (24 females and 6 males). The genetic diversity varies among cultivars ranging from 0.168 to 0.923 at loci mPdCIR057 and PDCAT 18, respectively, with an average of 0.695.

8. By using 22 primers, the results show a total of 188 alleles and ranged from 3 to 21 alleles with an average of 8.545 alleles per locus. However, the major allele frequency ranged from 0.140 to 0.909 for PDCAT18 and mPdCIR057, respectively, with an average of 0.436. High heterozygosity is shown in female cultivars with an average of 0.503.

10. The two male cvs., Ghanami Akhder and Ghanami Ahmer, are highly divergent from other cultivars and each other and are not involved in any cluster.

11. Analyze data using PCA based on 22 SSR loci representing 57% of the total variation. The results of the first two PC1 and PC2 axes exhibit the similar clusters of cultivars as in the dendrogram.
Acknowledgment

This project was supported by a grant from Tottori University and funds from Bundesministerium für Wirtschaftliche Zusammenarbeit und Entwicklung (BMZ), Germany, and the State Board of the Agriculture Research, Ministry of Agriculture, Iraq. We acknowledge the technical support of A. Sabbagh of the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

References


mohan.jain@helsinki.fi
Assessing Date Palm Genetic Diversity Using Different Molecular Markers

Mohamed A.M. Atia, Mahmoud M. Sakr, and Sami S. Adawy

Abstract

Molecular marker technologies which rely on DNA analysis provide powerful tools to assess biodiversity at different levels, i.e., among and within species. A range of different molecular marker techniques have been developed and extensively applied for detecting variability in date palm at the DNA level. Recently, the employment of gene-targeting molecular marker approaches to study biodiversity and genetic variations in many plant species has increased the attention of researchers interested in date palm to carry out phylogenetic studies using these novel marker systems. Molecular markers are good indicators of genetic distances among accessions, because DNA-based markers are neutral in the face of selection. Here we describe the employment of multidisciplinary molecular marker approaches: amplified fragment length polymorphism (AFLP), start codon targeted (SCoT) polymorphism, conserved DNA-derived polymorphism (CDDP), intron-targeted amplified polymorphism (ITAP), simple sequence repeats (SSR), and random amplified polymorphic DNA (RAPD) to assess genetic diversity in date palm.

Key words Genetic diversity, AFLP, SCoT, CDDP, ITAP, SSR, RAPD

1 Introduction

Date palm (Phoenix dactylifera L.) is one of the most successful fruit crops in arid and semi-arid tropical and subtropical habitats. The progress of any genetic preservation is dependent on understanding the amount and distribution of the genetic variation present in the genetic pool [1, 2]. Assessing the genetic diversity and population structure of date palm germplasm is important not only for developing strategies for date palm conservation but also for broadening the genetic base for date palm breeding. DNA fingerprinting in plants is primarily used for identification of genetic diversity, protection of biodiversity or germplasm conservation, and identifying markers associated with specific traits [3]. Many molecular markers are highly polymorphic, and they are not influenced by the environment or management practices [4]. Molecular markers based on amplified fragment length polymorphism (AFLP) [5, 6], random
amplified polymorphic DNA (RAPD) [1, 6, 7], and simple sequence repeats (SSR) [8] are powerful techniques which can be used to identify and determine plant genomes or to estimate the phylogenetic relationship among date palm genotypes [4, 6, 9, 10]. Recently, three novel developed gene-targeting marker approaches were introduced: start codon targeted (SCoT) polymorphism [11], conserved DNA-derived polymorphism (CDDP) [12], and intron-targeted amplified polymorphism (ITAP) [13]; these have proved powerful in estimating the phylogenetic relationship among genotypes and to identify sex-specific markers in date palm [14].

Molecular marker technologies continue to improve with development of simpler protocols with greater reliability and lower cost. This makes them increasingly practical for routine applications to tropical or subtropical species for which very limited research resources are available. It is worth mentioning that different marker systems vary in their mechanisms of detecting polymorphisms, genome coverage, and ease of application. Therefore, they could complement each other to draw conclusions that are more accurate.

The protocols presented in this chapter explain the uses of different molecular marker approaches to assess the genetic diversity among date palm genotypes belonging to different cultivars. Procedures include genomic DNA isolation, applying different PCR-based marker techniques, molecular marker scoring and data analysis, calculation of genetic similarity, and cluster analysis.

## 2 Materials

### 2.1 Plant Materials

Fresh leaf samples from date palm female trees of cvs. Bentamoda, Hayany, Samany, Siwy, and Zagloul and from male seedling progeny of these cultivars.

### 2.2 Reagents and Buffers

**of Amplified Fragment Length Polymorphism (AFLP)**

#### 2.2.1 Restriction Digestion and Ligation of Adapters

1. Highly purified DNA from male and female date palm trees (100 ng/μL) *(see Note 1)*.

2. *EcoRI*/*MseI* enzyme mix.

3. 5× reaction buffer.


5. T4 DNA ligase.

6. TE buffer.

#### 2.2.2 PCR Amplification of the Restriction Fragments

1. *EcoRI*/*MseI* preamp primer mix.

2. 10× PCR buffer plus Mg: 200 mM Tris–HCl (pH 8.4), 15 mM MgCl₂, and 500 mM KCl.

3. *EcoRI* primers (27.8 ng/μL).
4. *Mse*I primers (6.7 ng/μL, plus dNTPs).
5. *Taq* DNA polymerase (recombinant; 5 U/μL).

### 2.2.3 Denaturing Polyacrylamide Gel of the Amplified Fragments

1. Formamide loading buffer: 10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF.
2. TBE buffer (10×): 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
3. 30% acrylamide solution (see Note 2).
4. 6% urea solution.
5. Binding solution: 2 μL Bind-silane in 1 mL (0.5% acetic acid in 99% ethanol) (see Note 3).
7. 10% ammonium persulfate solution.
8. N,N,N,N'-Tetramethylethylenediamine (TEMED): Store at 4°C (see Note 4).
10. A 50 bp DNA ladder.
11. Fix/stop solution (10% glacial acetic acid): 100 mL glacial acetic acid and 900 mL ultrapure water.
12. Staining solution: 2 g silver nitrate (AgNO₃) and 3 mL 37% formaldehyde in 2 L ultrapure water.
13. Developer solution: 3% anhydrous sodium carbonate, 5.6% formaldehyde, and 0.002 mg/mL sodium thiosulfate solution, in deionized water.

### 2.3 Reagents and Buffers of Start Codon Targeted (SCoT) Polymorphism

1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. SCoT primers (10 pmol/μL).
3. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix: dATP, dCTP, dGTP, dTTP (2 mM each).
5. MgCl₂ solution (25 mM).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.
2.4 Reagents and Buffers of Conserved DNA-Derived Polymorphism (CDDP)

1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. CDDP primers (10 pmol/μL).
3. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
5. MgCl₂ solution (25 mM).
6. Taq DNA polymerase (recombinant; 5 U/μL).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.

2.5 Reagents and Buffers of Intron-Targeted Amplified Polymorphism (ITAP)

1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. ITAP forward primers (10 pmol/μL).
3. ITAP reverse primers (10 pmol/μL).
4. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
5. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
6. MgCl₂ solution (25 mM).
7. Taq DNA polymerase (recombinant; 5 U/μL).
8. Nuclease-free water.
9. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
10. A 100 bp plus DNA ladder.
11. 1 kb DNA ladder.

2.6 Reagents and Buffers of Random Amplified Polymorphic DNA (RAPD)

1. Highly purified date palm DNA samples (10 ng/μL).
2. Operon RAPD primers (10 pmol/μL).
3. 5× green PCR buffer: (20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
5. MgCl₂ solution (25 mM).
6. Taq DNA polymerase (recombinant; 5 U/μL).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.
2.7 Reagents and Buffers of Simple Sequence Repeats (SSR)

1. Highly purified DNA from date palm trees (adjust concentration to 100 ng/μL).
2. SSR forward primer (10 pmol/μL).
3. SSR reverse primer (10 pmol/μL).
4. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
5. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
6. MgCl₂ solution (25 mM).
7. Taq DNA polymerase (recombinant; 5 U/μL).
8. Nuclease-free water.
9. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
10. Agarose.
11. A 100 bp plus DNA ladder.
12. TE buffer: 10 mM Tris–HCl and 1 mM EDTA, pH 8.0.
13. 30% acrylamide solution (see Note 2).
14. 10% ammonium persulfate solution (see Note 5).
15. N,N,N,N′-Tetramethylethylenediamine (TEMED).
16. Running buffer (1× TBE).
17. 6% polyacrylamide gel (see Note 6).
18. Protein cell electrophoresis system.
19. Staining solution: 0.5 mg/mL ethidium bromide in 100 mL dH₂O.

2.8 Equipment

1. Microcentrifuge tubes.
2. Filter paper Whatman No. 1.
3. Kimwipes® tissue.
5. Centrifuge.
6. Thermal cycler.
7. Ice bath (4 °C).
8. Stainless-steel tray.
10. Electrophoresis system.
11. UV transilluminator.
12. Gel Doc™ xR+ System with Image Lab™ Software.
13. SPSS® v16 statistical software.
3 Methods

3.1 Amplified Fragment Length Polymorphism (AFLP)

3.1.1 DNA Restriction Digestion and Ligation of Adapters

1. Restriction digestion of genomic DNA: Add 400 ng date palm genomic DNA, 5 μL 5× reaction buffer, 2 μL EcoRI/MseI enzyme mix, and 15.5 μL distilled water to a 1.5 mL microcentrifuge tube.

2. Mix gently and settle the mixture at the bottom of the tube by centrifugation at 7200 × g for 10 s.

3. Incubate the mixture for 2 h at 37 °C, then incubate the mixture for 15 min at 70 °C to inactivate the restriction endonucleases, and collect pellet by centrifugation at 7200 × g for 10 s.

4. Ligation of adapters: Add 24 μL adapter ligation solution and 1 μL T4 DNA ligase to the digested DNA from the previous step. Mix gently at room temperature, centrifuge briefly to collect the reaction mixture, and incubate at 20 °C for 2 h.

5. Dilution of ligation mixture in 1:10 ratio: Take 10 μL reaction mixture and transfer to a 1.5 mL microcentrifuge tube, and then add 90 μL TE buffer and mix well (see Note 7).

3.1.2 PCR Amplification of the Restriction Fragments

1. Pre-amplification reaction: Add 2.5 μL diluted template DNA 1:10 from step 3 of Subheading 3.1.1, 20 μL EcoRI/MseI preamp primer mix, 2.5 μL 10× PCR buffer plus MgCl₂, and 0.2 μL (1 unit) of Taq DNA polymerase to a 0.2 mL thin-walled microcentrifuge tube.

2. Mix gently and centrifuge briefly to settle the mixture at the bottom of the tube. Put in the thermocycler and perform 20 cycles at 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s, then terminate the reaction by soaking in ice bath (4 °C).

3. Perform a 1:50 dilution as follows: transfer 3 μL to a 0.2 mL microcentrifuge tube containing 147 μL TE buffer. Both unused diluted and undilated reactions can be stored at −20 °C.

4. Selective amplification reaction: Add 5 μL diluted template DNA 1:50 from previous step, 0.5 μL EcoRI primer, 4.5 μL MseI primer (include dNTPs), 2 μL 10× PCR buffer plus MgCl₂, 0.2 μL (1 unit) Taq DNA polymerase, and 7.8 μL distilled water to a 0.2 mL thin-walled microcentrifuge tube.

5. Mix gently and centrifuge briefly to collect reaction.

6. Put 0.2 mL tubes in the thermocycler and perform touchdown profile as one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, lower the annealing temperature each cycle to 0.7 °C during 12 cycles (this gives a touchdown phase of 13 cycles), and then perform 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s.
3.1.3 Preparation of the Sequencing Plates and Gel Analysis

1. Short glass plate preparation: Clean the short glass plate three times with saturated Kimwipes® tissue with ultrapure water, then three times with absolute ethanol, and finally with fresh 1 mL binding solution (see Note 8).

2. Long glass plate preparation: Clean the long glass plate three times using saturated Kimwipes® tissue with ultrapure water, then three times with absolute ethanol, and finally with 1 mL SigmaCote® solution (see Note 9).

3. Allow both short and long plates 10 min to dry. Then assemble glass plates by placing 0.4 mm side spacers between the plates and side arms to hold them in place (see Note 10).

4. Preparation of 6% urea solution: To prepare 75 mL urea solution, mix the following in 250 mL graduated beaker containing 21.5 mL deionized water: 48 g urea, 7.5 mL 10× TBE buffer, 15 mL 30% acrylamide solution. Mix with magnetic stirrer for 30 min. Make up the volume to 75 mL with deionized water and filter through Whatman paper No. 1. Store at 4 °C, in a bottle wrapped with aluminum foil.

5. 10% ammonium persulfate solution: dissolve 0.1 g ammonium persulfate in 1 mL deionized water (see Note 11).

6. To prepare the gel mix, add 75 mL 6% urea solution, 100 μL TEMED, and 100 μL ammonium persulfate (NH₄)₂S₂O₈, and mix gently. Pour the solution carefully between the glass plates. Then insert the well-forming comb between the glass plates. The polymerization process will take at least 2 h.

7. Run a small aliquot (3 μL) of the selective PCR product on a 1.5% (w/v) agarose gel to check the DNA amplification.

8. Prepare the selective AFLP product for loading in denaturing gel by mixing an equal volume (2 μL) of the selective PCR product and formamide loading buffer. Denature the mix sample by incubating at 92 °C for 3 min and quickly cool on ice.

9. Pre-run the denaturing gel at 60 W to achieve a gel surface at approximately 50–55 °C, and then load 4 μL of each denatured sample into respective well.

10. Load a 50 bp DNA ladder and 100 bp DNA ladder as molecular size standards at the first and last well. On completion of loading, run the gel at 55 W (2000–3000 V) until xylene cyanol (slower dye) is about two-thirds down the length of the gel (see Note 12). Then, process the gel to the silver staining steps.

3.1.4 Silver Staining Procedure

1. Separate the plates: After electrophoresis, carefully separate the plates using a plastic wedge. The gel should be attached strongly to the short glass plate.
2. Fix the gel: Place the gel (plate) in a shallow stainless-steel tray, cover with fix/stop solution and agitate well for 20 min or until the tracking dyes are no longer visible (see Note 13).

3. Wash the gel: Rinse the gel every 2 min three times with ultrapure water (see Note 14).

4. Stain the gel: Transfer the gel to a staining solution and agitate well on orbital shaker for 30 min. Remove the gel from the staining solution and set it aside. Transfer the staining solution into a dark flask or beaker and rinse the tray using ultrapure water.

5. Wash and gel development: Dip the gel briefly into the tray containing ultrapure water for 5–10 s, and transfer the gel immediately into the tray of chilled developing solution (dissolve 30 g sodium carbonate (Na₂CO₃) in 1 L ultrapure water. Chill the ultrapure water flask in an ice bath to 10 °C. Then, immediately before use, add 1.5 mL 37% formaldehyde and a 200 μL aliquot of sodium thiosulfate (10 mg/mL) and agitate well on orbital shaker until the first bands are visible. Continue developing for an additional 2–3 min or until all bands become visible.

6. Stop development: To terminate the developing reaction and fix the gel, add 1 L fix/stop solution directly to the developing solution and incubate by shaking on orbital shaker for 2–3 min (see Note 15).

7. Remove the gel from the developer solution and rinse the gel twice for 2 min each time in ultrapure water. Dry the gel at room temperature (Fig. 1a).

3.2 Start Codon Targeted (SCoT) Polymorphism

3.2.1 SCoT PCR Reaction

1. In a sterile nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL SCoT primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the mixture by centrifugation at 7200 × g for 10 s.

3.2.2 Thermocycling Profile and Detection of the SCoT Products

1. Put the microcentrifuge tubes in the thermocycler and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and the final extension step 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gels stained with ethidium bromide in 1× TBE running buffer (dilute the 10× TBE to 1× TBE through adding 100 mL

mohan.jain@helsinki.fi
Fig. 1 Profiles of the date palm collected plants as revealed by AFLP. (a) SCoT, (b) CDDP, (c) ITAP, (d) SSR (e), and RAPD (f) marker systems. Sample order (from left to right): male Zagloul-1, male Zagloul-2, male Hayany-1, male Hayany-2, male Samany-1, male Samany-2, female Zagloul-1, female Zagloul-2, female Hayany-1, female Hayany-2, female Samany-1, female Samany-2, Bulk of males, and Bulk of females. The standard DNA marker is 100 bp ladder. Arrows show some polymorphic bands between the tested samples.
10× TBE to graduated cylinder containing 900 mL water). Load 5–10 μL of each sample into the respective wells.

3. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards in the first and last well.

4. On completion of loading, run the gel at 100 V until the dye is two-thirds down the length of the gel.

5. Visualize the SCoT PCR products under UV light and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software (Fig. 1b).

3.3 Conserved DNA-Derived Polymorphism (CDDP)

3.3.1 CDDP PCR Reaction

1. In a sterile nuclease-free microcentrifuge tube, mix on ice 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL CDDP primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the reaction mixture by brief centrifugation.

3.3.2 Thermocycling Profile and Detection of the CDDP Products

1. Put the microcentrifuge tubes in the thermocycler and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and the final extension step 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gels stain with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards in the first and last well.

3. On completion of loading, run the gel at 100 V until the dye is about two-thirds down the length of the gel.

4. Visualize the CDDP PCR products on UV light and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software (Fig. 1c).

3.4 Intron-Targeted Amplified Polymorphism (ITAP)

3.4.1 ITAP PCR Reaction

1. In a sterile, nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL ITAP forward primer, 3 μL ITAP reverse primer, 0.2 μL Taq DNA polymerase, and 6.8 μL distilled water.

2. Mix gently and collect the reaction mixture by centrifugation at 7200 × g for 10 s.
3.4.2 Thermocycling Profile and Detection of the ITAP Products

1. Put the microcentrifuge tubes in the thermocycler and program the thermocycler using the following cycling parameters: an initial denaturation step at 94 °C for 4 min, followed by five cycles of 1 min denaturing at 94 °C, 1 min annealing at 35 °C, and 1 min elongation at 72 °C. In the following 35 cycles, increase the annealing temperature by 0.4 °C, each cycle up to 50 °C. Then final extension step at 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gels stain with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards at the first and last well.

3. On completion of loading, run the gel at 100 V until the dye is about two-thirds down the length of the gel.

4. Visualize the ITAP PCR products under UV light and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software (Fig. 1d).

3.5 Random Amplified Polymorphic DNA (RAPD)

3.5.1 RAPD PCR Reaction

1. In a sterile nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL operon RAPD primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the reaction mixture by centrifugation at 7200 × g for 10 s.

3.5.2 Thermocycling Profile and Detection of the RAPD Products

1. Put the microcentrifuge tubes in the thermocycler and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, then at 36 °C for 1 min, and then at 72 °C for 90 s, with a final extension at 72 °C for 7 min.

2. Separate all PCR amplification products on 1.5% agarose gels stained with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards at the first and last well.

3. On completion of loading, run the gel at 100 V until the dye is two-thirds down the length of the gel.

4. Visualize the RAPD PCR products under UV light and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software (Fig. 1e).
3.6 Simple Sequence Repeats (SSR)

3.6.1 SSR PCR Reaction

1. In a sterile, nuclease-free microcentrifuge tubes, combine the following components on ice for each sample: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL SSR forward primer, 3 μL SSR reverse primer, 0.2 μL of Taq DNA polymerase, and 6.8 μL distilled water.

2. Mix gently and collect the reaction mixture by centrifugation at 7200 × g for 10 s.

3.6.2 Thermocycling Profile of SSRs

1. Put the microcentrifuge tubes in the thermocycler and perform touchdown profile, following an initial denaturation step at 94 °C for 4 min, followed by one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, lower the annealing temperature each cycle to 0.7 °C during 12 cycles (this gives a touchdown phase of 13 cycles), and then perform 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, with a final extension step 72 °C for 5 min (see Note 16).

2. Below are the methods used in SSR product resolving/detection, i.e., agarose gel and non-denaturing polyacrylamide gel electrophoresis (PAGE) (see Note 17).

3.6.3 Resolving of the SSRs Products on Agarose Gels

1. Separate all PCR amplification products on 2.0–2.5% agarose gel stain with ethidium bromide in 1× TBE running buffer.

2. Load 5–10 μL of each sample into a respective well. Load a 100 bp plus DNA ladder as molecular size standards at the first and last well.

3. At completion of loading, run the gel at 100 V until the dye is about two-thirds down the length of the gel.

4. Visualize the SSR PCR products on UV light, and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software (Fig. 1f).

3.6.4 Resolving of the SSRs Products on Non-denaturing Polyacrylamide Gels

1. Clean both the short and long glass plates three times using saturated Kimwipes® tissue with ultrapure water and then three times with absolute ethanol.

2. Allow both short and long plates 5–10 min to dry. Then assemble the gel sandwich (glass plates and spacers) held in place by the side arms.

3. Fix the gel sandwich into the alignment slot of the casting stand.

4. Pour the non-denaturing polyacrylamide gel solution to fill the space between the two glass plates.

5. Insert the appropriate comb immediately in a manner that does not allow air bubbles to become trapped under the teeth.

mohan.jain@helsinki.fi
6. Allow the gel to polymerize for 30–60 min at room temperature.

7. Place the plates with gels in the apparatus. One electrophoresis tank requires about 1 L of 1× TBE (see Note 18).

8. Remove the combs and flush out the wells using a syringe (see Note 19).

9. Load 10 μL of each sample (diluted 5:1 in loading dye) into the gel wells and load a 50 bp DNA ladder and 100 bp DNA ladder as molecular size standards at the first and last well.

10. Run gels at constant 250 V for 2–5 h, depending on the acrylamide concentration (see Note 20). Continue the run until the lower dye escaped from the gel.

11. Remove gels from plates and cut one or more corners of the gels, so the direction of the gel, the gel number, and sequence of samples wells can be identified after staining.

12. The gel is gently submerged in staining solution for 5–10 min at room temperature.

13. Briefly wash the gel in deionized water for 5 min.

14. Visualize the SSR PCR products on UV light and photograph the gel using a Gel Doc™ xR+ System with Image Lab™ Software.

### 3.7 Molecular Marker Scoring and Data Analysis

1. Score the clear and distinct amplification products generated from all primers visually as 1 for presence and 0 for absence bands to create a binary data set and save the scored binary data in Microsoft Excel file.

2. Calculate and count the number of monomorphic and polymorphic bands for each primer.

3. Calculate the percentage of polymorphism by dividing number of polymorphic bands by the total number of amplified bands by the same primer or primer combination.

### 3.8 Genetic Similarity and Cluster Analysis

1. Use the SPSS® V16 statistical software to perform the cluster analysis according to the following parameters:
   - Select the Dice’s coefficient to estimate the genetic similarity between studied individuals.
   - Select the unweighted pair group method of the arithmetic averages (UPGMA) (see Note 21) to generate the dendrogram (hierarchical clustering) for all different marker systems.

2. Open the SPSS® v16 statistical software, and then copy and paste the binary data from the Microsoft Excel file to the SPSS blank sheet (Fig. 2a).
Fig. 2 Steps of calculating the genetic similarity and cluster analysis using SPSS® V16 statistical software.
(a) Binary data entry, (b) selection of hierarchical cluster analysis, (c) variable selection, (d) adjusting the statistics parameters, (e) checking of proximity matrix parameter, (f) adjusting the plot parameters, (g) checking the dendrogram parameter, (h) adjusting the method parameters, (j) selection of the dice coefficient method, (k) calling of results, (l) proximity matrix table, (m) dendrogram results as phylogenetic tree.
3. Click on the drop-down menu Analysis, then select Classify, and then select Hierarchical Cluster (Fig. 2b).

4. Check the highlighted Variables check-box found under the Cluster. Then, select all variables existing in the left list, and move them under Variables through clicking on the highlighted arrow button (Fig. 2c).

5. Click on the highlighted Statistics button (Fig. 2d). Then, check the highlighted Proximity matrix check-box, and then click on the highlighted Continue button (Fig. 2e).

6. Click on the highlighted Plots button (Fig. 2f). Then, check the highlighted Dendrogram check-box, and be sure that the None check-box was checked before clicking on the highlighted Continue button (Fig. 2g).

7. Click on the highlighted Methods button (Fig. 2h). Then, select the Dice coefficient from the drop list of the highlighted Binary check-box, and then click on the highlighted Continue button (Fig. 2j).
8. Once you finish all previous steps, click on the highlighted OK button (Fig. 2h). Both the proximity matrix (Fig. 2i) and dendrogram (Fig. 2m) results will pop out immediately.

4 Notes

1. For optimal results, high-quality, intact DNA must be used as starting material for molecular marker analysis. DNA can be isolated using CTAB method or any other plant genomic DNA purification commercial kit.

2. To prepare 30% acrylamide, weigh 29.2 g acrylamide powder and 0.8 g bis-acrylamide and transfer to 250 mL graduated beaker containing about 40 mL water. Add a magnetic stirrer then leave solution to mix for about 30 min. Make up to 100 mL with water and filter twice through two layers of Whatman paper No. 1. Wear a mask when weighing acrylamide. Unpolymerized acrylamide is a neurotoxin, and care should be exercised to avoid skin contact. Cheaper grades of acrylamide and bis-acrylamide are often contaminated with metal ions. Stock solutions of acrylamide made with these grades can easily be purified by stirring overnight with approx. 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No. 1 paper. In our laboratory, we prepare the acrylamide solution fresh every week when we cast our own gels. Store at 4 °C, in a bottle wrapped with aluminum foil, and acrylamide and bis-acrylamide are slowly converted to acrylic acid and bis-acrylic acid. This deamination reaction is catalyzed by light and alkali.

3. Always freshly prepare the binding solution.

4. To reduce the pungent smell of TEMED solution, store at 4 °C.

5. To prepare ammonium persulfate solution, dissolve 0.1 g ammonium persulfate in 1 mL deionized water. It is best to prepare this fresh each time.

6. Combine the following components together in a graduated beaker: 20 mL 30% acrylamide solution, 69.3 mL water, 10 mL 10× TBE, 0.2 μL 10% APS, and 0.2 μL TEMED.

7. The unused portion of the ligation mixture may be stored at −20 °C.

8. Rubbing hard will remove too much of the Bind-silane and the gel may not adhere well.

9. Change gloves before preparing the long glass plate to prevent cross-contamination with binding solution. If the plate becomes contaminated with Bind-silane, soak it in 10% NaOH for 30–60 min.
10. Gels thinner than 0.4 mm may give weak signals.
11. We find that it is best to prepare the ammonium persulfate solution fresh each time.
12. Do not exceed settings that yield a gel surface temperature of 45–50 °C. Glass plates or gel will crack as a result of increase in gel temperature during electrophoresis.
13. The gel may be stored in fix/stop solution overnight (without shaking). Save the fix/stop solution to terminate the developing reaction (step 8).
14. Remove the gel (plate) out of the wash and allow it to drain 10–20 s before next wash.
15. The developed bands appear fairly light. Prolonged development time results in high background. It is better to stop development early than to overdevelop the gel.
16. The touchdown program may eliminate some unspecific bands compared to the standard program.
17. Resolve the PCR products of the microsatellites initially on agarose gel (2.0–2.5%). Due to that microsatellite alleles may vary in length by only few base pairs which are difficult to be detected by agarose. Therefore, use 8% non-denaturing polyacrylamide gels for exact allele sizing of the SSR loci.
18. The same stock of TBE should be used to prepare both the gel and the running buffer. To dilute the 10× TBE to 1× TBE, add 100 mL 10× TBE to graduated cylinder containing 900 mL water.
19. This is a critical step, because unpolymerized acrylamide solution polymerizes at the bottom of the wells and affects the migration of the fragments.
20. The run time for the gel depends on the acrylamide concentration, generally, 2 h for 8%, 3 h for 12%, and 5 h for 16% gels.
21. Unweighted pair group method of the arithmetic averages (UPGMA) is a simple agglomerative (bottom-up) hierarchical clustering method.

Acknowledgment

This work was carried out in Molecular Genetics and Genome Mapping (MGGM) Laboratory, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC). This work was funded by the Science and Technology Development Fund (STDF), Ministry of Scientific Research, Egypt (Projects No. 406 and 4607).
References


Chapter 13

Molecular Analysis of Date Palm Genetic Diversity Using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSRs)

Sherif F. El Sharabasy and Khaled A. Soliman

Abstract

The date palm is an ancient domesticated plant with great diversity and has been cultivated in the Middle East and North Africa for at least 5000 years. Date palm cultivars are classified based on the fruit moisture content, as dry, semidry, and soft dates. There are a number of biochemical and molecular techniques available for characterization of the date palm variation. This chapter focuses on the DNA-based markers random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) techniques, in addition to biochemical markers based on isozyme analysis. These techniques coupled with appropriate statistical tools proved useful for determining phylogenetic relationships among date palm cultivars and provide information resources for date palm gene banks.

Key words Isozymes, ISSR, Molecular identification, Polymorphism, RAPD

1 Introduction

The date palm (Phoenix dactylifera L., Arecaceae: Coryphoideae) was among the first crops domesticated in the Old World [1]. The date palm is an ancient plant with great diversity and has been cultivated in the Middle East and North Africa for at least 5000 years [2]. The fruits of the date palm are sweet berries with a sugar content of more than 50% [3] and considered an important nutritional food source for inhabitants of the Middle East where it is thought to originate [1, 2].

The genetic improvement of a crop species depends on the ability to select promising plant material; to facilitate the selection process, molecular markers that are associated with important traits can be used as selection tools. The markers can then be used to establish genetic maps, which in turn are important tools for more refined marker-assisted selection in breeding programs as well as for in-depth genetic and systematic analyses [2, 3]. Clearly, an
integrated approach is needed incorporating morphological and genetic studies to improve the knowledge of date palm taxonomy and diversity. Proteins and/or DNA attributes can be used successfully for cultivar identification, as a source of information of date palm gene banks and for studying the genetic diversity among cultivars. Date palm can be promoted best through better characterization and evaluation [4].

Protein electrophoresis profiles have been found to be valid evidence for taxonomic treatment of several plant groups [5–8]. Isozymes are successfully used for genetic differentiation and conservation purposes, where there is a basic need to assess some measures of genetic variability within and among populations [9, 10].

There are a number of molecular techniques available for characterization of the variation at the DNA level, e.g., random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs). Furthermore, they are able to reveal a virtually unlimited number of markers. Both ISSR [4, 11, 12] and RAPD [2, 13] proved useful to study genetic polymorphism among date palm cultivars.

This chapter describes biochemical and molecular genetic fingerprint for date palm cultivars using SDS-PAGE, isozymes, RAPD, and ISSR polymorphism and elucidates relationships among these species in order to achieve useful information for germplasm resources.

## 2 Materials

### 2.1 Plant Material

Leaf tissue of various date palm cultivars including Karamat, Siwy, Holow Ghanem, Agua, Saidy, Barhee, Aglani, Amri, and Deglet Noor.

### 2.2 SDS-PAGE Electrophoresis Stocks and Buffers

1. 1 M Tris (pH 8.8): Tris(hydroxymethyl)aminomethane (see Note 1).
2. 0.25 M EDTA: Ethylenediaminetetraacetic acid.
3. Sodium dodecyl sulfate (SDS 10% W/V).
4. Sample buffer solutions: 10 mL 10% SDS, 20 mL glycerol, 1.6 mL 0.25 M EDTA, 1.6 mL and deionized distilled water (dd water) up to 100 mL.
5. Resolving gel buffer: 1.5 M Tris (pH 8.8).
6. Stacking gel buffer: 0.5 M Tris (pH 6.8).
7. Acrylamide stock (30%): 30 g acrylamide, 1 g N,N-methylenedisacrylamide (see Note 2) and dd water up to 100 mL.
8. Ammonium peroxide sulfate solution (APS, 10% w/v).

mohan.jain@helsinki.fi
9. 12% v/v polyacrylamide gel: 40 mL acrylamide stock, 33.5 mL resolving gel buffer, 25 mL dd water, 1 mL 10% SDS, 750 μL 10% APS, and 60 μL TEMED.
10. Electrophoresis buffer (running buffer): 72 g glycine, 15 g Tris, 5 g SDS, and 5 L dd water.
11. Coomassie blue gel staining solution.
12. Gel destaining solution: 40 mL methanol, 10 mL glacial acetic acid, and dd water up to 100 mL.
13. TEMED: N, N', N'-tetramethylethylenediamine.
14. Polyacrylamide gel electrophoresis tank and electrophoresis power supply.

2.3 Agarose Gel Electrophoresis Stocks and Buffers

1. Tris-EDTA buffer (TE buffer): 0.121 g Tris-base (10 mM), 0.029 g Na₂-EDTA (1 mM), and dd water up to 100 mL, pH 8.
2. Tris-borate-EDTA (TBE) buffer: 5.4 g Tris-base (0.01 M), 2.75 g boric acid (0.89 M), 2 mL EDTA (0.002 mM), and dd water up to 100 mL, pH 8.
3. DNA running buffer (electrode buffer): 1× TBE (pH 8).
4. 5× sample loading dye: 2 mL EDTA (500 mM, pH 8), 5 mL glycerol, 0.75 mL bromophenol blue (2% w/v), 0.75 mL xylene cyanol (2% w/v), and 1.5 mL dd water.

2.4 Isozyme Electrophoresis Solutions

1. Extraction buffer—prepare the extraction buffer solutions for extract of the different enzymes as follows: 1 g Tris-base (pH 7.5), 5 mL glycerol, 100 μL mercaptoethanol, and dd water up to 100 mL.
2. Acrylamide stock solution (30% w/v): 29 g acrylamide and 1 g bis-acrylamide in up to 100 mL dd water.
3. Tris-borate buffer (pH 8.4): 2.12 g Tris and 0.62 g boric acid in 50 mL dd water.
4. Ammonium per sulfate solution (APS 10% w/v): 1 g ammonium persulfate in 10 mL dd water.
5. Electrophoretic buffer solution: 27.25 g Tris and 8.5 mL boric acid in 5 L dd water.
6. 100 mM phosphate buffer (pH 5.5): 61 mL of 0.2 M disodium phosphate (Na₂HPO₄) and 39 mL 0.2 M monosodium phosphate (NaH₂PO₄).
7. 50 mM Tris–HCl buffer (pH: 8.5): 0.605 g Tris in 50 mL water, adjust to pH 8.5 using HCl, and complete the solution up to 100 mL by dd water.
8. α-Naphthyl acetate.
9. β-Naphthyl acetate.
10. α-Naphthyl phosphate.
11. Fast Blue RR salt.
12. Fast Blue BB salt.
13. Malic acid.
15. Nitro blue tetrazolium (NBT).
16. Phenazine methosulfate (PMS).
17. MnCl$_2$ manganese (II) chloride.
18. MgCl$_2$ magnesium chloride.
19. Acetone.

3 Methods

3.1 Preparation of Buffers and Solutions

1. 10% sodium dodecyl sulfate (SDS): Dissolve 10 g SDS in 100 mL distilled water. Store the solution at room temperature (see Note 3).

2. Resolving gel buffer (1.5 M Tris–HCl pH 8.8): Dissolve 18.15 g Tris in 75 mL dd water and shake well with a magnetic stirrer, then add 3.5 mL concentrated HCl, adjust pH to 8.8 by HCl and complete up to 100 mL with dd water, and store at 4°C.

3. Stacking gel buffer (1 M Tris–HCl pH 6.8): Dissolve 12.11 g Tris in 75 mL H$_2$O, shake well with a magnetic stirrer and adjust pH to 6.8 by 1.5 mL with HCl, and complete up to 100 mL with dd water. Store in a dark bottle at 4°C.

4. 30% acrylamide: Dissolve 30 g acrylamide and 1 g N,N-methylenebisacrylamide in 50 mL dd water, and shake well with a magnetic stirrer, and then complete up to 100 mL with dd water.

5. Ammonium peroxide sulfate: Dissolve 0.1 g in 1 mL dd water; this solution is unstable and must be prepared fresh before use.

6. Coomassie blue: Dissolve 1 g in 455 mL methanol then mix well with a magnetic stirrer for 20 min before adding 90 mL acetic acid glacial and complete with water up to 1000 mL. This stock solution is mixed well with a magnetic stirrer then filtered through filter paper and stored at room temperature in a dark bottle.

7. Sample buffer solutions: Mix 20 mL SDS (10%), 10 mL glycerol, 6 mL 1 M Tris (pH 8.8), 800 μL 0.25 M EDTA, and water up to 100 mL; store this solution at 4°C until usage.

8. Ethidium bromide: Dissolve 0.1 g of ethidium bromide in 10 mL dd water with thorough mixing using a magnetic stirrer;
after that transfer the solution to a dark bottle and store at room temperature.

9. Tris-glycine electrophoresis buffer (run buffer): Weigh 15.1 g Tris and 94 g glycine; dissolve in 900 mL deionized water; then add 50 mL 10% (w/v) SDS and deionized water to 1000 mL. Dilute fivefold when ready to use. The final concentration would be Tris, 25 mM; glycine, 250 mM; and SDS, 0.1%. The pH of the buffer is 8.3.

3.2 SDS-PAGE
Electrophoresis

1. Perform total protein electrophoresis (SDS-PAGE) on a vertical slab (6 × 8 cm) using the electrophoresis apparatus manufactured by Bio-Rad mini gel. Samples of protein extracts are applied on 12% polyacrylamide gel.

2. Wash glass plates of electrophoresis (6 × 8 cm) with tap water and water and allow to dry. Place spacers between glass plates and then fix with clamps. Pour resolving gels quickly after preparation between the two glass plates at the two sides of the electrophoresis apparatus to a distance of 1.5 cm below the comb bottom and overlaid with isopropanol. Allow the gels to stand for at least 30 min to polymerize.

3. Pour the stacking gels similarly, after preparing, over the resolving gels, and place a 10-well comb immediately. Leave the gels about 30 min to polymerize. Remove the combs and then fill the upper and lower buffer tanks with running buffer (see Note 4).

4. Protein extraction: Grind plant tissue using mortar and pestle in liquid nitrogen to a fine powder. Weigh 0.25 g of each sample powder in 0.9 mL extraction buffer and shake thoroughly. Transfer the extracts to Eppendorf tubes and centrifuge for 10 min at 8000 × g under cooling conditions. Transfer the supernatant to new tubes.

5. Application of samples: Add a volume of 10 μL of sample buffer separately to equal volume of the protein extraction in Eppendorf tube; add 2 μL mercaptoethanol to the each tube. Boil samples for 5 min in a water bath. Add 2 μL bromophenol blue and load 10 μL of sample on the gel. Load control wells with protein standards (see Notes 5 and 6).

6. Running conditions: Fill the lower tank of the electrophoresis apparatus with running buffer, and assemble to the upper tank, so the gels are completely covered with the running buffer. Connect the electrodes to the power supply at 15 mA until the sample has entered the gel and then continue at 25 mA until the dye front reaches the bottom of the gel. After removing the staining solution, add 100 mL destaining solution and agitate gently for 1 h. Change the destaining solution several times until the gel background becomes clear (see Note 7).
3.3 Isozyme Analysis

1. Extraction of isozymes: Grind 1 g of each leaf sample with liquid nitrogen in a mortar and pestle, and add 2 mL isozyme extraction buffer. Transfer samples to Eppendorf tubes and shake for 2 h under cool conditions. Centrifuge the samples for 10 min at 10,000 \(\times g\) at 4 °C. Transfer supernatants to new tubes and store at −20 °C until use (see Notes 8 and 9).

2. Gel preparation: Prepare the gel solution by mixing 32 mL 30% acrylamide stock solution, 26 mL Tris-borate buffer, 47 mL water, 1 mL APS, and 100 μL TEMED. Pour the gel solution immediately between the two glass plates and place the comb immediately. Let the gel stand undisturbed to polymerize.

3. Application of samples: Extract the samples and mix a volume of 50 μL with 10 μL bromophenol blue, and load a volume of 60 μL from this mixture into each well.

4. Running conditions: Cover the gels completely with electrophoretic buffer; connect the apparatus to the power supply at 100 V (see Note 7).

5. Esterase gel staining: Soak the gel after electrophoresis in 100 mL of 100 mM phosphate buffer (pH 5.5) containing 50 mg α-naphthyl acetate, β-naphthyl acetate, and 100 mg Fast Blue RR salt in 2 mL acetone, and pour into the staining box (see Notes 10–14).

6. Malate dehydrogenase (E.C.1.1.1.37) gel staining: Soak the gel after electrophoresis in 100 mL 50 mM Tris–HCl (pH 8.5) containing 20 mg NAD, 300 mg malic acid, 20 mg NBT, and 4 mg PMS.

7. Acid phosphatase (E.C. 3.1.3.2) gel staining: Soak the gel after electrophoresis in 100 mL of 100 mM phosphate buffer (pH 5.5) containing 100 mg α-naphthyl phosphate, 100 mg Fast Blue BB salt, 100 mg MgCl₂, and 100 mg MnCl₂.

8. Alkaline phosphatase (E.C. 3.1.3.1) gel staining: Soak the gel after electrophoresis in 100 mL of 100 mM phosphate buffer (pH 5.5) containing 100 mg α-naphthyl phosphate, 100 mg Fast Blue BB salt, 100 mg MgCl₂, 50 mg MnCl₂, NaCl, and 1 g and 250 mg polyvinylpyrrolidone (PVP). Incubate at 37 °C in a dark room for complete staining (see Notes 15 and 16).

3.4 RAPD-PCR Analysis

1. DNA extraction: Collect young and fresh leaf samples separately from ten trees for each date palm cultivar; all the selected leaves should be normal and free from any pathogenic symptoms. Store in an icebox and quickly transport to the laboratory. Grind plant tissue using mortar and pestle in liquid nitrogen to a fine powder. Extract bulked DNA using DNeasy plant Mini Kit (QIAGEN).

2. Polymerase chain reaction (PCR)—amplification conducted in 25 μL reaction volume containing the following reagents:
2.5 μL of dNTPs (2.5 mM), 2.5 μL MgCl₂ (2.5 mM), and 2.5 μL 10x buffer, 3.0 μL primer (10 pmol), 3 μL template DNA (25 ng/μL), 1 μL Taq polymerase (1 U/μL), and 10.5 μL sterile dd water (see Note 17).

3. Use automated thermal cycle (model Techno 512) program for 1 cycle at 94 °C for 4 min followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. Store the reaction at 72 °C for 10 min.

4. Amplified products are size fractioned (using 1 Kbp ladder marker) by electrophoresis in 1.5% agarose gels in TBE buffer at 120 V for 1 h; the bands are visualized by ethidium bromide under UV florescence and photographed.

### 3.5 ISSR-PCR Analysis

1. Mix polymerase chain reaction (PCR) components (25 μL): 2.5 μL dNTPs (2.5 mM), 2.5 μL MgCl₂ (2.5 mM), and 2.5 μL of 10x buffer, 3 μL primer (10 pmol), 3 μL template DNA (25 ng/μL), 1 μL Taq polymerase (1 U/μL), and 12.5 μL sterile dd water.

2. Run the thermal cycler program: One cycle at 94 °C for 4 min followed by 45 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. Store the reaction products at 72 °C for 10 min. Separate the PCR products on a 1.5% agarose gel and estimate fragments sizes based on the 100 bp ladder marker (see Note 18) (Fig. 1).

**Fig. 1** DNA polymorphism based on ISSR-PCR analysis of the semidry date palm in Egypt M: 100 bp ladder marker. Lanes 1–9 refer to date palm cultivars: (M) ladder marker, (1) Karamat, (2) Siwy, (3) Holow Ghanem, (4) Agua, (5) Saidy, (6) Barhee, (7) Aglani, (8) Amri, and (9) Deglet Noor

mohan.jain@helsinki.fi
3. We obtain the linkage between RAPD and ISSS polymorphism per primer by computer coordinates for plotting MDPREF (Biplot) mapping with perceptual mapping (PERMAP), using polymorphism per primer as shown in Fig. 2.

4. **Notes**

1. All solutions prepared using dd water and can be stored at 4 °C for 1 month maximum except ammonium per sulfate solution must be freshly prepared before use. Some of these chemicals are toxic and/or mutagenic; lab safety roles must be followed.

2. Use high-quality acrylamide and bis-acrylamide.

3. Use fresh high-quality SDS to sharpen protein bands.

4. The stacking gel length should be 1 cm from the well bottom to the top of the separating gel for proper stacking of the protein sample.

5. Never overfill wells with sample; this could lead to artifacts.
6. Check the protein concentration before loading; mini gel wells should not contain more than 150 μg protein.

7. Cut a tiny wedge from the bottom left side of the marker lane of the gel for orientation purposes.

8. Centrifuge all samples in a microfuge tube at 10,000 × g for 5 min prior to loading (to remove any aggregates).

9. It is important that the samples are kept cold during the process in order to minimize the loss of enzyme activity.

10. The staining agent for different enzyme systems is varied, be aware that many of the substrates and stains used in this part of the electrophoretic procedure are toxic or carcinogenic; care must be taken to avoid direct contact with these chemicals.

11. The stain boxes should be labeled in advance.

12. Write a recipe card for a particular enzyme and place it near the stain box so that reagent amounts can be followed explicitly.

13. Do not pour dry chemicals or stains directly on the gel; tip the stain box and pour the chemical in the buffer, mixing well afterwards.

14. Chemicals are usually added in the order given on the card, substrates first, stains last.

15. Avoid any unnecessary exposure to light; this is accomplished by placing the box in the incubator (37 °C).

16. Put the stained gel on a light table so that a photograph can be taken when contrast is optimal.

17. Random 10-mer arbitrary primers synthesized by (Operon Biotechnologies, Inc., Germany) with the following sequences:
   - A-07: 5'-GAAACGGGTG-3',
   - A-12: 5'-TCGGCGATAG-3',
   - L-13: 5'-ACCGCCTGCT-3',
   - L-16: 5'-AGGTTGCAGG-3',
   - L-20: 5'-TGGTGGACCA-3'.

18. ISSR specific primers with the following sequences: 17899B (CA) 6GG, HB-10 (GA) 6CC, HB-11 (GT) 6CC, HB-13 (GAG) 3GC, HB-15 (GTG) 3GC.

References


mohan.jain@helsinki.fi
(Phoenix dactylifera L.) cultivars in Egypt. Egypt J Genet Cytol 38:269–284


Chapter 14

Determining Phylogenetic Relationships Among Date Palm Cultivars Using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) Markers

Nadia Haider

Abstract

Investigation of genetic variation and phylogenetic relationships among date palm (Phoenix dactylifera L.) cultivars is useful for their conservation and genetic improvement. Various molecular markers such as restriction fragment length polymorphisms (RFLPs), simple sequence repeat (SSR), representational difference analysis (RDA), and amplified fragment length polymorphism (AFLP) have been developed to molecularly characterize date palm cultivars. PCR-based markers random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are powerful tools to determine the relatedness of date palm cultivars that are difficult to distinguish morphologically. In this chapter, the principles, materials, and methods of RAPD and ISSR techniques are presented. Analysis of data generated from these two techniques and the use of these data to reveal phylogenetic relationships among date palm cultivars are also discussed.

Key words Random amplified polymorphic DNA (RAPD), Inter-simple sequence repeat (ISSR), Molecular marker, Phylogenetic relationships

1 Introduction

DNA-based markers provide useful information on genetic diversity and the relatedness of date palm (Phoenix dactylifera L.) cultivars that are difficult to distinguish morphologically. Two molecular markers, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR), have been used widely to evaluate phylogenetic relationships among date palm cultivars [1].

RAPD involves the use of single short synthetic oligonucleotide primers (generally 10 bases in length) of arbitrary sequence to amplify under low annealing temperatures [2] by PCR small portions of nanogram amounts of the target genome (DNA) that are randomly distributed throughout the genome [3]. This means that the amplified fragments generated depend on the size and length of both the primer and the target genome. The assumption
is that a given DNA sequence which is complementary to that of the primer will occur in the genome, on opposite DNA strands, and in opposite orientation, within a distance that is readily amplifiable by PCR [4]. Sets of arbitrary primers are commercially available from various sources (e.g., Operon Technologies Inc.). Haider et al. [1] provided names and sequences of 35 decamer oligonucleotide primers used for RAPD analysis of Syrian date palm cultivars. The amplified fragments of up to 3 kilo base pairs (kb or kbp), referred to as bands, due to their appearance on the gel, are usually separated on agarose gels (1.5–2.0%) and visualized by ethidium bromide (EtBr) staining [4].

Microsatellites, simple sequence repeats (SSRs) or short tandem repeats (STRs), are regions in the genome that consist of short DNA motifs (usually 2–5 nucleotides long) repeated multiple times in a row (e.g., GTGTGTGTGTGTGT) [5]. ISSR [6] is a multilocus, mostly dominant marker that exploits the abundance of SSR motifs in eukaryotic organisms [7]. ISSR uses primers (14–17 base pairs (bp), [5]) that are complementary to SSR sequences with or without short (1–3 bp) oligonucleotide anchors at the 3' or 5' end of the primer that discontinues the repeat array (e.g., CACACACACACAGT). The anchors ensure that primers target either terminus of the SSR. Products from a 5'-anchored primer include the targeted SSR sequences themselves and the region of DNA between them, whereas those from 3'-anchored primer consist primarily of the region between targeted SSRs. The PCR yields, in a single reaction, multiple DNA fragments (each of which is considered a locus) of variable length without prior knowledge of the DNA sequences of the target regions. The resulting fragments are resolved on agarose or polyacrylamide gels. The number and size of these fragments can be used as a basis for genetic variation studies.

Several studies inferred RAPD for the molecular characterization of date palms of Tunisia, Morocco, Saudi Arabia, Egypt, Algeria, and Syria [1] and efficiently estimated phylogenetic relationships among date palm cultivars. The ISSR markers have been applied to investigate the phylogenetic relationships among a set of Tunisian [8] and Saudi Arabian [9] date palm cultivars.

In this chapter, the principles, materials, and methods of RAPD and ISSR techniques are presented. Analysis of data generated from these two techniques, and the use of these data for revealing phylogenetic relationships among date palm cultivars, is also discussed.

2 Materials

2.1 Plant Material

Young leaves from 18 commercial female date palm cultivars and 5 prominent male genotypes (see Table 1).
2.2 Buffers and Reagents

1. 2× hexadecyltrimethylammonium bromide (CTAB) extraction buffer: 2% w/v CTAB, 100 mM Tris–HCl [pH = 8], 20 mM EDTA, 1.4 M NaCl, 0.1% β-mercaptoethanol (added just before use), and 1% polyvinylpyrrolidone (PVP). After autoclaving 2× CTAB extraction buffer, store at room temperature.

2. Nucleotides in dNTP mix: 0.6 mM each of dATP, dCTP, dGTP, and dTTP.

3. 5× Tris-borate-EDTA (TBE) buffer: 54 g Tris base, 27.5 g boric acid, and 100 mL of 0.5 M EDTA.

---

Table 1

Names of date palm female cultivars and male genotypes used and their origin as provided by the Ministry of Agriculture in Syria

<table>
<thead>
<tr>
<th>Number</th>
<th>Cultivar and male name</th>
<th>Geographical origin (i.e., introduced to Syria from)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Deglet Noor</td>
<td>Tunisia</td>
</tr>
<tr>
<td>2</td>
<td>Medjool</td>
<td>Morocco</td>
</tr>
<tr>
<td>3</td>
<td>Zahidi</td>
<td>Iraq</td>
</tr>
<tr>
<td>4</td>
<td>Birbin</td>
<td>Iraq</td>
</tr>
<tr>
<td>5</td>
<td>Ashrasi</td>
<td>Syria</td>
</tr>
<tr>
<td>6</td>
<td>Maktoom</td>
<td>Syria</td>
</tr>
<tr>
<td>7</td>
<td>Khastawi</td>
<td>Syria</td>
</tr>
<tr>
<td>8</td>
<td>Barhee</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>9</td>
<td>Khalas</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>10</td>
<td>Khadrawy</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>11</td>
<td>Nabtat Saif</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>12</td>
<td>Lolo</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>13</td>
<td>Gish Rabi</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>14</td>
<td>Khineze</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>15</td>
<td>Zagloul</td>
<td>Egypt</td>
</tr>
<tr>
<td>16</td>
<td>Shahabi</td>
<td>Iran</td>
</tr>
<tr>
<td>17</td>
<td>Kabkab yellow</td>
<td>Iran</td>
</tr>
<tr>
<td>18</td>
<td>Kabkab red</td>
<td>Iran</td>
</tr>
<tr>
<td>19</td>
<td>Male 1</td>
<td>Syria</td>
</tr>
<tr>
<td>20</td>
<td>Male 2</td>
<td>Syria</td>
</tr>
<tr>
<td>21</td>
<td>Male 3</td>
<td>Syria</td>
</tr>
<tr>
<td>22</td>
<td>Male 4</td>
<td>Syria</td>
</tr>
<tr>
<td>23</td>
<td>Male 5</td>
<td>Syria</td>
</tr>
</tbody>
</table>
2.3 Equipment

1. Sterile mortar and pestle.
2. Shaking water bath.
3. Centrifuge.
4. Micropipette.
5. Pipette tips.
6. Autoclave.
7. Sterile 1.5 microcentrifuge tubes.
8. Quartz cuvettes.
10. PCR machine.
11. Vortex.
12. Magnetic stirrer and magnetic bars.
13. Thin walled 0.2 mL PCR tubes.
14. Microwave oven.
15. Microwavable flask.
16. Gel casting trays and sample combs.
17. Electrophoresis chamber and power supply.
18. Transilluminator.

3 Methods

All steps to be carried out at room temperature unless otherwise indicated (see Notes 1–3).

3.1 Preparation of Plant Material

1. Wash collected leaves three times in sterile distilled water.
2. Immerse collected leaves in liquid nitrogen.
3. Keep collected leaves at $-60 \, ^\circ C$ until use.

3.2 DNA Extraction

The extraction of genomic DNA (see Note 4) is from the leaves of a date palm cultivar by using the modified CTAB method of Doyle and Doyle [10] as follows:

1. Place a piece of fresh or frozen 500 mg leaf material of each date palm cultivar in a sterile mortar.
2. Crush the leaf sample using liquid nitrogen (LN $-196 \, ^\circ C$) and a pestle (see Note 5).
3. Transfer 0.5 g of crushed leaf sample into a 2 mL Eppendorf tube.
4. Add 1 mL $2 \times$ CTAB (heated to $65 \, ^\circ C$; see Notes 6 and 7) and vortex briefly (see Note 8).
5. Keep the tube in a water bath at 65 °C for 1 h and vortex after 30 min while in the bath.
6. Place the tube on ice for 5 min.
7. Add 900 μL chloroform/isoamyl alcohol (24:1) and manually, gently shake the tube for 20 min (see Note 9).
8. Centrifuge at 10,000 × g for 10 min (see Note 10).
9. Transfer 1 mL supernatant to a new 2 mL tube.
10. Repeat steps 7 and 8.
11. Transfer the supernatant to a new 2 mL tube.
12. Add two-third volume cold (−20 °C) isopropanol, vortex briefly, and keep the tube at 4 °C for 30 min (see Note 11).
13. Centrifuge at 8000 × g for 5 min.
14. Discard the resulting supernatant and add 1 mL 75% cold ethanol (stored at −20 °C) and vortex briefly (see Note 12).
15. Centrifuge at 8000 × g for 5 min.
16. Discard the supernatant and add 1 mL 75% cold ethanol.
17. Discard the supernatant and air dry the tube contents under a flame hood for 10 min.
18. Elute DNA with 66 μL sterile double-distilled water and store the tube overnight at 4 °C.
19. Add 2 μL RNase A (10 mg/mL) and incubate the tube at 37 °C for 30 min (see Note 13).
20. Add sodium acetate (4 M; 15 μL) and gently mix for 1 min (see Note 14).
21. Add 200 μL absolute ethanol (see Note 15), and shake by inverting the tube upside down a few times.
22. Centrifuge at 8000 × g for 5 min.
23. Discard the supernatant, and add 200 μL 75% ethanol.
24. Centrifuge at 8000 × g for 5 min.
25. Repeat previous two steps.
26. Discard the supernatant and dry the pellet (a white cottony mass visible with the naked eye) for 10 min under the flame hood.
27. Elute the DNA pellet with 60–100 μL sterile double-distilled water (see Note 16).
28. Store the tube overnight at 4 °C.

### 3.3 DNA Quantification

The most common technique to determine concentration of nucleic acids in a sample is measurement of UV absorption by the nucleotides (absorbance method) (see Note 17).

1. Warm up the spectrophotometer for 15 min before reading samples (see Note 18).

mohan.jain@helsinki.fi
2. Take 500 μL sterile double-distilled water in a quartz cuvette (see Note 19) and calibrate the spectrophotometer at 260 nm as well as 280 nm.

3. Add 5 μL DNA sample to 500 μL sterile double-distilled water and mix well.

4. Note the A260 and A280 values on the spectrophotometer.

5. Note the A260/A280 ratio which is used to assess the purity of the sample as follows: (1) a ratio between 1.8 and 2.0 denotes that the absorption in the UV range is due to nucleic acid, (2) a ratio < 1.8 indicates the presence of proteins and/or other UV absorbers, and (3) a ratio > 2.0 indicates that the sample may be contaminated with chloroform (see Note 20).

6. Calculate the DNA concentration in μg/mL by multiplying A260 × 100 (dilution factor).

7. Dilute the DNA sample in double-distilled deionized water (final volume 100 μL) to a working concentration of approximately 10 ng/μL using the following equation: \( V_1 C_1 = V_2 C_2 \), where \( V_1 \) = volume of starting solution needed to make the new solution, \( C_1 \) = concentration of starting solution, \( V_2 \) = final volume of new solution, and \( C_2 \) = final concentration of new solution.

8. Prepare a pool of DNA extracted from samples of each genotype and use it as a template to represent the genotype for later amplifications.

### 3.4 RAPD Analysis

RAPD requires only 1 primer with an arbitrary sequence for amplification. Therefore, amplification in the RAPD process occurs anywhere along a genome that contains 2 complementary sequences to the primer which are within the length limits of PCR (~3 kb).

1. Add components of each PCR reaction of targeted samples in 25 μL volumes containing the following (see Note 21):

<table>
<thead>
<tr>
<th>Materials</th>
<th>μL</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>2.5</td>
<td>7.5 mM Tris–HCl, 50 mM KCl, 2 mM (NH₄)₂SO₄</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1.5</td>
<td>3 mM</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1.5</td>
<td>0.6 mM each dNTP</td>
</tr>
<tr>
<td>20 ng primer</td>
<td>4</td>
<td>80 ng</td>
</tr>
<tr>
<td>5 units/μL Taq polymerase</td>
<td>0.3</td>
<td>1.5 units</td>
</tr>
<tr>
<td>10 ng/μL genomic DNA</td>
<td>2</td>
<td>20 ng</td>
</tr>
<tr>
<td>Sterile ddH₂O</td>
<td>13.2</td>
<td></td>
</tr>
</tbody>
</table>
2. Vortex the components for approximately 20 s to mix well.
3. Place samples in a thermal cycler (also known as a thermocycler, PCR machine, or DNA amplifier) in which amplification is performed.
4. Subject PCR reactions to the following PCR program:
   (a) An initial denaturation at 94 °C for 1 min.
   (b) 45 cycles of:
      - 94 °C for 10 s for denaturation.
      - 35 °C for 10 s for annealing of primers (see Note 22).
      - 72 °C for 10 s for extension.
   (c) A final extension cycle is performed at 72 °C for 2 min.
   (d) Hold at 4 °C until the reaction tubes are removed from the machine in order to maintain product integrity.
5. Store reactions short-term in the refrigerator (4 °C) or long-term in the freezer (−20 °C) until ready to load onto agarose gel.

3.5 ISSR Analysis

ISSR is carried out on date palm cultivar DNA samples using appropriate primers (see Haider et al. [1]).

1. Mix the PCR reaction components in a 25 μL reaction volume containing the following components (see Note 21):

<table>
<thead>
<tr>
<th>Materials</th>
<th>μL</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>2.5</td>
<td>7.5 mM Tris–HCl, 50 mM KCl, 2 mM (NH4)2SO4</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>1.5</td>
<td>3 mM</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1.5</td>
<td>0.6 mM each dNTP</td>
</tr>
<tr>
<td>50 pmol/μL primer</td>
<td>4</td>
<td>200 pmol</td>
</tr>
<tr>
<td>5 units/μL Taq polymerase</td>
<td>0.3</td>
<td>1.5 units</td>
</tr>
<tr>
<td>10 ng/μL genomic DNA</td>
<td>2</td>
<td>20 ng</td>
</tr>
<tr>
<td>Sterile ddH2O</td>
<td>13.2</td>
<td></td>
</tr>
</tbody>
</table>

2. Vortex for approximately 20 s to mix.
3. Place ISSR reactions in a thermocycler.
4. Subject PCR reactions to the following PCR program:
   (a) Initial denaturation at 94 °C for 5 min.
   (b) 40 cycles of:
      - 94 °C for 10 s for denaturation.
      - 50–60 °C for 10 s for annealing of primers.
      - 72 °C for 10 s for extension.
A final extension cycle is performed at 72 °C for 7 min.

Hold at 4 °C until the reaction tubes are removed from the machine.

5. Store reactions in the refrigerator or freezer until ready to load onto agarose gel.

3.6 Gel Electrophoresis and Visualization of PCR Products

1. Size separate generated amplification products by standard horizontal electrophoresis (see Note 23) in 1.2% (for RAPD) or 1.8% (for ISSR) ethidium bromide-stained agarose gels in 0.5× TBE.

2. Use a 1 kb (for RAPD) or 100 bp (for ISSR) DNA ladder (a collection of DNA fragments of known lengths) to estimate the approximate molecular weight of bands observed in amplification products.

3. Photograph amplification profiles generated from RAPD and ISSR under UV light (see Note 24).

3.6.1 Preparation of TBE Buffer

1. To prepare 1000 mL 5× TBE buffer, weigh 54 g Tris base (C₄H₁₁NO₃; molecular weight, 121.4) and 27.5 g boric acid (H₃BO₃; molecular weight, 61.83). Transfer them to 2 L beaker/conical flask. Add 800 mL sterile double-distilled water. Add 100 mL 0.5 M EDTA solution (pH 8). Mix until all ingredients dissolve completely.

2. Adjust the solution volume to 1000 mL with sterile double-distilled water. Mix it again. Filter the solution to remove any undissolved materials [11].

3. Transfer the solution to an autoclavable bottle.

4. Sterilize the solution by autoclaving 20 min at 121–124 °C and 1.1 kg/cm² (liquid cycle). Solution can be stored at 15–25 °C (room temperature) for several months.

3.6.2 Gel Preparation

1. Weigh 1.2 g (for RAPD) or 2.5 g (for ISSR) of agarose (see Notes 25–27).

2. Pour agarose powder into microwavable flask along with 100 mL 0.5× TBE electrophoresis buffer.

3. Microwave for 1–3 min until the agarose is completely dissolved (see Note 28).

4. Let agarose solution cool to about 40 °C on the benchtop for 5 min (see Note 29).

5. Add 5 μL ethidium bromide (10 μg/μL) to the gel (final concentration 0.5 μg/mL) and swirl to mix (see Note 30).

6. Prepare plastic casting tray (mold) by wrapping masking tape around the sides, making sure to fold the tape to cover the bottom of the casting tray to prevent leakage.
7. Pour the agarose into the gel casting tray (see Note 31) with the well comb in place (see Note 32). Make sure no bubbles are caught.

8. Place newly poured gel at 4 °C for 10–15 min or let set at room temperature for 20–30 min, until it has completely solidified (see Note 33).

9. Remove the comb gently after gel hardens by pulling straight up using care not to rip the bottom of the wells. Remove tape, but leave the gel slab in the tray.

3.6.3 Loading Samples, Running an Agarose Gel, and Visualization of PCR Products

1. Prepare samples of PCR products by mixing the 25 μL-PCR product with 4 μL of the 6× loading buffer (see Note 34) in fresh Eppendorf tubes.

2. Insert the gel, still in its plastic tray, horizontally into the electrophoresis chamber and add sufficient TBE buffer to cover it to a depth of about 1 mm (see Note 35).

3. Carefully load a molecular weight ladder into the first lane of the gel (1 kb for RAPD or 100 bp for ISSR).

4. Carefully load ½ of each sample containing PCR product mixed with loading buffer into the additional wells of the gel with a micropipette (use a new tip with each sample), taking care not to cross-contaminate the wells (see Note 36).

5. Place the lid, making sure that the positive and negative poles are placed correctly (see Note 37) and power leads are connected to the apparatus.

6. Apply a current (see Note 38) to run the gel at 85 V for RAPD and ISSR (for about 2.30 h; see Note 39) until the bromothymol blue marker dye line is approximately 75–80% of the way down the gel (see Note 40).

7. Turn off power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.

8. Visualize amplification profiles by removing the gel slab from the tray and placing it on a UV transilluminator (see Note 41).

9. Record the pattern by photography or computer image capture. The gel can be photographed using UVPGDS 8000 complete gel documentation and analysis system and analyzed further.

3.7 Analyzing Agarose Gels and Construction of Dendrograms

3.7.1 Scoring RAPD and ISSR Bands

1. Use the DNA ladder in the first lane as a guide (the manufacturer’s instruction will give the size of each band) to screen amplification profiles of PCR product samples (see Note 42), and compile them into a binary data matrix (e.g., Table 2).

2. Assess only the presence or absence of a particular band (e.g., see Figs. 1 and 2) (see Note 43).
3. Numerically record only distinct, reproducible, and well-resolved bands in individual lanes as (1) present or (0) absent.

### 3.7.2 Analysis of RAPD and ISSR Data

Use the unweighted pair-group method with arithmetic mean (UPGMA) method (see Notes 44 and 45) and percent disagreement values (PDV) of the STATISTICA program [12] to construct

---

### Table 2

An illustrative example of a binary data matrix generated for four date palm samples (1–4) based on RAPD or ISSR bands scored

<table>
<thead>
<tr>
<th>Band</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

---

Fig. 1 RAPD banding profiles generated using one of RAPD primers (OP-F 03) for date palm genotypes (lanes, 1–23). Lanes 13 and 14, standard 1-kb DNA ladder [1]
the binary data matrix (Table 3) and the dendrogram (Fig. 3) based on the collective data generated from RAPD or ISSR markers. See Ng and Tan [5] for detailed description on how RAPD and ISSR bands are scored and their data are analyzed.

4 Notes

1. Wear gloves throughout the entire protocol.
2. Work in a clean location, either on fresh blue bench paper, in the hood, or on a freshly ethanol-treated bench top.
3. Be careful with pipette tips to avoid cross contamination of samples or the solutions.
4. Sometimes, DNA extraction from plants is difficult due to the existence of high levels of endogenous phenolics, polysaccharides, or other substances that may interfere with DNA isolation [13]. Moreover, date palm leaves are hard, fibrous, and difficult to grind. Haymes et al. [13] described and tested four procedures for extraction of DNA from date palm leaves, three of which were conducted using commercial kits. Most of the published DNA extraction procedures for date palm use the conventional phenol-chloroform or the CTAB extraction method.
5. Grinding the plant sample in LN breaks the cells wall and exposes the DNA within. This is referred to as cell lysis or cell disruption.
6. Prepare CTAB extraction buffer immediately before use.
7. The detergent CTAB disrupts the cell membranes, so that the DNA is released into the extraction buffer. Presence of polyphenolic compounds and polysaccharides in the leaves of date
## Table 3
Matrix of PDVs generated from combined data of RAPD and ISSR conducted on Syrian female and male genotypes [1]

<table>
<thead>
<tr>
<th>Deglet Noor</th>
<th>Medjool</th>
<th>Zahidi</th>
<th>Birbin</th>
<th>Ashrasi</th>
<th>Maktoom</th>
<th>Khastawi</th>
<th>Barhee</th>
<th>Khalas</th>
<th>Khadrawy seyf</th>
<th>Gish Lolo</th>
<th>Rabi</th>
<th>Khineze</th>
<th>Zagloul</th>
<th>Shahabi (yellow)</th>
<th>Kabkab (red)</th>
<th>Male 1</th>
<th>Male 2</th>
<th>Male 3</th>
<th>Male 4</th>
<th>Male 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deglet Noor</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medjool</td>
<td>0.22</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zahidi</td>
<td>0.26</td>
<td>0.12</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birbin</td>
<td>0.32</td>
<td>0.20</td>
<td>0.14</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ashrasi</td>
<td>0.28</td>
<td>0.20</td>
<td>0.15</td>
<td>0.11</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maktoom</td>
<td>0.25</td>
<td>0.25</td>
<td>0.27</td>
<td>0.33</td>
<td>0.30</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khastawi</td>
<td>0.24</td>
<td>0.29</td>
<td>0.29</td>
<td>0.35</td>
<td>0.33</td>
<td>0.13</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barhee</td>
<td>0.31</td>
<td>0.23</td>
<td>0.22</td>
<td>0.18</td>
<td>0.18</td>
<td>0.33</td>
<td>0.32</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khalas</td>
<td>0.28</td>
<td>0.17</td>
<td>0.16</td>
<td>0.18</td>
<td>0.19</td>
<td>0.29</td>
<td>0.30</td>
<td>0.23</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khadrawy</td>
<td>0.32</td>
<td>0.21</td>
<td>0.19</td>
<td>0.18</td>
<td>0.19</td>
<td>0.37</td>
<td>0.37</td>
<td>0.19</td>
<td>0.12</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nabetat Saif</td>
<td>0.31</td>
<td>0.21</td>
<td>0.18</td>
<td>0.13</td>
<td>0.14</td>
<td>0.33</td>
<td>0.36</td>
<td>0.20</td>
<td>0.14</td>
<td>0.17</td>
<td>0.08</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lolo</td>
<td>0.32</td>
<td>0.23</td>
<td>0.20</td>
<td>0.12</td>
<td>0.13</td>
<td>0.32</td>
<td>0.37</td>
<td>0.20</td>
<td>0.18</td>
<td>0.17</td>
<td>0.08</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gish Rabi</td>
<td>0.28</td>
<td>0.20</td>
<td>0.18</td>
<td>0.18</td>
<td>0.17</td>
<td>0.32</td>
<td>0.34</td>
<td>0.22</td>
<td>0.14</td>
<td>0.18</td>
<td>0.16</td>
<td>0.15</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khineze</td>
<td>0.28</td>
<td>0.22</td>
<td>0.21</td>
<td>0.17</td>
<td>0.18</td>
<td>0.32</td>
<td>0.33</td>
<td>0.17</td>
<td>0.17</td>
<td>0.16</td>
<td>0.16</td>
<td>0.15</td>
<td>0.17</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zagloul</td>
<td>0.30</td>
<td>0.21</td>
<td>0.20</td>
<td>0.18</td>
<td>0.16</td>
<td>0.34</td>
<td>0.36</td>
<td>0.16</td>
<td>0.20</td>
<td>0.20</td>
<td>0.19</td>
<td>0.20</td>
<td>0.19</td>
<td>0.17</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shahabi 0.31</td>
<td>0.22</td>
<td>0.20</td>
<td>0.16</td>
<td>0.15</td>
<td>0.36</td>
<td>0.36</td>
<td>0.16</td>
<td>0.19</td>
<td>0.15</td>
<td>0.14</td>
<td>0.16</td>
<td>0.19</td>
<td>0.15</td>
<td>0.13</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kabkab</td>
<td>0.30</td>
<td>0.19</td>
<td>0.17</td>
<td>0.16</td>
<td>0.16</td>
<td>0.31</td>
<td>0.34</td>
<td>0.18</td>
<td>0.16</td>
<td>0.17</td>
<td>0.16</td>
<td>0.17</td>
<td>0.15</td>
<td>0.16</td>
<td>0.13</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(yellow)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kabkab</td>
<td>0.28</td>
<td>0.18</td>
<td>0.16</td>
<td>0.13</td>
<td>0.15</td>
<td>0.31</td>
<td>0.34</td>
<td>0.17</td>
<td>0.15</td>
<td>0.17</td>
<td>0.16</td>
<td>0.16</td>
<td>0.15</td>
<td>0.15</td>
<td>0.10</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(red)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 1</td>
<td>0.28</td>
<td>0.20</td>
<td>0.17</td>
<td>0.14</td>
<td>0.14</td>
<td>0.31</td>
<td>0.35</td>
<td>0.20</td>
<td>0.19</td>
<td>0.19</td>
<td>0.15</td>
<td>0.15</td>
<td>0.17</td>
<td>0.19</td>
<td>0.18</td>
<td>0.15</td>
<td>0.15</td>
<td>0.14</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Male 2</td>
<td>0.25</td>
<td>0.30</td>
<td>0.30</td>
<td>0.35</td>
<td>0.33</td>
<td>0.23</td>
<td>0.22</td>
<td>0.34</td>
<td>0.31</td>
<td>0.37</td>
<td>0.36</td>
<td>0.30</td>
<td>0.27</td>
<td>0.34</td>
<td>0.34</td>
<td>0.31</td>
<td>0.31</td>
<td>0.33</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Male 3</td>
<td>0.26</td>
<td>0.21</td>
<td>0.21</td>
<td>0.22</td>
<td>0.22</td>
<td>0.29</td>
<td>0.29</td>
<td>0.24</td>
<td>0.18</td>
<td>0.23</td>
<td>0.23</td>
<td>0.22</td>
<td>0.16</td>
<td>0.22</td>
<td>0.23</td>
<td>0.23</td>
<td>0.22</td>
<td>0.19</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>Male 4</td>
<td>0.22</td>
<td>0.18</td>
<td>0.18</td>
<td>0.20</td>
<td>0.22</td>
<td>0.24</td>
<td>0.27</td>
<td>0.23</td>
<td>0.18</td>
<td>0.21</td>
<td>0.21</td>
<td>0.22</td>
<td>0.19</td>
<td>0.21</td>
<td>0.21</td>
<td>0.20</td>
<td>0.19</td>
<td>0.18</td>
<td>0.19</td>
<td>0.26</td>
</tr>
<tr>
<td>Male 5</td>
<td>0.25</td>
<td>0.21</td>
<td>0.20</td>
<td>0.18</td>
<td>0.21</td>
<td>0.32</td>
<td>0.31</td>
<td>0.21</td>
<td>0.22</td>
<td>0.21</td>
<td>0.19</td>
<td>0.20</td>
<td>0.21</td>
<td>0.20</td>
<td>0.18</td>
<td>0.18</td>
<td>0.19</td>
<td>0.19</td>
<td>0.19</td>
<td>0.28</td>
</tr>
<tr>
<td>Average</td>
<td>0.28</td>
<td>0.21</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.30</td>
<td>0.31</td>
<td>0.22</td>
<td>0.20</td>
<td>0.21</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.21</td>
<td>0.20</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.21</td>
<td>0.20</td>
<td>0.19</td>
<td>0.19</td>
<td>0.30</td>
<td>0.32</td>
<td>0.22</td>
<td>0.19</td>
<td>0.21</td>
<td>0.19</td>
<td>0.20</td>
<td>0.19</td>
<td>0.20</td>
<td>0.21</td>
<td>0.20</td>
<td>0.19</td>
<td>0.18</td>
<td>0.19</td>
<td>0.31</td>
</tr>
</tbody>
</table>
palm may interfere with the successful extraction of PCR amplifiable DNA [14]. Inclusion of NaCl and PVP with the extraction buffer is used to remove polysaccharides and polyphenolic compounds, respectively.

8. To avoid nucleolytic degradation of the DNA, minimize the time between thawing of frozen tissue and its exposure to the extraction buffer.

9. DNA is insoluble in alcohol. Therefore, adding chloroform/isoamyl alcohol (CIA) makes the DNA float up, while all other cell components precipitate at the bottom of the test tube. CIA also helps to isolate the CTAB solution so it is easy to obtain the DNA (http://www.answers.com/Q/What_is_the_function_of_Chloro-Isoamyl_Alcohol_in_DNA_extraction).

10. Perform all centrifugations with the cap of the tube pointing up.

11. When isopropanol is added, DNA aggregates together, giving a pellet upon centrifugation because DNA is insoluble in isopropanol.

12. Ethanol is used to wash the DNA after precipitation.

13. RNase A is an important enzyme for the removal of RNA for RNA-free DNA.

**Fig. 3** Dendrogram of date palm cultivars grown in Syria based on combined data of RAPD and ISSR [1]
14. The function of adding the acetate solution (either sodium acetate or ammonium acetate) is to raise the salt concentration and therefore cause the nucleic acid to precipitate out of solution.

15. The ethanol wash is primarily to remove excess salts, which are soluble in 75% ethanol, but DNA is not.

16. The DNA pellet is dissolved in a slightly alkaline buffer, usually in the Tris-EDTA (TE) buffer or in ultrapure water. TE buffer can be used to elute DNA when you want to store it because its pH (near 8) stabilizes the DNA for a longer time. In addition, DNA is more stable and less subject to degradation in TE than in water. To store extracts in sterile ddH2O water, they should be at −20 °C rather than −4 °C. Avoid excessive freeze-thaw of the DNA samples. Water tends to be acidic even as low as pH 4–5. DNA will undergo acid hydrolysis over time and become degraded in water (http://forums.biotechniques.com/viewtopic.php?f=2&t=4143).

17. In the absorbance method, the more concentrated the DNA solution, the more UV light it will absorb. This method is simple, is accurate, and only requires a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument), and a solution of purified DNA. In this method, DNA is quantified in solution by measuring the absorbance of UV light at 260 nm (where DNA absorbs light most strongly) in a spectrophotometer. The purity of isolated DNA is also determined spectrophotometrically. DNA absorbs UV light at 260 and 280 nm, and aromatic proteins absorb UV light at 280 nm. The most common purity calculation is the ratio of the absorbance at 260 nm divided by the reading at 280 nm. However, this method is limited by the quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too small. In the estimation of total genomic DNA, for example, the presence of RNA and sheared DNA can interfere with the accurate estimation of total high molecular weight genomic DNA [15].

18. Be sure that there is no cuvette in the well as the spectrophotometer goes through its self-diagnosis.

19. Quartz cuvettes are very expensive so be careful with them. The blank is used as a standard for calibrations of the spectrophotometer at 260 and 280 nm. Since there is no DNA in this cuvette, the reading should be close to 0. If not, set the spectrophotometer to 0. The machine is then calibrated. TE buffer should be used for DNA quantification instead of water if the DNA was eluted in TE buffer.
20. If the ratio is $<$1.8 or $>$2.0, it is advisable to re-precipitate the DNA [15].

21. To reduce the possibility of cross contamination in RAPD and ISSR amplification reactions, (1) prepare a master reaction mix (a premixed, ready-to-use solution containing Taq DNA polymerase, dNTPs, MgCl$_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates) and use a negative control which consists of the reaction mixture excluding any DNA and (2) repeat RAPD and ISSR analyses twice for all samples and score only clear bands produced in both replicates.

22. There is no exact annealing temperature of a PCR reaction. The annealing temperature chosen for PCR relies directly on length and composition of the primers. The annealing temperature is normally defined as 5–10 $^\circ$C below the melting temperature (Tm) of the primer used. Employ the following equation to calculate the optimal melting temperature.

\[
\text{Melting temperature} = 4(G + C) + 2(A + T),
\]

where G + C is the number of G or C nucleotides in the primer and A + T is the number of A or T nucleotides. There are two other options. First, use the annealing temperature of the primers as noted on their vials. Second, run a temperature gradient PCR to find the best annealing temperature.

23. Gel electrophoresis is the standard lab procedure for separating DNA by size (i.e., length in base pairs) for visualization. Electrophoresis uses an electrical field to move the negatively charged DNA toward a positive electrode through an agarose gel matrix. The gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. Thus, the length of a DNA fragment can be accurately determined by running it on an agarose gel alongside a DNA ladder [16].

24. Problems that can be encountered in PCR amplification and suggested solutions:

(a) Erratic or no amplification: (1) a component is missing from the reaction; repeat the reaction, (2) inhibitors of PCR may have cp-purified with the DNA; vary DNA concentration, include a cleanup set during DNA extraction, (3) make new primer stock, (4) use a different primer, (5) increase concentration of Taq polymerase per reaction [17].

(b) Fuzzy/indistinct amplification: (1) replace Taq polymerase buffer, (2) check the primer; ensure primer is properly stored, (3) use another primer; all primers may not give amplification products, (4) check activity of Taq polymerase by comparing between different batches, (5) vary DNA concentration [17].
(c) A single, monomorphic intense band that may be observed in all samples and in the control may be a primer artifact; decrease primer concentration [17].

(d) High molecular weight smears >4 kb: (1) decrease DNA concentration, (2) decrease Taq polymerase concentration, (3) ensure gels are made with the correct buffer, (4) conduct electrophoresis at lower voltage [17].

(e) Gel has strong background after staining: (1) decrease staining time, (2) destain gel for longer, (3) too much Taq or DNA in the PCR reaction [17].

(f) Inadequate separation of low molecular weight products, separate products on higher concentrations of agarose gels, specialist agaroses or polyacrylamide gels [17].

25. Avoid making a large gel if there is only a small sample lot to run or if there is no need to run them that far, because agarose is expensive (http://labs.mcdb.lsa.umich.edu/labs/maddock/protocols/DNA/agarose_gels.ht).

26. The percentage measurement is a weight/volume. Simply add the required amount of agarose to 100 mL TBE buffer; for example, 1.2 g/100 mL equals 1.2%.

27. The percentage of the gel prepared for visualizing RAPD or ISSR profiles depends on how clear the separation of fragments needs to be.

28. While microwaving agarose gel, do not stir because eruptive boiling can occur. Microwave for 30–45 s, stop and swirl, and then continue toward a boil. The initial boil has a tendency to boil over so pay close attention to it [16].

29. Leave the gel on the bench top for a few min to cool, or run water over the surface of the flask for quicker cooling.

30. EtBr is a chemical that intercalates DNA and makes it visible under UV light after electrophoresis. If EtBr is not added to the gel and buffer, place the gel into a container filled with 100 mL of TBE running buffer and 5 μL of EtBr, place on a rocker for 20–30 min, replace EtBr solution with water, and destain for 5 min. EtBr is a known mutagen: wear gloves, eye protection, and a lab coat when working with this chemical. Do not get any of it on your fingers, on yourself, or around the lab [16].

31. Immediately before pouring the gel into the tray, swirl the flask to make sure it is mostly all at the same temperature; otherwise the gel will harden in a weird manner and will be ruined. Pour slowly to avoid bubbles which will disrupt the gel. A pipette tip can be used to push away any bubbles from the well comb or toward the sides/edges of the gel. After pouring the gel, rinse out the flask well with water before putting it in the sink,
because hardened agarose on glassware is very difficult to clean ([16], http://labs.mcdb.lsa.umich.edu/labs/maddock/protocols/DNA/agarose_gels.ht).

32. Determination of the size of the comb depends on the desired width of the wells and on the number of samples to be loaded into the gel.

33. To set the gel more quickly, cool the gel tray to 4 °C in advance to make it easier to pour the gel into it [16].

34. Loading buffer contains something dense (e.g., glycerol) to allow the sample to fall into the sample wells and one or two tracking dyes, which migrate in the gel and allow visual monitoring on how far the electrophoresis has proceeded (http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.ht). Because the loading buffer contains a high percentage of glycerol, adding it to the sample makes the latter heavier than water so the sample settles to the bottom of the gel well instead of diffusing in the buffer. Loading buffer also provides a visible dye that helps with gel loading and allows gauging how far the gel has run [16].

35. Use TBE for long runs and repeated electrophoresis because it is a better conductive medium than TAE (Tris-acetate EDTA) (https://www.researchgate.net/post/What_is_difference_between_TAE_and_TBEBuffers_and_their_properties_regarding_use_in_agarose_gel_electrophoresis). For small DNA fragments (< 1 kb), TBE is recommended as the image resolution is better. TAE has a lower buffering capacity compared to TBE [18]. Avoid using buffers more than three times. Over time water evaporates increasing the concentration of the buffer components [18].

36. To prevent bubbles or buffer from entering the tip when loading the sample in the well, place the very top of the tip of the pipette into the buffer just above the well. Push the sample out very slowly and steadily and watch as the sample fills the well [16]. One-half of the sample containing PCR product mixed with loading buffer is loaded for visualization of amplification products, while the other half is loaded for final imaging of the gel.

37. Red is positive and black is negative. Wells are closest to the negative pole. The DNA is negatively charged and will run toward the positive electrode.

38. Observing bubbles coming off the electrodes confirms that current is flowing. DNA will migrate toward the positive electrode, which is usually colored red (http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.ht).

39. The run-time depends on the gel concentration and voltage.
40. Fragments of linear DNA migrate through agarose gels with a mobility which is inversely proportional to the log$_{10}$ of their molecular weight (http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.ht).

41. When using UV light, wear a face shield or safety goggles, a lab coat, and gloves for skin protection.

42. DNA polymorphism among date palm cultivar samples can be due to (a) mismatches at the primer site, (b) appearance of a new primer site, and (c) length of the amplified region between primer sites [5].

43. Only the presence or absence of a particular band can be assessed because of the nature of RAPD and ISSR markers. Criteria for selecting scoring bands are (a) reproducibility, (b) thickness, and (c) size. Fragments with the same mobility are considered as identical, irrespective of fragment intensity.

44. UPGMA is a simple agglomerative (bottom-up) hierarchical clustering method. It is one of the most popular methods in ecology for the classification of sampling units (such as vegetation plots) on the basis of their pairwise similarities in relevant descriptor variables (such as species composition). In a phylogenetic context, UPGMA assumes a constant rate of evolution (molecular clock hypothesis) and is not a well-regarded method for inferring relationships unless this assumption has been tested and justified for the data set being used. UPGMA was initially designed for use in protein electrophoresis studies, but is currently most often used to produce guide trees for more sophisticated phylogenetic reconstruction algorithms. The UPGMA algorithm constructs a rooted tree (dendrogram) that reflects the structure present in a pairwise dissimilarity matrix (or a similarity matrix).

45. Clustering methods such as the UPGMA use a sequential clustering algorithm. A dendrogram is built in a stepwise manner, by grouping sequences or groups of sequences—usually referred to as operational taxonomic units (OTUs)—that are most similar to each other, that is, for which the genetic distance is the smallest. When 2 OTUs are grouped, they are treated as a new single OUT. From the new group of OTUs, the pair for which the similarity is highest is again identified, and so on, until only 2 OTUs remain [19].

Acknowledgments

Many thanks to the Director General of AEC of Syria and Head of Department for their support.
References

Genotyping and Molecular Identification of Date Palm Cultivars Using Inter-Simple Sequence Repeat (ISSR) Markers

Basim M. Ayesh

Abstract

Molecular markers are credible for the discrimination of genotypes and estimation of the extent of genetic diversity and relatedness in a set of genotypes. Inter-simple sequence repeat (ISSR) markers rapidly reveal high polymorphic fingerprints and have been used frequently to determine the genetic diversity among date palm cultivars. This chapter describes the application of ISSR markers for genotyping of date palm cultivars. The application involves extraction of genomic DNA from the target cultivars with reliable quality and quantity. Subsequently the extracted DNA serves as a template for amplification of genomic regions flanked by inverted simple sequence repeats using a single primer. The similarity of each pair of samples is measured by calculating the number of mono- and polymorphic bands revealed by gel electrophoresis. Matrices constructed for similarity and genetic distance are used to build a phylogenetic tree and cluster analysis, to determine the molecular relatedness of cultivars. The protocol describes 3 out of 9 tested primers consistently amplified 31 loci in 6 date palm cultivars, with 28 polymorphic loci.

Key words  Fingerprinting, Genetic distance, Genotyping, ISSR, Molecular markers, Polymorphism

1 Introduction

More than 3000 date cultivars have been developed by thousands of years of seedling selection worldwide [1]. The date elite cultivars with ideal traits are faced with difficulties in propagation, improvement of the existing cultivars, or selection of new ones with superior features. Date palm gender cannot be reliably distinguished morphologically before the reproductive age (5–10 years). Therefore, there is a need to apply precise techniques for the discrimination of different date palm cultivars.

DNA-based molecular markers have been employed to provide reliable useful information on the genetic diversity, relationship between cultivars, DNA fingerprinting, and pedigree analysis [2–4]. The most common molecular markers used are polymerase chain reaction (PCR)-based multilocus-dominant markers.
including random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) [5–12]. In general, PCR-based genotyping assays require target DNA sequence information for primer design. ISSR amplifies the sequences flanked by two inversely oriented microsatellite repeats using a single primer specific to the repeated sequence [12–14]. Microsatellite regions are abundant throughout the eukaryotic genome, highly polymorphic in length, and interspersed with sequences having polymorphic lengths [15–17]. ISSR genotyping provides information on many loci and is suitable for the identification and discrimination between closely related variants [7, 9, 14].

This protocol describes genotyping procedures of six date palm cultivars using ISSR primers with standardized genomic DNA extraction and PCR amplification details.

2 Materials

2.1 Plant Material

Leaf tissue obtained from six date palm cultivars (Ameri, Halawy, Zahidi, Barhee, Bentaisha, and Hayany) grown in the Gaza Strip, Palestine, and authenticated by fruit production upon consultation with the Palestinian Agricultural Relief Committees (PARC).

2.2 Extraction of Plant Genomic DNA

1. Tris–Cl buffer (pH 8): 1 M aqueous solution (see Notes 1 and 2).
2. EDTA buffer (pH 8): 0.5 M aqueous solution (see Note 3).
3. Extraction buffer: 100 mM Tris–Cl (pH 8), 50 mM EDTA (pH 8), 500 mM NaCl, and 10 mM β-mercaptoethanol (see Note 4).
4. Sodium dodecyl sulfate (SDS) solution: 20% (w/v) aqueous solution (see Note 5).
5. Ammonium acetate (5 M): 385.4 g/L ammonium acetate.
6. Resuspension buffer: 50 mM Tris–Cl (pH 8) and 10 mM EDTA (pH 8) (see Note 6).
7. RNase A solution: 100 mg/mL aqueous solution (see Note 7).
8. TE buffer: 10 mM Tris–Cl (pH 8) and 1 mM EDTA (pH 8) (see Note 8).
9. Sodium acetate (3 M): 40.8 g/L sodium acetate trihydrate.
10. Isopropanol: Prechilled at –20 °C.

2.3 PCR Amplification of ISSR

1. Primers: 10 μM working aqueous solution. The sequences of 3 ISSR primers are listed in Table 1 (see Notes 9 and 10).
2. GoTaq Green Master Mix 2× (see Note 11).
2.4 Agarose Gel Electrophoresis

1. 1× TAE electrophoresis buffer: Tris/acetate/EDTA buffer (see Note 12).
2. Agarose.
3. Ethidium bromide solution: 10 mg/mL aqueous solution (see Note 13).
4. 100 bp DNA ladder.

3 Methods

3.1 Plant Tissue Preparation

1. Weigh 100 mg freshly collected young date palm leaves and place into a prechilled mortar and pestle (see Note 14).
2. Rapidly freeze the plant material in a proper amount of liquid nitrogen and grind to a powder as the liquid nitrogen boils off. More liquid nitrogen may be added as needed to keep the powder frozen while grinding (see Note 15).
3. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and leave it open to allow the liquid nitrogen to evaporate without thawing the ground tissue.
4. Immediately store the tissue powder at −70 °C or pass for DNA preparation.

3.2 DNA Extraction

1. Extract plant DNA from 100 mg ground young date palm leaves using the manual protocol described here [5] (see Note 16).
2. Lyse the ground plant tissue with 1.5 mL extraction buffer, while keeping the tubes on ice.
3. Add 100 μL 20% SDS, mix thoroughly, and incubate at 65 °C for 10 min.
4. Add 500 μL 5 M potassium acetate, mix thoroughly, and place on ice for at least 20 min or until insoluble complexes are formed from proteins, polysaccharides, and potassium dodecyl sulfate.
5. Centrifuge the tubes at 25,000 × g for 20 min at 4 °C.
6. Pour the supernatant through a sterile small funnel containing two layers of gauze or Miracloth filter to a new tube containing 1 mL prechilled isopropanol and 1 mL 5M ammonium acetate.

7. Mix gently by inverting the tubes a number of times and incubate at −20 °C for 20 min.

8. Pellet the DNA by 15 min centrifugation at 20,000 × g, gently discard the supernatant, and air-dry the pellet for 5 min. At this stage, the pellet should be visible and clear.

9. Redissolve the pellet in 700 μL resuspension buffer and 4 μL 100 mg/mL RNase A. Incubate at 37 °C for 1 h to degrade contaminating RNA.

10. Add 75 μL 3 M sodium acetate and centrifuge for 15 min to pellet insoluble debris. Transfer the supernatant to a clean tube containing 500 μL isopropanol, at room temperature for 5 min.

11. Pellet the DNA by centrifugation at 20,000 × g for 15 min, and rinse the pellet with 500 μL 70% ethanol. Air-dry for 10 min.

12. Resuspend the DNA pellet in 100 μL TE buffer and incubate for 1 h with gentle mixing at 4 °C.

13. Determine the quality and concentration of DNA by measuring its absorbance at 260, 280, and 230 nm (see Note 17).

### 3.3 PCR Amplification of ISSR

1. Thaw 2 × GoTaq Green Master Mix, primer solutions, RNase-free water, and template DNA. Mix the vials thoroughly and keep on ice until use (see Note 18).

2. Calculate the number of samples including positive controls, negative controls, and blanks.

3. Prepare a master mix by multiplying the amounts of reagents (see Table 2) by the number of samples plus one extra sample, to compensate for possible losses of reagents during pipetting (see Note 19).

### Table 2

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial concentration</th>
<th>Volume per sample (μL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq Green Master Mix</td>
<td>2×</td>
<td>12.5</td>
<td>1×</td>
</tr>
<tr>
<td>Primer</td>
<td>10 μM</td>
<td>5.0</td>
<td>2 μM</td>
</tr>
<tr>
<td>Sterile nuclease free water</td>
<td></td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>15 ng/μL</td>
<td>2.0</td>
<td>30 ng</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>25.0</strong></td>
<td></td>
</tr>
</tbody>
</table>
4. Assemble a separate reaction master mix for each of the primers (ISSR04, ISSR05, and ISSR07) on ice. Do not introduce the templates into the reaction mixture components.

5. Mix the reaction components thoroughly and dispense 23.0 \( \mu L \) volumes into 0.2 mL PCR tubes labeled with a proper identifier.

6. Add 2.0 \( \mu L \) template DNA (15 ng/\( \mu L \)), the positive controls, negative controls, and blank samples each to its corresponding reaction tube. Mix thoroughly and spin briefly if necessary to collect down any droplets.

7. Program the thermal cycler according to Table 3. Place the PCR tubes into the thermal cycler, and start the PCR cycling program (see Note 20).

### Table 3

Thermal cycling conditions

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>57</td>
<td>90 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>90 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

3.4 Gel Electrophoresis

1. Prepare a 1.4% (w/v) agarose gel in 1 \( \times \) TAE buffer and allow it to solidify.

2. Load 10 \( \mu L \) from each sample along with a 100 bp DNA ladder (see Note 21).

3. Stain the amplification products with ethidium bromide (0.5 \( \mu g/mL \)) and visualize by UV trans-illumination. Photograph a representative agarose gel electrophoresis (Fig. 1, see Note 22).

3.5 Fingerprinting

1. Use a high-quality gel electrophoresis photograph to construct a binary matrix by visually scoring the presence of an amplification fragment as (1) and its absence as (0) (see Note 23).

2. Use the Dice similarity coefficient to calculate a pairwise genetic similarity matrix (see Note 24).

3. Calculate the genetic distance between each pair of cultivars by the following formula:

\[
\text{distance} = 1 - \text{similarity}_{\text{Dice}}
\]
4. Using the genetic distance matrix, run the neighbor-joining (NJ) analyses to build a phylogenetic tree for cluster analysis and determination of the molecular relatedness of the cultivars (Fig. 2) [18] (see Notes 25 and 26).

4 Notes

1. All buffers, solutions, and reagents must be prepared in a nuclease- and contaminant-free environment and using molecular biology-grade materials. If the use of disposables is not
possible, all equipment, pipettes, and other plastic/glassware should be sterilized by autoclaving. Water should be double distilled or deionized, sterilized by autoclave, and stored at 2–8 °C.

2. Prepare 1 M Tris–Cl buffer (pH 8) by dissolving 121 g Tris base in 800 mL H₂O and adjust to pH 8 with 1 N HCl. Mix and add H₂O to 1 L. Approximately 42 mL HCl is needed to achieve pH 8. It is important to adjust the pH of Tris–Cl buffer solution at room temperature, because the pH of Tris buffers changes significantly with temperature. Complete the volume to 1 L with H₂O.

3. A 0.5 M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8) can be prepared by dissolving 186.1 g Na₂EDTA·2H₂O in 700 mL H₂O, adjusting to pH 8 with 10 M NaOH (~50 mL), and then increasing the volume to 1 L with H₂O. The EDTA will slowly dissolve as the pH of the solution reaches 8. Sterilize by autoclave.

4. Mix 10 mL 1 M Tris–Cl (pH 8), 10 mL 0.5 M EDTA (pH 8), 10 mL 5 M NaCl, 70 μL β-mercaptoethanol (from 14.3 M stock), and 70 mL H₂O. The buffer is stable up to 1 month at room temperature.

5. The 20% SDS solutions may be purchased from different suppliers or alternatively prepared by dissolving 20 g SDS powder in 100 mL H₂O. Heating to 68 °C facilitates dissolving SDS in water, thus helps to prepare concentrated SDS aqueous solution. The solution cannot be autoclaved but may be sterilized by filtration through 0.2 μm filters.

6. Mix 5 mL 1 M Tris–Cl (pH 8), 2 mL 0.5 M EDTA (pH 8), and 93 mL H₂O.

7. Either purchase ready-made solution or prepare by dissolving 500 mg lyophilized RNase A powder into 5 mL distilled H₂O. The solution is filter sterilized through 0.45 μm membrane microfilter. The sterilized RNase A solution may be stored at 2–8 °C for up to 6 months.

8. Mix 1 mL 1 M Tris–Cl (pH 8), 0.2 mL 0.5 M EDTA (pH 8), and 99 mL H₂O.

9. Prepare a 100 μM stock solution of each primer and then prepare 10 μM aliquots as needed by 1:10 dilution of the stock primer solution. The stock solutions are stable for several months when stored at −20 °C.

10. The validity of ISSR primers is determined by analyzing three different samples from each of six date palm cultivars and repeating the experiments three separate times for each primer. Primers with identical electrophoresis profiles are considered reproducible.
11. This described protocol is optimized using the GoTaq Green Master Mix premixed ready-to-use solution containing optimal concentrations of Taq DNA polymerase, dNTPs, MgCl₂, and reaction buffers. The use of PCR master mixes from other suppliers must be validated.

12. Prepare 1 L 50× TAE electrophoresis buffer stock solution by mixing 242 g Tris base, 57.1 mL glacial acetic acid, and 37.2 g Na₂EDTA·2H₂O and add distilled water to raise final volume to 1 L. Prepare 1 L 1× TAE working solution by diluting 20 mL 50× stock solution with 980 mL H₂O.

13. Ethidium bromide is classified by the Hazard Communication Standard (HCS) as a health hazard and is suspected of causing genetic defects. It must be handled using personal protective equipment as required.

14. New tender-white to pale-yellow leaves are recommended to increase the DNA yield and avoid high levels of endogenous phenolic compounds, polysaccharides, or other substances present in the green leaves that may interfere with DNA extraction.

15. Make sure to protect your eyes from splashes of liquid nitrogen by wearing protecting goggles. Many other strategies can be followed to disrupt the plant tissue, provided that they preserve the purity and quality of DNA and prevent premature release of nucleases before addition of the extraction buffer.

16. DNA may be extracted from 100 mg ground young date palm leaves using the DNeasy Plant Mini Kit, following the manufacturer’s recommendations. Both extraction procedures give comparable results in terms of quantity and quality of the extracted DNA [5]. The DNA concentration is 25–65 ng/μL by using the DNeasy Plant Mini Kit and 35–125 ng/μL using the manual protocol.

17. The concentration of DNA can be spectrophotometrically determined by reading the absorbance of DNA at 260 nm (dsDNA concentration = 50 μg/mL × A₃₆₀ × dilution factor). The quality of DNA can be assessed spectrophotometrically by calculating the A₃₆₀/A₂₈₀ ratio (a ratio of ≥1.8 is generally accepted as “pure” for DNA; lower ratios may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm). Absorbance at 230 nm reflects contamination of the sample by phenol or urea.

18. ISSR-PCR used in this protocol is less susceptible to variation in reaction conditions than other similar genotyping techniques. Consistent results are obtained using a ready-to-use Master Mix and kits with separately provided reaction components.
19. Always introduce a blank negative control to assess for contamination of PCR reactions. Contaminated PCRs give an amplification product in the blank and should be repeated with newly aliquoted reagents. Contamination usually results from carryover of amplification products of previous PCRs or from genomic DNA of a highly concentrated sample. To minimize the risk of PCR contamination, the reactions must be assembled in a separate dedicated area, preferably within a PCR work station provided with positive pressure, HEPA filter, and a UV lamp.

20. Use a thermal cycler with a heated lid in order to minimize evaporation of the reaction mixture that lead to results discrepancy.

21. The GoTaq Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. Furthermore, the reactions assembled with GoTaq Green Master Mix or equivalent kit have sufficient density for direct loading onto agarose gels, and thus the samples need not be mixed with loading samples prior to loading.

22. Enhanced electrophoretic separation of the PCR products can be obtained on a vertical polyacrylamide gel using a standard procedure. Even better separation could be obtained with capillary electrophoresis and fragment analysis provided that the primers are fluorescently labeled.

23. There are a number of commercial and free gel analysis software packages, which can automatically detect lanes and bands with precise determination of the amplification fragment size. An example of a freely downloadable software is the GelAnalyzer freeware (Fig. 3).

Fig. 3 An example of automatic determination of gel electrophoresis profile. A plot of band intensity (Y-axis) versus distance migrated by the band (X-axis), detected by the automatic gel analysis software, GelAnalyzer. The analyzed lane is shown below
Normally, only those bands consistently scored in at least three independent runs are considered for analysis.

24. The Dice/Sørensen similarity coefficient for a pair of individuals $i$ and $j$ is defined as

$$S_{ij, \text{Dice}} = \frac{2n_{11}}{2n_{11} + n_{01} + n_{10}}$$

where $n_{xy}$ is the number of characters that have state $x$ in individual $I$ and state $y$ in individual $J$. Possible character states are band presence (1), band absence (0) [19].

The Jaccard coefficient can also be used to calculate the similarity matrix.

25. A number of freeware packages are available that can be used to perform the NJ analysis, to construct a phylogenetic tree, as well as to perform different tests for cluster analysis. An example of such software is the Fingerprint Analysis with Missing Data 1.3 Software (FAMD) [20].

26. One benefit of NJ analyses using the Dice coefficient is the exclusion of shared–absence characters. It is highly recommended to run a bootstrap test (1000 replicates each) and consider values greater than 50%.

References


diversity-in-microorganisms/dna-based-techniques-for-studyinggenetic-diversity


Chapter 16

Molecular Identification of Date Palm Cultivars Using Random Amplified Polymorphic DNA (RAPD) Markers

Nasser S. Al-Khalifah and A.E. Shanavaskhan

Abstract

Ambiguity in the total number of date palm cultivars across the world is pointing toward the necessity for an enumerative study using standard morphological and molecular markers. Among molecular markers, DNA markers are more suitable and ubiquitous to most applications. They are highly polymorphic in nature, frequently occurring in genomes, easy to access, and highly reproducible. Various molecular markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), and random amplified polymorphic DNA (RAPD) markers have been successfully used as efficient tools for analysis of genetic variation in date palm. This chapter explains a stepwise protocol for extracting total genomic DNA from date palm leaves. A user-friendly protocol for RAPD analysis and a table showing the primers used in different molecular techniques that produce polymorphisms in date palm are also provided.

Keywords  Electrophoresis, Genomic DNA extraction, Polymerase chain reaction, Random amplified polymorphic DNA

1 Introduction

Cultivar identification of date palm based on morphological characters is an intricate, empirical exercise due to narrow variations among the cultivars and the presence of large numbers of homonyms and synonyms. Compared to morphological markers, molecular markers are more stable and ideally neutral to environmental effects, management practices, and other influential parameters. Among the molecular markers, DNA markers are more suitable and ubiquitous to most applications. They are highly polymorphic in nature, frequently occurring in genomes, easy to access, and highly reproducible [1].

DNA markers are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are generated by hybridizing restriction-digested target DNA by a labeled probe of known
sequence, for example, restriction fragment length polymorphism (RFLP) and restriction landmark genomic scanning (RLGS). In PCR-based assays, in vitro amplification of a particular region or loci is done using a specific or arbitrarily chosen oligonucleotide sequence called a primer. The amplified products are separated electrophoretically and banding patterns determined, for example, amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and inter-simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), single nucleotide polymorphism (SNP), and sequence-characterized amplified regions (SCAR).

Molecular markers have been extensively used in date palms (*Phoenix dactylifera* L.) for detecting phylogenetic relationships of cultivars, somaclonal variation, cultivar identification, and sex determination using different markers such as RFLP [2], AFLP [3, 4], SSR [5–8], ISSR [7, 9], and RAPD [10–13].

A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. Various protocols for DNA extraction have successfully been applied to many plant species [14–17]. The major differences in these protocols mainly concern the ingredients and the pH of the extraction buffer. For example, Dellaporta et al. [14] used 5 M potassium acetate to separate polysaccharides, polyphenols, and proteins from plant tissue. Doyle and Doyle [15] employed CTAB to isolate DNA with the reducing agent 2-mercaptoethanol in addition to proteinase K which removes protein. Others like Edwards et al. [16] utilized SDS and phenol instead of CTAB as a detergent for the same function of pure DNA isolation. Reichardt and Rogers [17] used high CTAB concentration, which is an active detergent to deter DNase activity and removes polysaccharides.

This chapter provides detailed protocols for DNA extraction and molecular analysis techniques using RAPD markers for the identification of date palm cultivars.

## 2 Materials

### 2.1 Plant Material

Leaf samples from young, yellow emerging leaves of various date palm cvs.: Barney, Shehel, Om-Kobar, Ajwa, Om-Hammam, Sukkari Hamra, Sukkari Asfar, Bareem, Nabtet Saif, Rabeeha, Shishi, Sugai, and Nabtet Sultan (*see Notes 1 and 2*).

### 2.2 DNA Extraction

1. EDTA: 0.1 mM diaminoethane-tetraacetic acid (pH 8 using NaOH).

2. Tris: 10 mM Tris (hydroxymethyl) aminomethane (pH 7.5–9 using HCl).
3. Extraction buffer: 50 mM EDTA pH 8, 100 mM Tris pH 8, 500 mM NaCl, and 10 mM mercaptoethanol.
4. TE buffer (0.1×): 10 mM Tris and 0.1 mM EDTA.
5. Sodium dodecyl sulfate (SDS, 10% w/v).
6. Potassium acetate (5 M).
7. Ice cold isopropanol (see Note 3).
8. Sodium acetate (3 M).
9. Proteinase K.
10. Ethanol (70% v/v).
11. RNase solution A.
12. Liquid nitrogen (see Note 4).

2.3 Polymerase Chain Reaction (PCR)

1. Deionized water: Autoclaved deionized distilled water kept in 100 mL bottles.
2. Primers: Decamer OPERON primers are available as oligo (small) 10-mer Kit series A, B, C, to Z (20 primers in each series) (see Note 5).
3. Taq DNA polymerase (5 U/mL).
4. PCR buffer (10×): 500 mM KCl, 5 mM MgCl₂, and 100 mM Tris (pH 9).
5. Gene amplifying dNTP: Blend of 10 mM each of dATP, dCTP, dGTP, and dTTP in a solution of 0.6 mM Tris (pH 7.5).

2.4 Agarose Gel Electrophoresis

1. Tris-Borate-EDTA buffer (TBE buffer, 10×): 135 mM Tris, 68.75 mM boric acid, and 11.63 mM EDTA.
2. Ethidium bromide solution (10 mg/mL) (see Note 6).
3. Loading dye (10×): sucrose (60% w/v), bromophenol blue (0.25% w/v), and xylene cyanol (0.25% w/v).
4. Agarose.

2.5 Equipment

1. Instruments: Thermal cycler (see Note 7), DyNA Quant 200 fluorometer, centrifuge, vortex, water bath, centri-evaporator, electrophoresis tank, UV-transilluminator, and gel documentation system.
2. Glassware and plastic ware: Mortar and pestle, Eppendorf tubes, casting trays, combs, and micropipettes.
3. Supplies: Primers, Taq DNA polymerase, PCR buffer, and dNTPs.
4. Software: Diversity Data Base.
3 Methods

3.1 Preparation of Buffers and Solutions

1. Extraction buffer: For making 500 mL extraction buffer, add 50 mL 1 M Tris, 50 mL 0.5 M EDTA, and 50 mL 5 M NaCl into a beaker and make up the volume to 500 mL by adding deionized distilled water and then autoclave for 20 min at 121 and 1.1 kg/cm². Allow the solution to cool to room temperature and add 10 μL 10 mM β-mercaptoethanol.

2. TE buffer (0.1×): Add 500 μL 2 M Tris (pH 8) and 20 μL 0.5 M EDTA, and then raise the volume to 100 mL with deionized distilled water and autoclave for 20 min.

3. Sodium dodecyl sulfate (SDS, 10%): Dissolve 10 g sodium dodecyl sulfate in 100 mL distilled water, with gentle heating (65 °C).

4. Potassium acetate (5 M): Dissolve 29.5 g potassium acetate in 11.5 mL glacial acetic acid and raise the volume up to 100 mL with distilled water.

5. Sodium acetate (3 M): Dissolve 40.8 g sodium acetate in 70 mL distilled water and raise the volume to 100 mL.

6. Proteinase K: 20 mg proteinase K in 1 mL distilled water; store at 20°C.

7. RNase solution A (1 mg/mL): Dissolve 20 mg RNase A in a mixture of 5 μL 2M Tris, 3 μL 5 M NaCl, and 992 μL distilled water by heating in a water bath for 15 min for inactivating DNase and then cooled slowly. Dilute the stock solution 20 times in distilled water to get a final concentration of 1 mg/mL. Store at −20 °C.

8. Primers: Add equal volume of TE buffer (pH 8) to each tube of lyophilized primers to make a stock concentration of 1 mg/mL. The amount of TE buffer to be added is specified in a list accompanying the primers supplied by the company. Make a dilution of 100 times of the stock solution by adding 198 mL TE buffer and 2 mL of the stock solution (1 mg/mL) in a new tube. The working solution will be of a final concentration 10 ng/mL.

9. Taq DNA polymerase: Centrifuge tube before opening to improve recovery of contents. Always keep the enzyme at −20 °C. One unit will catalyze the incorporation of 10 μmol of total nucleotide into acid-insoluble product in 30 min at 70 °C utilizing M13 mp 18 DNA as the template.

10. Gene amplification dNTPs: Make a dilution of 10 mM concentrated dNTPs to a final concentration of 2.5 mM and aliquot 50–75 μL in 0.5 mL microfuge tubes and keep frozen until further use.
11. Ethidium bromide solution (10 mg/mL): Dissolve 10 mg ethidium bromide tablets in 1 mL distilled water and stir for 1 h to ensure that the dye has dissolved evenly. Keep the solution in dark bottles.

12. Loading dye 10×: Add 60 g sucrose, 0.25 g bromophenol blue, and 0.25 g xylene cyanol in 100 mL deionized distilled water and keep stirring overnight to dissolve completely.

13. 10× TBE buffer (Tris-Borate-EDTA): Add 108 g Tris, 55 g boric acid, and 9.3 g EDTA in 600 mL of deionized distilled water and then make up to 1 L (pH to 8). Take 100 mL stock solution and add 900 mL water to make the final working solution (1×), for making agarose gel and filling electrophoresis tank.

3.2 Genomic DNA Extraction and Purification

3.2.1 Extraction of DNA

1. Turn on the water bath and set at 65 °C.

2. Dispense 600 μL extraction buffer into the required number of 1.5 mL microtubes and preheat in a water bath.

3. Precool the autoclaved mortar and pestle with liquid nitrogen.

4. Cut 150–250 mg young leaves into small pieces, wash with distilled water, blot dry, and grind into a fine powder in liquid nitrogen.

5. Transfer the powdered tissue to a 1.5 mL microtube containing 600 μL (2× weight of grindate) of the preheated extraction buffer before the frozen powder starts thawing.

6. Mix gently by inverting the tube several times and then add 50 μL 10% SDS and 4 μL 20 mg/mL proteinase K, and mix gently.

7. Incubate at 65 °C in water bath for 30 min with occasional swirling.

8. Add 200 μL of 5 M potassium acetate, vortex gently, and keep it on ice for 10–20 min or alternatively at −20 °C for 2 min.

9. Centrifuge at 2000 × g for 20 min at 4 °C and transfer the supernatant of the solution (the top aqueous phase) to a new tube.

10. Add equal volume of cold 2-propanol (−20 °C) to the supernatant and mix gently to precipitate nucleic acids.

11. Centrifuge at 2000 × g for 20 min at 4 °C and pour off the supernatant (precipitated nucleic acids form the pellet). Wash the pellet twice with 70% ethanol. Air dry at room temperature for 30 min or in centri-evaporator for 5 min and resuspend in 50 μL 0.1× TE buffer (pH 8).

12. Add 2.5 μL (10 mg/mL) of RNase A solution (1 mg/mL) and incubate for 1 h at 37 °C. Store at −20 °C for further use.

mohan.jain@helsinki.fi
3.2.2 Purification of DNA

Add 1/10th volume of the suspension of 3 M sodium acetate, mix gently, and then add equal volume of absolute ethanol to precipitate the purified DNA.

1. Spin at 2000 \( \times g \) for 10 min, discard the supernatant, and wash the pellet with 70% ethanol.
2. Air-dry or centri-evaporate for 5 min and resuspend in 0.1 \( \times \) TE buffer (pH 8).
3. Measure the concentration of the DNA by using DyNA Quant 200 fluorometer. Store at \(-20^\circ C\) until use.

3.3 Polymerase Chain Reaction (PCR) for RAPD Analysis

The significance of PCR lies in its ability to amplify a specific DNA or cDNA sequences from trace amounts in a complex mixture of templates. It is possible to amplify specific DNA or cDNA sequences from as short as 50 bp to over 10,000 bp in length, more than a million fold in a few hours in a reaction that is carried out in an automated DNA thermal cycler [18]. For any PCR-related molecular analysis, the isolated, purified template DNA has to be amplified using specific primers.

1. Master Mix: For PCR reactions, combine the reaction components on ice in the order 17.2 \( \mu L \) dH2O, 2.5 \( \mu L \) 10 \( \times \) PCR buffer, 1 \( \mu L \) dNTPs (2.5 mM), 1.6 \( \mu L \) Operon Primer (10 \( \eta g/\mu L \)), 0.2 \( \mu L \) Taq polymerase (5 U/\( \mu L \)), 2.5 \( \mu L \) template DNA (10 \( \eta g/\mu L \)); total volume 25 \( \mu L \). Total volume of the master mix required can be calculated by multiplying each unit value in the master mix table by the number of samples (see Note 8).

2. Combine all the reagents except template DNA in one tube and then aliquot 23 \( \mu L \) in PCR tubes on ice.

3. Template DNAs are added into each tube separately. The concentration and volume of the DNA samples can vary. For example, in the case of PCR amplification of date palm, the DNA concentration should be 25 \( \eta g/\mu L \) in each reaction. Any increase or decrease in the volume of DNA is compensated by a decrease or increase in the volume of distilled water.

4. Write labels on all the tubes indicating the name of primer and sample.

5. Arrange all PCR tubes on to the metal block of PCR machine. Thermal cycler has a metal block which can be heated and cooled to a desirable temperature very precisely within a short period of time. Usually for DNA amplification by RAPD technique, 35–40 cycles are run (see Note 9). Each cycle comprises of three steps:
   (a) Denaturation of double-stranded DNA at 94 \( ^\circ C \),
   (b) Annealing of primers at 36 \( ^\circ C \),
   (c) Extension or formation of new strand of DNA at 72 \( ^\circ C \),
3.4 Agarose Gel Electrophoresis

1. To make 1.4% gel, add 2.8 g agarose to 200 mL 1×TBE buffer and heat in microwave oven until agarose is fully dissolved.
2. Cool down to room temperature and then add 6 μL ethidium bromide solution (10 mg/mL).
3. Mix well and pour it on a casting tray fitted with a comb of 15 or 30 teeth depending on the number of wells required. When the gel solidifies, remove the comb vertically and then dip the gel in the electrophoresis tank filled with 1×TBE buffer.
4. After PCR add 5 μL loading dye in each reaction tube and load the gel into each well with the help of a micropipette. Load at least 10–15 μL of the samples.
5. Connect the electrophoresis tank with a power supply unit and run at 80 V for 4 h or as required, i.e., until the loading dye reaches the end of the gel.

3.5 Documentation and Analysis

1. After electrophoresis, the gels are placed on an UV-transilluminator to visualize the DNA band profiles or the PCR amplified fragments.
2. The gels are then documented by taking photographs either by using gel documentation system or simply a Polaroid camera. Examples of resultant RAPD profiles are shown for tissue culture-derived plants of Barhee cv. (Fig. 1) and comparison of field-grown popular date palm cultivars (Fig. 2).
3. The documented profiles of the DNA samples are then subjected to different statistical analyses using commercial software like NTSys. For DNA fingerprinting and finding the phylogeny among the samples, Diversity Data Base software is used. Genetic similarities between the genotypes are estimated on the basis of shared amplification patterns [19, 20] (see Note 10).

4 Notes

1. When collecting leaf samples from the field-grown date palms, care should be taken to collect samples from the mother plant only. Small plants around the base of the mother plant may be seedlings rather than offshoots.
2. It is better to collect young, yellow-colored leaflets from the emerging leaves. Keep the collected samples in an ice box and shift to the laboratory at the earliest and store it in a deep freezer. Before grinding, leaf samples should be washed carefully to remove any surface contaminants that may affect the integrity of the DNA samples. Mature leaves are difficult to grind, and the chlorophyll content will affect the quality of DNA. Hundreds of cultivars have been collected and their DNA isolated by using this protocol.
3. Isopropanol should be kept in a freezer (−20 °C) until use.
4. Always use gloves while extracting DNA using liquid nitrogen.
5. Primers used in different molecular techniques that produce polymorphisms in the DNA profiles of date palms are also provided (Table 1).
6. Ethidium bromide is highly mutagenic. Discard the used microtips, strictly following hazardous waste disposal protocols.
7. A machine used for the PCR reaction is also commonly known as a PCR machine. Thermal cycler has a metal block, which can be heated and cooled to a desirable temperature very precisely within a short period of time. Usually for DNA amplification by RAPD technique, 35–40 cycles are run.
8. While dispensing master mix, PCR tubules must be kept in dry ice.

Fig. 1 RAPD profiles of 30 tissue culture-derived plants of Barhee cv. using OPA12 primer showing no marked variations among the different batches of cultures. M-100KBase DNA Marker, Nos 1–30 represent different batches of TC-derived Barhee cv. Source: Reproduced from [11] with permission
Fig. 2 RAPD profiles of 13 date palm cultivars using primers OPC10 (a) and OPD15 (b). Lanes: M-100KBase DNA Marker, 1 Barney, 2 Shehel, 3 Om-Kobar, 4 Ajwa, 5 Om-Hammam, 6 Sukkari Hamra, 7 Sukkari Asfar, 8 Bareem, 9 Nbtet Saif, 10 Rabeeha, 11 Shishi, 12 Sugai, 13 Nbtet Sultan. Source: Reproduced from [10] with permission
Table 1
Primers produced polymorphisms in different types of molecular analyses in date palms

<table>
<thead>
<tr>
<th>RAPD</th>
<th>SSR</th>
<th>ISSR</th>
<th>AFLP</th>
</tr>
</thead>
</table>

Numbers within brackets refer to the reference numbers.
9. The time for each step of the cycle depends on the nature of DNA. Before starting with new DNA samples, an optimization of the conditions for PCR reaction is recommended.

10. A similarity matrix obtained gives an idea of the similarity and/or diversity among the individual samples and in general the whole population. On the basis of the similarity matrix, a phylogenetic tree or dendrogram can be constructed using unweighted paired group method of arithmetic means (UPGMA) for the cluster analysis.

Acknowledgments

The authors express thanks to the King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia, for providing support and infrastructure facilities.

References


mohan.jain@helsinki.fi
Part IV

Gender Identification
Chapter 17

Early Sex Identification in Date Palm by Male-Specific Sequence-Characterized Amplified Region (SCAR) Markers

Pushpa Kharb and Charu Mitra

Abstract

Date palm (*Phoenix dactylifera* L.) is a dioecious plant, and sex of the seedlings can be determined only at the time of first flowering which takes 4–5 years. Female date palm plants are of economic importance as they bear the fruit. Therefore, sex identification at an early stage is highly desirable. DNA-based markers are useful for early sex detection. In this chapter, we describe male-specific sequence-characterized amplified region (SCAR) markers to identify sex in date palm at the seedling stage. Genomic DNA is isolated separately from both male and female date palm genotypes. Amplification of this genomic DNA isolated from male and female plants using the SCAR primers results in an amplicon of 406 bp in both female and male samples and a unique amplicon of 354 bp only in male samples. Based on this amplification pattern, the sex of date palm seedlings can be predicted.

Key words  Dioecious, Molecular markers, Primers, Sex identification, Sex-specific marker, Scar

1 Introduction

Date palm is a dioecious plant of great economic importance owing to the delicious fruits borne by the female plants. For commercial production of date palm, selection of seedlings for female plants producing fruits is critical as only 8–10% of male plants are required in the field to produce enough pollen. Unfortunately, the gender of seedlings cannot be determined until flowering which usually takes place after 4–5 years in the field. This represents a serious inconvenience to plant breeders who have to retain large number of males for several years.

Currently, there is no reliable method to identify sex of the date palm seedlings at the early stages, though there has been significant progress in our understanding of sex determining mechanisms in date palm using traditional means. There are no easily distinguishable sex chromosomes in date palm, despite some cytological evidence [1]. As biochemical studies have yielded little insight for the gender identification of immature plants [2], the identification
of DNA sequences or sequence polymorphisms is gender specific offering a promising alternative to efficiently determine date palm gender [3–8].

In this chapter, we describe a robust male-specific SCAR marker (patent filed in India; application no. 1513/DEL/2010 dated 29/6/10) that can discriminate male and female date palm plants quite accurately.

2 Materials

2.1 Plant Material

Date palm young leaves (10–15 g samples), collected from six different cultivars of date palm trees (Hilawy, Zahidi, Khadrawy, Zagloul, Medjool, and Shamran) belonging to both sexes.

2.2 DNA Isolation

1. Liquid nitrogen.

2. Cetyltrimethylammonium bromide (CTAB) extraction buffer: 0.2 M Tris (pH 8.0), 0.02 M EDTA (disodium, pH 8), 1.4 M sodium chloride, 1.5% CTAB, and 2% β-mercaptoethanol (Table 1; see Notes 1–4).

3. Chloroform: isoamylalcohol (24:1 v/v) mixture (see Note 5).

4. Ice cold isopropanol.

5. Wash I solution: 94.4 mL 76% ethanol and 6.6 mL 3 M sodium acetate (pH 6.8) (see Note 6).

6. Wash II solution: 99 mL 76% ethanol and 1 mL 1 M ammonium acetate (pH 7.7) (see Note 7).

7. Tris–EDTA (TE) buffer: 1 mL 1 M Tris solution (pH 8) and 200 μL 0.5 M EDTA solution (see Note 8).

8. RNase A solution, 50 μg/mL.

9. Absolute alcohol (ethanol).

Table 1
Composition of CTAB extraction buffer

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Stock</th>
<th>To make 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 M Tris</td>
<td>20.0 mL</td>
</tr>
<tr>
<td>2</td>
<td>0.5 M EDTA</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>3</td>
<td>5 M NaCl</td>
<td>28.0 mL</td>
</tr>
<tr>
<td>4</td>
<td>β-mercaptoethanol</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>5</td>
<td>CTAB</td>
<td>1.5 g</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water</td>
<td>Volume to make 100 mL</td>
</tr>
</tbody>
</table>
Table 2
Details of SCAR primers

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Annealing temperature</th>
<th>Expected amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward primer</td>
<td>5’TTTTGGGCTTGTCTAGCATC3’</td>
<td>55 °C</td>
<td>354 and 406</td>
</tr>
<tr>
<td>2</td>
<td>Reverse primer</td>
<td>5’GTTCTGCAAAATTAAGAGAAAAGGT 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3 Polymerase Chain Reaction (PCR) Amplification with SCAR Marker

1. PCR stock solutions: 10× PCR buffer, 25 mM MgCl2, 10 μM forward primer, 10 μM reverse primer, 2 mM deoxyribonucleotides (dNTPs) (mixture of dATP, dCTP, dGTP, and dTTP), and Taq DNA polymerase (5 unit/μL) (Table 2; see Note 9)

2.4 Gel Electrophoresis

1. 10× Tris-Borate-EDTA (TBE) buffer: 108 g Tris, 55 g boric acid, 40 mL 0.5 M EDTA (pH 8), and water for a final volume of 1000 mL (see Note 10).
2. 6× loading dye solution: 4 g sucrose, 0.025 g bromophenol blue, 0.025 g xylene cyanol, and water for a final volume of 10 mL (see Note 11).
3. Ethidium bromide solution: 10 mg/mL (see Note 12).
4. Agarose powder.

2.5 Equipment

1. Pestle and mortar.
2. Hot air oven.
3. Water bath.
4. Autoclave.
5. Centrifuge machine.
6. Thermocycler (PCR).
7. UV-transilluminator.
8. Electrophoretic unit.
9. Sterile glassware.
10. Micropipettes.
11. Eppendorf tubes, centrifuge tubes (50 mL).
12. PCR tubes.

3 Methods

3.1 Extraction and Isolation of DNA

According to the CTAB method by Murray and Thompson [9], and modified by Saghai-Maroof et al. [10] and by Xu et al. [11], carry out the following steps:
1. Take 10 g fresh young leaves of date palm, remove the midrib, and cut remaining leaf tissue into small pieces (see Note 13).

2. Grind leaf samples to fine powder in a sterile pestle and mortar using liquid nitrogen.

3. Take 10 mL CTAB buffer (maintained at 65 °C in a water bath) in a sterilized 50 mL polypropylene tube. Add the finely powdered leaf tissue to this buffer. Mix the contents gently.

4. Incubate tubes in water bath at 65 °C for 90 min. Contents of the tubes require thorough mixing by inverting them at 15-min regular intervals.

5. Cool samples to room temperature and add 10 mL chloroform: isoamylalcohol (24:1;v/v) mixture. Mix the samples by gently inverting the tubes several times. Centrifuge the samples for 10 min at 4217 × g, at room temperature (25 °C).

6. Transfer upper aqueous phase to presterilized clean centrifuge tube and again extract with 10 mL chloroform: isoamylalcohol (24:1;v/v) mixture.

7. Add equal volume of ice cold isopropanol to re-extract upper aqueous phase and let the DNA precipitate out, and spool out using sterile glass hooks.

8. After drying DNA at room temperature for 2–3 h, mix it with 1 mL Wash I solution and keep for 20 min at room temperature. Then wash the DNA samples with 1 mL Wash II solution for 2 min. Dry the DNA samples at room temperature for 2–3 h and subsequently dissolve in an appropriate volume of TE buffer.

9. Add RNase A solution (50 μg/mL) to DNA samples and incubate in a water bath at 37 °C for 3–4 h to remove RNA contamination. Now again extract DNA by adding equal volume of chloroform: isoamylalcohol (24:1; v/v) mixture, and centrifuge at 6589 × g for 10 min (see Note 14).

10. Transfer the supernatant to Eppendorf tube. Add 1/10th volume of 3 M sodium acetate (pH 6.8) and two volumes of chilled absolute alcohol. Centrifuge this mixture to pellet down the DNA. Wash the pellet with 70% alcohol, air dry, and finally dissolve in TE buffer.

3.2 Quantitative and Qualitative Estimation of DNA

It is important to know the quality as well as amount of DNA before proceeding further for any molecular techniques such as carrying out PCR or performing restriction digests, or any other marker-based techniques. DNA can be assessed quantitatively as well as qualitatively by various methods, the most widespread being (a) using gel electrophoresis and (b) spectrophotometric determination. We use gel electrophoresis method as follows:
1. Prepare 0.8% (w/v) agarose gel suspension by dissolving 0.8 g agarose powder in 100 mL 1× TBE buffer (see Note 10). Microwave or stir on a hot plate until agarose is dissolved and solution is clear. Allow solution to cool to about 55°C and add 5 μg/mL ethidium bromide.

2. Place the comb in gel tray and pour gel solution into tray to a depth of about 5 min. Allow the gel to solidify for about 20 min at room temperature.

3. Remove the comb. Place the solidified gel into the electrophoresis tank and pour 1× TBE until the gel is completely submerged.

4. Mix 4 μL sterilized distilled water, 1 μL loading dye, and 1 μL DNA sample solution and load into the well. Load 2 μL standard DNA of known concentration (25, 50, 100 ng) into the side wells (see Note 15).

5. Connect the apparatus to power supply and run the gel at constant voltage (3 V/cm of gel) for 1 h or until the dye moves 3–4 cm from the wells.

6. Place the gel on UV-transilluminator. View and photograph the gel under UV light. Compare the intensity of the DNA bands of the samples with the intensity of the standard DNA bands. As the amount of DNA present in each standard DNA bands is known, the amount of DNA of each sample can be calculated by comparing the fluorescent yield of the sample with that of the standard, and dilution of DNA samples can be made accordingly.

7. Dilute the DNA samples to a concentration of ~50 ng/μL using TE buffer and store at −20°C.

### 3.3 PCR Amplification Using the SCAR Primers

1. For setting up PCR reaction, prepare master mix in 1.5 μL centrifuge tube of the following components except template DNA with final concentration 1× PCR buffer, 250 μM dNTP mix, 0.125 μM each of forward/reverse primer (Table 2), 1.5 mM MgCl2, and 1.5 U Taq DNA polymerase (Table 3; see Note 9).

2. Dispense 18 μL of above master mix in each of the 0.2 mL PCR tubes.

3. Add 2 μL template DNA and mix thoroughly and gently by pipetting 3–4 times.

4. Turn on the thermocycler and place samples in it. The following thermocycling steps are followed:
5. After the run is completed, turn off the machine and remove the samples.

6. Add 2.5 μL gel loading dye in each sample and carry out electrophoresis for separation of amplified products.

### Table 3
Composition for PCR reaction mixture

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume added (μL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer (10×)</td>
<td>2.0</td>
<td>1×</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>1.2</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>2.5</td>
<td>250 μM</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>0.25</td>
<td>0.125 μM</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>0.25</td>
<td>0.125 μM</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μL)</td>
<td>6.0</td>
<td>1.5 U/μL</td>
</tr>
<tr>
<td>Template DNA (50 ng/μL)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Sterilized water</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μL</td>
<td></td>
</tr>
</tbody>
</table>

5. (a) Initial denaturation 94 °C for 4 min
6. (b) Denaturation 94 °C for 1 min
7. (c) Annealing 55 °C for 1 min
8. (d) Extension 72 °C for 2 min
9. (e) Final extension 72 °C for 8 min

3.4 Agarose Gel Electrophoresis of Amplified DNA Products

1. Prepare 1.5% agarose gel by dissolving 1.5 g agarose in 100 mL 1× TBE and pour the gel in gel casting tray with appropriate comb with required well number and size.

2. Load 5–7 μL PCR amplified DNA samples per well. Also load the DNA size standards in one well to calculate the size of the amplified fragments.

3. Carry out electrophoresis at constant voltage (3 v/cm of gel) until the bromophenol blue dye has reached 3/4 s of the gel length.

4. Examine the gel under UV light in the gel documentation system (or transilluminator) and photograph the gel.
5. Amplification with SCAR primers produces a common 406 bp fragment in both female and male genotypes and a unique allele of 354 bp in male genotypes only (Fig. 1).

4 Notes

1. 1 M Tris (pH 8.0): Dissolve 121.14 g Tris base in 800 mL of distilled water. Adjust the pH by slowly adding concentrated HCl and make up final volume to 1000 mL. Sterilize by autoclaving and store at room temperature.

2. 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8): Prepare 0.5 M EDTA by dissolving 18.6 g EDTA (disodium salt) to 80 mL distilled water. Adjust to pH 8 by slowly adding sodium hydroxide (NaOH) pellets and make final volume 100 mL. Autoclave and store at room temperature.

3. 5 M NaCl: Dissolve 292.2 g of NaCl in 700 mL distilled water while stirring with a magnetic stirring bar and adjust the volume to 1 L. Sterilize it by autoclaving and store at room temperature.

4. β-mercaptoethanol should be added freshly.

5. Mix 24 volume of chloroform with 1 volume of isoamylalcohol and store in a dark bottle.

6. 3 M sodium acetate solution (pH 6.8) is prepared by adding 20.412 g sodium acetate in 40 mL water. Then add few drops of glacial acetic acid to adjust pH 6.8 and make the final volume to 50 mL with distilled water. Autoclave and store at 4 °C.

Fig. 1 Banding profile of amplified genomic DNA isolated from 10 male and 15 female genotypes of date palm using SCAR primers. The arrows indicate the sex-specific fragments. Lanes F1–F15, individual female genotypes; M1–M10, individual male genotypes; M, 100 bp DNA ladder
7. Prepare 1 M ammonium acetate (pH 7.7) by dissolving 7.71 g in 100 mL water. Sterilize by filtration and store at room temperature.

8. TE buffer: Mix 1 mL 1 M Tris solution (pH 8) and 200 μL 0.5 M EDTA solution and make the final volume to 100 mL with distilled water.

9. Take out MgCl₂, dNTPs, primers, and buffer from the deep freezer and allow them to thaw at room temperature. Tag DNA polymerase needs to be taken out only before use and put it back immediately to the deep freezer after use.

10. 10× TBE buffer: Dissolve 108 g Tris, 55 g boric acid, and 40 mL 0.5 M EDTA (pH 8.0) in water and make the final volume to 1000 mL. Preparation of 1 L 10× TBE is done by dissolving 100 mL 10× TBE buffer in 900 mL water.

11. 6× loading dye solution: Weigh 4 g sucrose, 0.025 g bromophenol blue, and 0.025 g xylene cyanol and mix in distilled water and make final volume 10 mL. Store it at 4 °C in the refrigerator.

12. Ethidium bromide solution (10 mg/mL): Add 0.2 g ethidium bromide in distilled water to make up the final volume 20 mL and wrap the tube in aluminum foil. Store it at 4 °C.

13. Removal of midrib helps in cutting the leaf into small pieces for easy and fast grinding.

14. During the DNA isolation, a considerable amount of RNA is often extracted. It usually appears as low molecular weight DNA in agarose gel electrophoresis. As RNA may cause suppression of PCR amplification, its removal from the samples is essential.

15. Uncut Lambda DNA of known concentration can be used to determine the concentration of DNA samples.

References


mohan.jain@helsinki.fi


Chapter 18

Gender Identification in Date Palm Using Molecular Markers

Faisal Saeed Awan, Maryam, Muhammad J. Jaskani, and Bushra Sadia

Abstract

Breeding of date palm is complicated because of its long life cycle and heterozygous nature. Sexual propagation of date palm does not produce true-to-type plants. Sex of date palms cannot be identified until the first flowering stage. Molecular markers such as random amplified polymorphic DNA (RAPD), sequence-characterized amplified regions (SCAR), and simple sequence repeats (SSR) have successfully been used to identify the sex-linked loci in the plant genome and to isolate the corresponding genes. This chapter highlights the use of three molecular markers including RAPD, SCAR, and SSR to identify the gender of date palm seedlings.

Key words DNA markers, Sex identification, SCAR, SSR, RAPD

1 Introduction

Date palm is a dioecious tree and produces male and female flowers on different trees. A major problem for breeders is the identification of sex at an early stage so they can cultivate a large number of productive female trees. It is very difficult to identify the gender of the trees up to the age of 5 years, until it reaches the reproductive stage, 5–10 years, as this species is very slow in flowering. Thus, early plant sex determination is very important to speed up breeding programs, and molecular tools have made this possible [1].

Sex determination separates physically male and female gamete-producing structures to different individual plant species. As date palm is a dioecious tree, sex chromosomes are observed in homomorphic form. An extra-heterochromatin, present on both arms of the male chromosomes, is sex determinant [2]. Chromosome manipulation is an important step for assured creation of female producing trees. Gender discrimination is highly desirable before flowering for speeding up breeding and screening for gender at an early developmental stage and would save time, cost, and other resources and be beneficial economically [3]. For plant breeders and geneticists, development of molecular markers is promising...
because plants exhibit unique systems for sex determination. The production of unisexual flowers in many species is independent, and it is operated by many and different novel mechanisms [4] and lacks a single mechanism for the sex determination. To carry on breeding programs, there is a lack of genetic diversity due to nonavailability of a simple and reliable method for gender differentiation before first flowering [5, 6]. Date palm progenies contain equal proportions of male and female plants that suggest the genetic control of sex determination [7]. Similarly, cytological studies have proposed the existence of sex chromosomes in date palm [2]; however, the genes associated with sex were not determined. Moreover, isozymes [8] and peroxidases [9] provide little information for sex identification in immature plants; however, gender-specific DNA sequences are suitable for gender determination [10].

Molecular tools have been used to comprehend the genetic basis of sex evaluation of plants at early stages of development [11, 12]. Arbitrary primers are used for the DNA template amplification in the random amplified polymorphic DNA (RAPD) technique [13]. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers are effectively used for molecular studies of date palm [14, 15] and endeavor to discriminate the sex-specific DNA with different molecular techniques like inter-simple sequence repeat (ISSR). However, RAPD technology has the shortcoming of relatively low reproducibility and reliability which is overcome by developing the sequence-characterized amplified regions (SCAR) markers from already-identified RAPD [16]. Microsatellites or simple sequence repeats (SSR) are also valuable tools for distinguishing male and female date palms [17]. SSR markers successfully characterized 1-year-old date palm seedlings as male or female and identified candidate markers involved in sex determination [1].

In this chapter, molecular markers RAPD, SCAR, and SSR are described with the objective to determine the gender of date palm seedlings, for efficient screening of breeding material and the management of date palm genetic resources.

2 Material

2.1 Plant Material

1. Young leaves of mature already known female date palm plants (Halawy and Khadrawy cvs.) and male plant of unknown origin.
2. Young leaves of 1-year-old progeny of cvs. Halawy and Khadrawy derived from seeds (see Note 1).

2.2 Genomic DNA Isolation

1. CTAB buffer: 100 mM Tris (pH 8), 20 mM EDTA (pH 8), 1.4 M NaCl, and 1% PVP (see Notes 2 and 3).
3. Chilled isopropanol (see Note 4).
5. Ethanol 70%.
6. Doubled-distilled deionized water (d$_3$H$_2$O) (see Note 5).
7. TAE solution (10×): 1 M Tris–HCl (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl, and 0.1 M ammonium acetate.

2.3 Random Amplified Polymorphic DNA (RAPD)

1. PCR buffer (10×): 100 mM Tris–HCl (pH 8.3) and 500 mM KCl.
2. Stoffel buffer (10×): 100 mM Tris–HCl (pH 8.3) and 100 mM KCl.
3. Standard PCR buffer (10×): 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM MgCl$_2$, and 0.01% w/v gelatin.
4. Deoxynucleoside triphosphates: 2 mM each of dGTP, dATP, dTTP, and dCTP. Ready-made solutions of dNTPs (see Note 6).
5. Primers: Decamer primers (see Note 7).
6. Magnesium chloride: 1 M stock (see Note 8).
7. Taq DNA polymerase.
8. Genomic DNA: 5–25 ng/μL stocks (see Note 9).
9. Loading dye: 0.25% bromophenol blue 10% glycerol, 0.1 M EDTA, and 2% SDS.

2.4 Sequence Characterized Amplified Regions (SCAR)

1. Gel Extraction Kit.
2. TA-cloning vector pTZ57R/T for cloning of PCR fragments.
3. E. coli strain (TOP10 cells).
4. IPTG stock solution: 0.1 M IPTG (isopropyl-thio-β-D-galactoside) (see Note 10).
5. X-gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside) solution: 40 mg/mL stock in N,N-dimethylformamide.
6. Miniprep solutions (plasmid isolation solutions):

<table>
<thead>
<tr>
<th>Solution I (suspension buffer):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tris (pH 7.4–7.6)</strong></td>
</tr>
<tr>
<td><strong>EDTA</strong></td>
</tr>
<tr>
<td><strong>RNase</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution II (denaturation solution):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NaOH</strong></td>
</tr>
<tr>
<td><strong>SDS</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution III (neutralization solution):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Potassium acetate (pH 4.8–5)</strong></td>
</tr>
</tbody>
</table>

mohan.jain@helsinki.fi
2.5 Simple Sequence Repeats

1. PCR buffer (10×): 750 mM Tris–HCl (pH 8.8), 200 mM (NH₄)₂SO₄, and 0.1% Tween 20.

2. Acrylamide/bis-acrylamide solution (29:1): 29 g acrylamide monomer and 1 g bis-acrylamide (cross-linker) in 100 mL water (see Notes 11 and 12).

3. Silver nitrate solution: 250 mg silver nitrate and 375 μL formaldehyde in 250 mL water.

4. Binding solution: 3 μL Bind Silane, 1 mL 95% ethanol, and 1 mL 0.5% glacial acetic acid.

5. Developer solution: 7.5 g sodium carbonate, 375 μL formaldehyde, and 50 μL sodium thiosulfate (10 mg) in 250 mL water.

2.6 Equipment

1. Pestle and mortar.

2. Thermal cycler (gradient).

3. Centrifuge.


5. Gel electrophoresis units, vertical and horizontal.

6. Pipette sets.

7. Gel documentation unit.

8. Water bath.

9. Incubation oven.

10. Transilluminator.

11. Vortex.

3 Methods

3.1 Seedling Establishment and Maintenance

1. Gather date palm fruits in summer after fully ripe. Wait until the fruit darkens and begins to wrinkle or exude a fine, sugary sap.

2. Tear the fruit in half by hand. Pick out the large, pointed seed. Soak the seed in a bowl of water for 24 h to soften the epicarp. Pick out and discard any seeds that float to the surface because they are probably not viable. Gently scrub and rinse the seeds that sink to the bottom.

3. Fill 15 cm-deep pots with sterile medium, such as a mix of ½ milled peat and ½ perlite or ½ seed-starting compost and ½ coarse sand. Add water until the medium feels moderately moist in the top 8 cm. Prepare 1 pot for each seed you want to plant.

4. Place 1 date palm seed in each container. Press the seed onto the surface of the growing medium so it is halfway buried.
Cover the seed with a thin layer of coarse sand. Mist the sand liberally after sowing to help settle it onto the seed.

5. Place the containers in a bright, sheltered spot with indirect sunlight. Warm the containers with a germination mat set to around 21 °C. Cover the containers with a propagator or a sheet of plastic wrap.

6. Maintain light moisture in the growing medium to prevent mold and bacterial growth while still providing the seed with the necessary moisture. Let the medium become nearly dry just beneath the surface, and then water to 2.5 cm depth.

7. Watch for the first grasslike sprouts in 3–8 weeks. Leave the propagator and germination mat in place until the date palm seedlings reach 5 cm in height, and then turn off the germination mat. Remove the propagator 2 weeks after germination.

8. Transplant the date palms into 4 L nursery containers filled with sand-based potting soil. Grow them under warm, bright conditions outdoors such as near a lightly shaded south-facing wall. Provide irrigation weekly during their first summer.

9. Acclimate the date palm seedlings to direct sun at the end of their first summer by placing them in an increasingly bright position for more hours each day. Grow them in full sun for 1 full year to ensure their root system is strong and well developed before outplanting them. Provide supplemental water during the summer and in winter if no rain falls for longer than 2 weeks. After 1 year these seedlings are ready for molecular studies.

3.2 Genomic DNA Extraction

1. Grind washed date palm leaves (3 g) to fine powder in pestle and mortar using liquid nitrogen and add 2.5 mL 2× CTAB already heated at 65 °C.

2. Transfer the mixture to 1.5 mL Eppendorf tubes and incubate at 65 °C for 30 min in a water bath. Slightly invert the tubes after 5–10 min during incubation time.

3. Add equal volume of supernatant, chloroform isoamyl alcohol (24:1) and mix gently to form an emulsion.

4. Centrifuge the mixture at 12,470 × g for 10 min. Transfer the supernatant solution (the top aqueous phase) to a new 1.5 mL Eppendorf tube.

5. Add isopropanol (pre-chilled) 0.6 volumes of the supernatant and mix gently by inverting the tubes. Precipitated DNA is visible at this step.

6. Centrifuge the mixture at 12,470 × g for 10 min to form the pellet, and then wash the pellet with 70% ethanol and air-dry the pellet.
7. Dissolve the DNA pellet in 100 μL d_3H_2O treated with RNase (1 μL of stock RNase per 20 μL DNA solution), and incubate at 37 °C for 1 h.

8. Repeat steps 7–9 and dissolve the pellet in 100 μL d_3H_2O.

9. Genomic DNA quality can be checked at 0.8% agarose gel electrophoresis.

3.2.1 DNA Quantification

1. The extracted genomic DNA is quantified by using Nanodrop ND-1000 spectrophotometer.

2. Make the working dilutions 15 ng/μL with d_3H_2O of all DNA samples for PCR reactions.

3.3 Random Amplified Polymorphic DNA (RAPD)

3.3.1 Assemble RAPD Reactions

1. Mix 5 μL DNA stock, 2.5 μL PCR buffer, 2.5 μL magnesium chloride stock for Taq polymerase enzyme, 2.5 μL primer stock (4 μM), 1.25 μL dNTPs stock (2 mM), 0.1–0.2 μL Taq enzyme, and sterile distilled water to a total reaction volume of 25 μL (see Note 13).

3.3.2 Amplify DNA in Thermal Cycler

1. Use average speed thermal cycler 40–45 cycles for 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C, followed by 1 cycle for 7 min at 72 °C and a 4 °C incubation. With faster thermal cyclers, a shorter protocol can be used: 40–45 cycles of 15 s at 94 °C, 30 s at 36 °C, and 1 min at 72 °C. An initial denaturation step for 5 min at 94 °C and/or a final 10 min extension at 72 °C can be added to the amplification protocol, depending on the templates used. Cycling conditions may be modified depending on the thermal cycler used (see Note 14).

3.3.3 Ingredients of RAPD Reaction Mixture

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d_3H_2O (deionized distilled water)</td>
<td>8.3</td>
</tr>
<tr>
<td>10× PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>0.2 mM dNTPs (deoxynucleotide triphosphate)</td>
<td>4</td>
</tr>
<tr>
<td>50 mM MgCl_2</td>
<td>3</td>
</tr>
<tr>
<td>0.025% gelatin</td>
<td>2.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (1 Unit/μL)</td>
<td>0.2</td>
</tr>
<tr>
<td>Primer (15 ng/μL)</td>
<td>2</td>
</tr>
<tr>
<td>Template DNA (15 ng/μL)</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>
3.3.4 Agarose Gel Electrophoresis

1. Assemble the combs and the gel casting tray in such a way that do not touch the tray.

2. Make 1.2% agarose gel: Pour 100 mL 0.5× TBE buffer in the flask and 2.5 g agarose in the same flask and mix gently. Wrap the flask loosely and boil in the microwave until the granules of agarose completely dissolve in buffer (0.5× TBE buffer) (see Note 15).

3. Cool the agarose solution to about 50 °C, add 0.5 μg ethidium bromide, and then pour the solution into the casting tray, ensuring that there are no bubbles in the tray gel.

4. Cool the gel until it became opaque, within 20–30 min. Submerge the gel by filling the tank with 1 L 0.5× TBE buffer which cover the gel by about 0.5 cm.

5. Remove the combs by lifting it at one end, tilting the comb as it comes out (see Note 16). Ensure that the wells are submerged and fill with buffer (see Note 17).

6. Add 3 μL loading dye and load the PCR product samples with micropipette into a single well in the gel.

7. After loading all samples, cover the gel tank with the lid. Connect the red wire with the positive charge and black wire with negative. Run the gel at 80 V.

8. When samples have covered the 75% distance, turn off the power supply.

9. Transfer the gel into a plastic tray (see Note 18). Visualize the bands on the ultra transilluminator to confirm the amplification of the primer and photograph with gel document system.

10. Dispose of the gel into the hazardous trash. Wash the gel tank and tray with distilled water and dry it for further use.

3.3.5 Identification of Unique Fragment

Identify the polymorphic RAPD fragments that are amplified only in either male samples or female samples (see Note 19).

3.4 Development of SCAR Marker from RAPD Fragment

3.4.1 Cloning of RAPD Fragment

1. Elute the DNA fragment from the gel using the Gel Extraction Kit.

2. Ligate the DNA fragment with T4 DNA ligase in TA cloning vector pTZ57R/T.

3. Transform the recombinant vector into E. coli strain (TOP10 cells).

4. Sequence the cloned fragment by dideoxynucleotide chain termination sequencing using PCR-based BigDye kit using M13 universal primers.

5. Design the SCAR primers of approximately 18–24 bp from a flanking sequence of cloned RAPD fragment sequence.
3.4.2 Preparation of Electrocompetent Cells

1. Pick a single colony from a freshly grown *E. coli* strain and transfer into 100 mL LB medium in 1 L flask and incubate at 37 °C overnight with vigorous shaking.

2. Take 2 mL of the overnight culture and dilute to 250 mL in 1 L flask and shake vigorously at 37 °C until OD of 0.5–1.0, for 2–3 h.

3. Chill on ice for 30 min and centrifuge cells at 664 × *g* for 5 min.

4. Discard supernatant and place tubes to ice and then add 5 mL of 0.1 M sterilized and precool MgCl$_2$ to each tube. Mix gently to resuspend the cells.

5. Centrifuge MgCl$_2$-treated cells at 664 × *g* for 5 min, discard supernatant and resuspend the pellet in 5 mL of 0.1 M pre-cooled CaCl$_2$, and leave on ice for 15–30 min.

6. Centrifuge again at 3664 × *g* for 5 min, discard supernatant, and resuspend the pellet in 2 mL of 1 M CaCl$_2$ and 0.7 mL of 100% glycerol.

7. Aliquot 200 μL in sterilized microfuge tubes and immediately freeze the competent cells by dipping each tube in liquid nitrogen for 5 s and then store at −70 to −80 °C.

3.4.3 Ligation of Eluted Fragment

1. Select the vector for the cloning of eluted PCR fragment, e.g., pTZ57R/T, provided by InsTAclone PCR Product Cloning Kit.

2. Ligate the RAPD fragment into the cloning vector using the following reaction mixtures.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (1×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector pTZ57R/T</td>
<td>1 μL</td>
</tr>
<tr>
<td>Eluted DNA fragment</td>
<td>6 μL</td>
</tr>
<tr>
<td>Ligase buffer (10×)</td>
<td>4 μL</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>d$_3$H$_2$O (deionized distilled water)</td>
<td>8.5 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

3. The ligation mixture is incubated overnight (approximately 14–16 h) at 14 °C in a water bath.

3.4.4 Transformation in *E. coli* 10b by Heat Shock

1. Add 5 μL ligated DNA into the aliquot containing 200 μL competent cells.

2. Put in the ice for 30 min.
3. Give heat shock for 2 min at 42 °C.
4. Put in ice for 2 min.
5. Add 1 mL LB media in the DNA and put it in the incubator for 37 °C for 1 h.
6. Spread 100 μL transformed culture and 30 μL IPTG and X-gal on solid LB medium having 100 ng/mL ampicillin.
7. After the liquid is absorbed completely, seal the plates with parafilm and incubate at 37 °C overnight.
8. Pick the white and transparent colonies with sterile toothpicks and culture in liquid LB medium containing 100 ng/mL ampicillin.
9. Keep the culture tubes at 37 °C in a water bath overnight with vigorous shaking.
10. Plasmid is isolated and checked on 1% agarose gel.

3.4.5 Plasmid Isolation from E. coli

1. Culture a single colony in 20 mL liquid LB medium containing 100 μg/mL ampicillin or 50 μg/mL kanamycin and grown overnight at 37 °C.
2. The E. coli culture is centrifuged in 1.5 mL tube at 12,470 × g for 5 min.
3. Discard the supernatant and the pellet is allowed to dry for 2 min.
4. Add 100 μL solution 1 to 1.5 mL tube and suspend the pellet in the solution with the help of vortex.
5. Add 150 μL solution II to 1.5 mL tube and mix well by inverting gently.
6. Add 200 μL solution III to 1.5 mL tube, mix well, and centrifuge at 12,470 × g for 5 min.
7. Take the supernatant in fresh 1.5 mL tube and add two volumes of 100% ethanol.
8. Discard the supernatant and the pellet is washed with 70% ethanol.
9. After centrifugation the supernatant is discarded and the pellet is vacuum dried.
10. Add 20 μL sterile distilled water to dissolve DNA and store at -40 °C.

3.4.6 Restriction Digestion

1. Cloned DNA is confirmed by restriction digestion analysis.
2. A single enzyme (Xba1) is used to linearize the recombinant vector (pTZ57R/ T + PCR eluted fragment).
3. Combinations of two enzymes are used to separate out the cloned DNA from vector.
4. The following reaction mixture is used for restriction of cloned DNA fragment:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (1×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d$_3$H$_2$O (deionized distilled water)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Xba1</td>
<td>6 μL</td>
</tr>
<tr>
<td>BamH1</td>
<td>4 μL</td>
</tr>
<tr>
<td>Buffer 10×</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>8.5 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

5. Incubate the digestion mixture at 37 °C overnight. Then load the digested reaction mixture in 1% agarose gel with the low molecular weight DNA ladder. The restricted DNA fragment can be visualized along with the plasmid DNA with two distinct separate bands.

3.4.7 Sequencing

The complete sequence of the cloned fragment is determined by dideoxynucleotide chain termination sequencing using PCR-based BigDye kit. The sequence can be used to identify the homology using BLAST (Basic Local Alignment Search Tool) from www.ncbi.nlm.nih.gov/BLAST/.

3.4.8 Primer Designing

1. The SCAR primers of approximately 18–24 bp are designed from flanking sequence of cloned RAPD fragment sequence using Primer3 software (http://primer3.ut.ee/).
2. These primers can be used to identify specifically the male or female date palms at seedling stage.

3.4.9 SCAR (PCR)

Use following composition of reaction mixture for SCAR (PCR) analysis:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (1×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d$_3$H$_2$O (deionized distilled water)</td>
<td>8.3 μL</td>
</tr>
<tr>
<td>10× PCR buffer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>0.2 mM dNTPs</td>
<td>4 μL</td>
</tr>
<tr>
<td>50 mM MgCl$_2$</td>
<td>3 μL</td>
</tr>
<tr>
<td>0.025% gelatin</td>
<td>2.5 μL</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (IUnit/μL)</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Primer (15 ng/μL)</td>
<td>2 μL</td>
</tr>
<tr>
<td>Template DNA (15 ng/mL)</td>
<td>2 μL</td>
</tr>
<tr>
<td>Total</td>
<td>25 μL</td>
</tr>
</tbody>
</table>
3.4.10 SCAR (PCR) Temperature Profile

The SCAR (PCR) amplification profile includes initial denaturation at 94 °C for 10 min and then 30 cycles comprising denaturation at 94 °C for 1:30 min, annealing of primers at 56 °C for 30 s, and extension at 72 °C for 2 min and then final extension at 72 °C for 10 min and store at 4 °C.

3.5 Simple Sequence Repeats (SSR)

1. Make the working dilutions of all DNA samples with 20 ng/μL stock DNA sample for SSR analysis.

2. Carry out the polymerase chain reaction (PCR) amplification using 20 μL reaction mixture containing 2 μL (40 ng) of total genomic DNA, 2 μL 10× PCR buffer, 6.4 μL dNTPs, 2.0 μL MgCl₂ (25 mM), forward and reverse primer (1.0 μL of each primer), 0.2 μL Taq DNA polymerase, and 5.8 μL nuclease-free water (see Note 20).

3. Now place this reaction mixture in a Veriti 96-well fast thermal cycler for amplification under the following conditions: initial DNA denaturation at 95 °C for 10 min, 35 cycles (denaturation at 95 °C for 30 s, annealing temperature depending upon the primer (see Note 21) for 30 s, and extension at 72 °C for 1 min), and final extension at 72 °C for 7 min.

4. After amplification, store the PCR tubes at 4 °C.

3.6 Agarose Gel Confirmation

Same as Subheading 3.3.4.

3.7 Polyacrylamide Gel Electrophoresis (PAGE)

3.7.1 Glass Plate Preparation

1. Wash both the glass plates (long and short) of electrophoresis apparatus, carefully with detergent, rinse meticulously with tap water, and remove detergent residues with deionized water (see Note 22). Clean plates systematically with 95% ethanol and wipe out with tissue paper.

2. Apply fresh binding solution on short glass plate to chemically cross-link gel to plate (see Note 23). Clean plates with fine tissue paper and cover back plate with binding solution.

3. After 5 min, wipe the plate three times gently, using fine tissue paper saturated with 95% ethanol in one direction and then perpendicular to the first direction (see Note 24).

4. Treat the long glass plate with SigmaCote® using a tissue saturated with it, and after 10 min remove excess SigmaCote® by wiping plate with a fine tissue (see Note 25).

5. Clump the both plates after placing spacers inside (0.4 mm-thick spacer) (see Note 26).

3.7.2 Polyacrylamide Gel Preparation

1. Use the freshly prepared 6% polyacrylamide gel (20:1, acrylamide/bis-acrylamide) to resolve amplicons of SSR primers. Cast the gels at least 90 min before use.
2. For 8% gel take 3.2 mL 30% (29:1) acrylamide, 2.4 mL (5× TBE), and 6.4 mL H2O in a conical flask. Add 200 μL freshly prepared 10% ammonium per sulfate (APS) and 10 μL TEMED just before pouring the gel (see Note 27).

3. Wear gloves and work quickly after addition of TEMED to complete the gel before the acrylamide polymerizes. Deliver the gel mix to plate assembly. Be sure that there are no air bubbles in the gel and no leakage of gel from the plates.

4. Immediately, insert the appropriate comb into the gel, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth should be slightly higher than the top of the glass.

5. Allow the acrylamide to polymerize for 30–60 min at room temperature.

6. Carefully clean spilled gel from the back of white plates and set the glass plates into the apparatus.

7. Add running buffer (1× TBE) and carefully pull the combs from the polymerized gel. It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

8. Use a Pasteur pipette or a syringe to flush out the wells with buffer, and pre-electrophorese the gel at 150 V for at least 5 min.

9. Mix the DNA samples with the appropriate amount of gel-loading buffer. The samples may be denatured by heating at 95 °C for 2 min and immediately chilled on ice. Load the mixture into the wells using a micropipette equipped with a drawn-out plastic tip (see Note 28).

10. Connect the electrodes to a power pack, turn on the power, and begin the electrophoresis run.

11. Run the gel until the marker dyes move from down to bottom of the gel. Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs.

12. Detach the glass plates. Lay the glass plates on the bench. Use a spacer or plastic wedge to lift a corner of the upper glass plate. Check that the gel remains attached to the lower plate. Pull away the upper plate smoothly. Remove the spacers.

13. Silver stain the gel (see Note 29) or dry the gel and expose to film or phosphorimager screen.

14. Immerse the gel in silver staining solution for 20 min.

15. Pour out the silver stain solution and wash the gel quickly with deionized water, within 10 s.
16. Immerse the gel in an ice-cold developer solution until optimal image intensity is obtained.

17. Stop the developing process by immersing the gel in 7.5% ice-cold glacial acetic acid.

18. Wash the gel extensively with water.

19. Transfer gel onto two layers of 55 mm Whatman no. 1 filter paper.

20. Air-dry the gel at room temperature for 1 h or dry using gel drier 70 °C for 30 min.

21. Band scoring is done by visual inspection. Bands are sized and matched directly on gels to identify the unique fragments present either in male or female date palm samples (see Note 30).

### 4 Notes

1. Pack the leaves in plastic zipper bags after cleaning with tissue paper and store them at –20 °C until DNA extraction.

2. Use sterile plastic and glassware to prepare working solutions. All reagents, chemicals, and enzymes should be of analytical grade and be stored at their recommended temperature requirements.

3. Make the final volume CTAB to 1 L and maintain at pH 8. Before starting extractions, add 2.5 μL β-mercaptoethanol.

4. Put the isopropanol in the freezer before extraction and use the chilled isopropanol in the extraction procedures.

5. Sterile, double-distilled deionized water (d\textsubscript{3}H\textsubscript{2}O) should be used for preparing all reagents and premixes.

6. Aliquot in 1.5 mL plastic tubes and store at –20 °C.

7. Dilute in sterile distilled water to produce a 15 ng/μL working dilution and store at –20 °C.

8. 25 mM solution prepared by dilution of a 1 M stock. Use sterile distilled water, aliquot, and store at –20 °C.

9. A sufficient quality of DNA is obtained by using CTAB DNA extraction procedure. If DNA isolation problems arise, phenol extraction followed by ethanol precipitation is frequently helpful. Contamination with DNA may cause appearance of bands in the no DNA controls.

10. Make an IPTG stock solution of 50 mg/mL in distilled water. Use 100 μL/100 mL.

11. Both acrylamide and bis-acrylamide are neurotoxic and can be absorbed through the skin. Wear gloves and a face mask when weighing out these two compounds in a fume cupboard.
The solution is as toxic as the compounds. Wear gloves at all times when handling the reagent. Wash hands prior to removing gloves. Wash any exposed skin that comes in contact with these compounds immediately.

12. Add a magnetic stirrer and mix for 30 min and make volume up using water. Store at 4 °C, in a bottle wrapped with aluminum foil. Acrylamide hydrolyzes to acrylic acid and ammonia, better storing it at 4 °C in the refrigerator. Normally acrylamide mixtures are frozen in aliquots, but it’s better to prepare the fresh acrylamide solution every month to cast the gel.

13. Wear gloves throughout the RAPD reaction preparation procedure. PCR buffer, dNTPs, MgCl₂ solution, and primer solutions are thawed from frozen stocks, mixed by vortexing, and placed on ice. Frozen DNA should also be thawed and mixed gently. The PCR tubes containing reaction mixtures are sealed, centrifuged, and placed in the thermal cycler for DNA amplification.

14. RAPD amplification is no longer reproducible at low concentration of genomic DNA and at higher DNA concentration produces smears or poor resolution. Generally, use 5–25 ng DNA per 25 μL reaction. However, it is advisable to amplify a dilution series of the template using one or two primers.

15. Cover the beaker with aluminum foil while heating to avoid evaporation loss and overflow.

16. Pulling the comb straight up creates a vacuum in the wells, which tends to lift the whole gel out of the tray.

17. If the wells are not properly submerged in buffer, the sample will not come out of the well and not run properly.

18. While transferring gel from plate to plastic tray and to transilluminator, use gloves as methyl blue dye is carcinogenic, so avoid skin contact.

19. As shown in Fig. 1a, b, two unique RAPD fragments of sizes 1.1 kb and 750 bp in male or female date palm samples, respectively, with primer GLB-6. These two gender-specific RAPD fragments were cloned and sequenced, and the specific SCAR (16–24 bp) primers were designed from flanking ends of the RAPD fragments and successfully amplified in all male date palm samples as shown in Fig. 2. These SCAR markers can be used to identify male plants at an early seeding stage which would lead to the improvement of genetic material and advancement of breeding programs in date palm. The SSR amplification also resulted in a unique fragment of 300 bp (Fig. 3) only amplified in male parents and the seedling as male plant while absent in female parents.
Fig. 1 Identification of unique RAPD fragments in mature male M1–M4 (unknown origin) and female date palm cultivars (Halawy F1 and F2 and Khadrawy F3 and F4). (a) A specific RAPD fragment of 1.1 kb is amplified in all four male samples (M1, M2, M3, M4). (b) A specific RAPD fragment of 750 bp is amplified in all four female samples (Halawy F1 and F2 and Khadrawy F3 and F4). M is a 1 kb ladder.

Fig. 2 PCR amplification using SCAR primers and a single fragment of 750 bp is amplified in nine mature male date palm samples (1–9) of unknown origin. M is a 1 kb ladder.
20. Before amplifying all the samples, it is necessary to optimize the volume of reagents in order to save time and expense.

21. When we select the SSR primers from literature, its annealing temperature is given, but it may vary under our lab conditions so it should be optimized.

22. Because detergent microfilms left on the glass plates may result in high brown-colored background upon staining the gel.

23. Binding solution avoided gel tearing during staining.

24. This step is performed to take away the excess binding solution to avoid long glass plate from contamination.

25. Excess SigmaCote® may cause inhibition of staining so it should be removed properly.

26. Hold the plates by the edges or wear gloves, so that oil from the hands does not become deposited on the working surfaces of the plates. Do not allow the treated surfaces to come into contact with one another.

27. Ammonium persulfate is used as a catalyst for the copolymerization of acrylamide and bis-acrylamide gels. The polymerization reaction is driven by free radicals that are generated by an oxidoreduction reaction in which a diamine (e.g., TEMED) is used as the adjunct catalyst.

28. Do not discard the entire sample from the loading device, as it could produce air bubbles and blow the sample out of the well. However, it is important not to take too long to complete loading the gel; otherwise, the samples will diffuse from the wells. The limit of detection upon visual inspection of double-stranded DNA is approximately 1 pg/mm². This is about 1000 to 10,000 times more sensitive than ethidium bromide staining.

---

**Fig. 3** SSR (PCR) analysis of male and female parents and their seedlings. A unique fragment of 300 bp is amplified in mature male date palm samples of unknown origin. The same fragment (300 bp) is absent in all known female samples of Halawy cv. (lanes 9, 10, and 11) and Khadrawy cv. (lanes 12, 13, and 14) while amplified in four samples of date palm progeny (lanes 20, 23, 25, and 30). M is a 50 bp ladder.
29. Use freshly prepared staining and developer solutions.
30. As shown in Fig. 3, specific locus of 300 bp is amplified in all eight plants, and the same is present in male seedling progeny.

Acknowledgment

This research work was funded by the Higher Education Commission, Pakistan, and supported by Plant Functional Genomics Lab., Centre of Agricultural Biochemistry and Biotechnology (CABB), and Plant Tissue Culture Cell, University of Agriculture Faisalabad, Pakistan.

References


mohan.jain@helsinki.fi
Development of Sex-Specific PCR-Based Markers in Date Palm

Mohamed A.M. Atia, Mahmoud M. Sakr, Morad M. Mokhtar, and Sami S. Adawy

Abstract
Molecular markers are used efficiently in the development and identification of gender-specific PCR-based markers in date palm. There is mounting evidence that different marker systems vary in their mechanisms of detecting polymorphism and genome coverage. Therefore, they could complement each other to generate accurate sex-specific markers in date palm. This chapter describes the uses of PCR-based molecular markers to develop and identify the gender in different date palm genotypes; these are amplified fragment length polymorphism (AFLP), start codon targeted polymorphism (SCoT), conserved DNA-derived polymorphism (CDDP), intron-targeted amplified polymorphism (ITAP), and random amplified polymorphic DNA (RAPD). Also described is how to characterize the identified markers by Sanger sequencing and to explore their functions through alignment of their sequences with the Genbank databases.

Key words AFLP, CDDP, ITAP, SCoT, RAPD, Sex-specific marker

1 Introduction
The date palm (Phoenix dactylifera L.) is a dioecious, long-lived monocotyledonous plant, with separate male and female trees. Date palm has been cultivated for some 7000 years and is believed to have originated in Mesopotamia [1]. Date palm trees are cultivated not only for their valuable fruits but also for producing fuel and fiber and as shade for ground crops. Therefore, it has a major socioeconomic importance, especially in the Middle East, in North Africa, and to a small extent in the USA and Mexico. This tree is highly valuable in the development of sustainable agriculture in drought and saline-affected regions worldwide [2, 3].

In date palm, long juvenility and dioecy pose a challenge in breeding because morphologically it is not possible to distinguish tree gender until flowering, which takes place 5–8 years after planting [4]. Early sex identification of female date palm trees, which
produce the fruits, would facilitate new breeding approaches via controlled crossings and marker-aided selection (MAS).

Over the recent decades, serious efforts have been made to understand the basis of sex determination/differentiation in date palm and to develop methods that could discriminate male and female trees at an early developmental stage. Technologies for genome fingerprinting (molecular marker detection) include random amplified polymorphic DNA (RAPD) [5], PCR-based restriction fragment length polymorphism (PCR-RFLP) [6], microsatellites [7], and, recently, the combination of two PCR-based approaches RAPD and start codon targeted polymorphism (SCoT) [8, 9]. Previous investigations employed different molecular marker systems including three novel gene-targeting marker approaches: conserved DNA-derived polymorphism (CDDP) [10], SCoT and intron-targeted amplified polymorphism (ITAP) [11], as well as amplified fragment length polymorphism (AFLP) [12]. These marker systems were utilized to develop a novel set of sex-specific PCR-based markers for early gender determination in date palm trees [13].

This chapter describes different molecular markers, SCoT, CDDP, ITAP, AFLP, and RAPD, to develop reliable sex-specific PCR-based markers for early gender determination in date palms. Also, it covers procedures for developing sex-specific PCR-based markers, including genomic DNA purification, applying different PCR-based marker approaches, molecular markers data analysis, gel purification, PCR product ligation, bacterial transformation, isolation of recombinant plasmid DNA, Sanger sequencing, and BLAST analysis (Fig. 1).

2 Materials

2.1 Plant Materials

Fresh leaf samples representing male and female seedlings of three date palm cvs. (Hayany, Samany, and Zagloul; two plants of each gender).

2.2 Amplified Fragment Length Polymorphism (AFLP)

2.2.1 Restriction Digestion and Ligation of Adapters

1. Highly purified DNA from male and female seedling date palm trees (100 ng/μL) (see Note 1).
2. EcoRI/MseI enzyme mix.
3. 5× reaction buffer.
5. T4 DNA ligase.
6. TE buffer.
2.2.2 PCR Amplification of the Restriction Fragments

1. EcoRI/MseI preamp primer mix.
2. 10× PCR buffer plus Mg (200 mM Tris–HCl (pH 8.4), 15 mM MgCl₂, and 500 mM KCl).
3. EcoRI primers (27.8 ng/μL).
4. MseI primers (6.7 ng/μL, plus dNTPs).
5. Taq DNA polymerase (recombinant, 5 U/μL).

2.2.3 Denaturing Polyacrylamide Gel of the Amplified Fragments

1. Formamide loading buffer: 10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF.
2. TBE buffer (10×): 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
3. Acrylamide solution, 30%.
4. Urea solution, 6%.
5. Binding solution: 2 μL Bind Silane in 1 mL (0.5% acetic acid in 99% ethanol) (see Note 2).
7. Ammonium persulfate solution, 10%.
8. N,N,N,N'-Tetramethylethylenediamine (TEMED) (see Note 3).
10. A 50 bp DNA ladder.
11. Fix/stop solution (10% glacial acetic acid): 100 mL glacial acetic acid and 900 mL ultrapure water.
12. Staining solution: 2 g silver nitrate (AgNO₃) and 3 mL 37% formaldehyde in 2 L ultrapure water.
13. Developer solution: 3% anhydrous sodium carbonate, 5.6% formaldehyde, and 0.002 mg/mL sodium thiosulfate solution, in deionized water.

2.3 Start Codon Targeted Polymorphism (SCoT)
1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. SCoT primers (10 pmol/μL).
3. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
5. MgCl₂ solution (25 mM).
6. Taq DNA polymerase (recombinant, 5 U/μL).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.

2.4 Conserved DNA-Derived Polymorphism (CDDP)
1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. CDDP primers (10 pmol/μL).
3. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
5. MgCl₂ solution (25 mM).
6. Taq DNA polymerase (recombinant, 5 U/μL).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.

2.5 Intron-Targeted Amplified Polymorphism (ITAP)

1. Highly purified date palm DNA samples (10 ng/μL).
2. ITAP forward primers (10 pmol/μL).
3. ITAP reverse primers (10 pmol/μL).
4. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
5. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
6. MgCl₂ solution (25 mM).
7. Taq DNA polymerase (recombinant, 5 U/μL).
8. Nuclease-free water.
9. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
10. A 100 bp plus DNA ladder.
11. 1 kb DNA ladder.

2.6 Random Amplified Polymorphic DNA (RAPD)

1. Highly purified date palm DNA samples (adjust concentration to 10 ng/μL).
2. Operon RAPD primers (10 pmol/μL).
3. 5× green PCR buffer: 20 mM Tris–HCl (pH 8.4) and 50 mM KCl.
4. PCR nucleotide mix (dATP, dCTP, dGTP, dTTP), 2 mM each.
5. MgCl₂ solution (25 mM).
6. Taq DNA polymerase (recombinant, 5 U/μL).
7. Nuclease-free water.
8. 10× TBE buffer: 750 mM Tris–HCl, 900 mM boric acid, and 2 mM Na₂-EDTA, pH 8.3.
9. A 100 bp plus DNA ladder.
10. 1 kb DNA ladder.

2.7 Molecular Marker Data Analysis

1. Light box.
2. Scoring sheet.

2.8 Gel Extraction of the Differential Products

1. 1.5 mL microcentrifuge tube.
2. Sterile razor blade.
3. QIAquick Gel Extraction Kit.
2.9 PCR Product Ligation

1. Sterile 0.2 mL PCR tube.
3. PCR purified insert DNA.
4. Ligase buffer: 300 mM Tris–HCl (pH 7.8), 100 mM MgCl₂, 100 mM DTT, and 10 mM ATP.
5. T4 DNA ligase (stored in 10 mM Tris–HCl (pH 7.4), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol).
6. Nuclease-free water.

2.10 Bacterial Transformation

1. DH5-alpha E. coli competent cells.
2. LB-agar/antibiotic/IPTG/X-Gal plates.
3. SOC media: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose.

2.11 Isolation of Recombinant Plasmid DNA

1. LB broth: 10 g peptone, 5 g yeast extract, and 10 g NaCl in 1 L ddH₂O.
2. Buffer 1: 50 mM Tris–HCl, 10 mM EDTA, and 100 μg/mL RNase A, pH 8.0.
3. Buffer 2: 1% SDS, 0.2 M NaOH.
4. Buffer 3: 3.0 M potassium acetate, pH 5.5.
5. Isopropanol.

3 Methods

3.1 Amplified Fragment Length Polymorphism (AFLP)

3.1.1 DNA Restriction Digestion and Ligation of Adapters

1. Restriction digestion of genomic DNA: Add 400 ng date palm genomic DNA, 5 μL 5× reaction buffer, 2 μL EcoRI/MseI enzyme mix, and 15.5 μL distilled water to a 1.5 mL microcentrifuge tube.
2. Mix gently and settle the mixture at the bottom of the tube by centrifugation at 7200 × g for 10 s.
3. Incubate the mixture for 2 h at 37 °C, then incubate the mixture for 15 min at 70 °C to inactivate the restriction endonucleases, and collect the mixture by centrifugation at 7200 × g for 10 s.
4. Ligation of adapters: Add 24 μL of adapter ligation solution and 1 μL T4 DNA ligase to the digested DNA from the previous step. Mix gently at room temperature, centrifuge briefly to collect the mixture, and incubate at 20 °C for 2 h.
5. Dilution of ligation mixture in 1:10 ratio: Take 10 μL of the reaction mixture and transfer to a 1.5 mL microcentrifuge tube, then add 90 μL TE buffer, and mix well (see Note 4).
3.1.2 PCR Amplification of the Restriction Fragments

1. Preamplification reaction: Add 2.5 μL diluted template DNA (1:10 from step 3 of Subheading 3.1.1), 20 μL EcoRI/MseI preamp primer mix, 2.5 μL 10× PCR buffer plus MgCl₂, and 0.2 μL (1 unit) Taq DNA polymerase to a 0.2 mL thin-walled microcentrifuge tube.

2. Mix gently and centrifuge briefly to settle the mixture at the bottom of the tube. Put in the thermocycler and perform 20 cycles at 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s, and then terminate the reaction by soaking in an ice bath (4 °C).

3. Perform a 1:50 dilution as follows: Transfer 3 μL to a 0.2 mL microcentrifuge tube containing 147 μL TE buffer. Both unused diluted and undiluted reactions can be stored at −20 °C.

4. Selective amplification reaction: Add 5 μL diluted template DNA 1:50 from previous step, 0.5 μL EcoRI primer, 4.5 μL MseI primer (include dNTPs), 2 μL 10× PCR buffer plus MgCl₂, 0.2 μL (1 unit) Taq DNA polymerase, and 7.8 μL distilled water to a 0.2 mL thin-walled microcentrifuge tube.

5. Mix gently and centrifuge briefly to collect reaction.

6. Put 0.2 mL tubes in the thermocycler and perform touchdown profile as one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, lower the annealing temperature each cycle 0.7 °C during 12 cycles (this gives a touchdown phase of 13 cycles), and then perform 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s.

3.1.3 Preparation of the Sequencing Plates and Gel Analysis

1. Short glass plate preparation: Clean the short glass plate three times with saturated Kimwipes® tissue with ultrapure water, then three times with absolute ethanol, and finally with fresh 1 mL binding solution (see Note 5).

2. Long glass plate preparation: Clean the long glass plate three times using saturated Kimwipes® tissue with ultrapure water, then three times with absolute ethanol, and finally with 1 mL SigmaCote® solution (see Note 6).

3. Allow both short and long plates 10 min to dry. Then assemble glass plates by placing 0.4 mm side spacers between the plates and side arms to hold them in place (see Note 7).

4. Prepare 30% acrylamide solution: Weigh 29.2 g acrylamide powder and 0.8 g bis-acrylamide and transfer to 250 mL graduated beaker containing 40 mL water. Mix with magnetic stirrer for 30 min. Make up volume to 100 mL with water and filter twice through Whatman paper No. 1 (see Note 8). Store at 4 °C, in a bottle wrapped with aluminum foil (see Note 9).

5. Prepare 6% urea solution: To prepare 75 mL urea solution, mix the following in 250 mL graduated beaker containing 21.5 mL
deionized water (48 g urea, 7.5 mL 10× TBE buffer, 15 mL 30% acrylamide solution). Mix with magnetic stirrer for 30 min. Make up the volume to 75 mL with deionized water and filter through Whatman paper No. 1. Store at 4 °C in a bottle wrapped with aluminum foil.

6. Prepare 10% ammonium persulfate solution: Dissolve 0.1 g ammonium persulfate in 1 mL deionized water (see Note 10).

7. Prepare the gel mix by adding 75 mL 6% urea solution, 100 μL TEMED, and 100 μL ammonium per sulfate, and mix gently. Pour the solution carefully between the glass plates. Then insert the well-forming comb between the glass plates. The polymerization process takes about 1 h.

8. Run a small aliquot (3 μL) of the selective PCR product on a 1.5% (w/v) agarose gel to check the DNA amplification.

9. Prepare the selective AFLP product for loading in denaturing gel by mixing an equal volume (2 μL) of the selective PCR product and formamide loading buffer. Denature the mix sample by incubating at 92 °C for 3 min and quickly cool on ice.

10. Pre-run the denaturing gel at 60 W to achieve a gel surface at approximately 50–55 °C, and then load 4 μL of each denatured sample into the respective well.

11. Load a 50 bp DNA ladder and 100 bp DNA ladder as molecular size standards at the first and last well. On completion of loading, run the gel at 55 W (2000–3000 V) until xylene cyanol (slower dye) is about two third down the length of the gel (see Note 11). Then, process the gel to the silver staining steps.

3.1.4 Silver Staining Procedure

1. Separate the plates: After electrophoresis, carefully separate the plates using a plastic wedge. The gel should be attached strongly to the short glass plate.

2. Fix the gel: Place the gel (plate) in a shallow stainless steel tray, cover with fix/stop solution, and agitate well for 20 min or until the tracking dyes are no longer visible (see Note 12).

3. Wash the gel: Rinse the gel every 2 min three times with ultrapure water (see Note 13).

4. Stain the gel: Transfer the gel to a staining solution and agitate well on orbital shaker for 30 min. Remove the gel from the staining solution and set it aside. Transfer the staining solution into a dark flask or beaker and rinse the tray using ultrapure water.

5. Wash and gel developing: Dip the gel briefly into the tray containing ultrapure water for 5–10 s, and transfer the gel immediately into the tray of chilled developing solution “(dissolve 30 g sodium carbonate (Na₂CO₃) in 1 L ultrapure water.

mohan.jain@helsinki.fi
Chill to 10 °C in an ice bath. Then, immediately before use, add 1.5 mL 37% formaldehyde and a 200 μL aliquot of sodium thiosulfate, 10 mg/mL, and agitate well on orbital shaker until the first bands are visible. Continue developing for an additional 2–3 min or until all bands become visible.

6. Stop development: To terminate the developing reaction and fix the gel, add 1 L fix/stop solution directly to the developing solution, and incubate with shaking on an orbital shaker for 2–3 min (see Note 14).

7. Remove the gel from the developer solution and rinse the gel twice for 2 min each time in ultrapure water. Dry the gel at room temperature.

3.2 Start Codon Targeted Polymorphism (SCoT)

3.2.1 SCoT PCR Reaction

1. In a sterile nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs, 2 mM), 1.5 μL MgCl2 solution (25 mM), 3 μL SCoT primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the mixture by centrifugation at 7200 × g for 10 s.

3.2.2 Thermocycling Profile and Detection of the SCoT Products

1. Put the microcentrifuge tubes in a thermocycler and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min and the final extension step 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gels stained with ethidium bromide in 1× TBE running buffer (dilute the 10× TBE to 1× TBE through adding 100 mL of 10× TBE to graduated cylinder containing 900 mL water). Load 5–10 μL of each sample into the respective well.

3. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards in the first and last well.

4. On completion of loading, run the gel at 100 V until the dye is two third down the length of the gel.

5. Visualize the SCoT PCR products under UV light and photograph the gel using a Gel Doc™ XR+ System with Image Lab™ Software.

3.3 Conserved DNA-Derived Polymorphism (CDDP)

3.3.1 CDDP PCR Reaction

1. In a sterile nuclease-free microcentrifuge tube, mix on ice 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs, 2 mM), 1.5 μL MgCl2 solution (25 mM), 3 μL CDDP primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the reaction by brief centrifugation.
3.3.2 Thermocycling Profile and Detection of the CDDP Products

1. Put the microcentrifuge tubes in thermocycler and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min and the final extension step 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gel stain with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into the respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards in the first and last well.

3. On completion of loading, run the gel at 100 V until the dye is about two third down the length of the gel.

4. Visualize the CDDP PCR products on UV light and photograph the gel using a Gel Doc™ XR+ System with Image Lab™ Software.

3.4 Intron-Targeted Amplified Polymorphism (ITAP)

3.4.1 ITAP PCR Reaction

1. In a sterile, nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs; 2 mM), 1.5 μL MgCl2 solution (25 mM), 3 μL ITAP forward primer, 3 μL ITAP reverse primer, 0.2 μL Taq DNA polymerase, and 6.8 μL distilled water.

2. Mix gently and collect the reaction by centrifugation at 7200 × g for 10 s.

3.4.2 Thermocycling Profile and Detection of the ITAP Products

1. Put the microcentrifuge tubes in thermocycler, and program the thermocycler using the following cycling parameters: an initial denaturation step at 94 °C for 4 min, followed by 5 cycles of 1 min denaturing at 94 °C, 1 min annealing at 35 °C, and 1 min elongation at 72 °C. In the following 35 cycles, increase the annealing temperature by 0.4 °C each cycle until reaching 50 °C and then a final extension step 72 °C for 5 min.

2. Separate all PCR amplification products on 1.2% agarose gel stain with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into the respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards at the first and last well.

3. On completion of loading, run the gel at 100 V until the dye was about two third down the length of the gel.

4. Visualize the ITAP PCR products under UV light and photograph the gel using a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad®).
3.5 Random Amplified Polymorphic DNA (RAPD)

3.5.1 RAPD PCR Reaction

1. In a sterile, nuclease-free microcentrifuge tube, combine the following on ice: 3 μL date palm genomic DNA (equal 30 ng), 5 μL 5× green PCR buffer, 2.5 μL PCR nucleotide mix (dNTPs, 2 mM), 1.5 μL MgCl₂ solution (25 mM), 3 μL operon RAPD primer, 0.2 μL Taq DNA polymerase, and 9.8 μL distilled water.

2. Mix gently and collect the reaction by centrifugation at 7200 × g for 10 s.

3.5.2 Thermocycling Profile and Detection of the RAPD Products

1. Put the microcentrifuge tubes in a thermocycler, and perform a standard PCR cycle as follows: an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, at 36 °C for 1 min, and then at 72 °C for 90 s, with a final extension at 72 °C for 7 min.

2. Separate all PCR amplification products on 1.5% agarose gel stain with ethidium bromide in 1× TBE running buffer. Load 5–10 μL of each sample into the respective well. Load a 100 bp plus DNA ladder and 1 kb DNA ladder as molecular size standards at the first and last well.

3. On completion of loading, run the gel at 100 V until the dye is two third down the length of the gel.

4. Visualize the RAPD PCR products under UV light, and photograph the gel using a Gel Doc™ XR+ System with Image Lab™ Software.

3.6 Molecular Marker Data Analysis

For all molecular marker techniques (SCoT, CDDP, ITAP, AFLP, and RAPD), score all amplicons generated from all primers.

1. Score the generated/amplified bands visually and detect the differential bands between male and female samples (Fig. 2).

3.7 Gel Purification

1. After running the gel, move it to an open UV box (see Note 15). Remove the gel from any gel tray as plastic will block much of the UV.

2. With a clean and sterile razor blade, slice the desired/differential DNA fragment from the gel.

3. Place the gel in a labeled microcentrifuge tube.

4. Weigh the tube with the gel fragment after zeroing the scale with an empty tube. The weight of the gel is directly proportional to its liquid volume, and this is used to determine the amount of each buffer to be added during the DNA isolation step.

5. Isolation of DNA from the gel is done with a commercial gel purification kit, such as the QIAquick Gel Extraction Kit (see Note 16). Always follow the manufacturer’s instructions.
3.8 PCR Product Ligation

1. For standard cloning, a 3:1 insert/vector ratio is preferred. Mix all the reagents in a PCR 0.2 mL tube: 25 ng vector DNA, 75 ng insert DNA, 1× ligase buffer, 0.5–1 μL T4 DNA ligase, and ddH2O to a total of 10 μL.

2. Incubate at room temperature for 2 h, or overnight at 16 °C, and proceed with bacterial transformation.

3.9 Bacterial Transformation

1. Take tubes containing competent cells out of −80 °C and thaw on ice, approximately 10–20 min.

2. Prepare two LB/antibiotic/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency. Take LB-agar plates out from storage at 4 °C to warm at the room temperature.

3. Mix 10 pg to 100 ng DNA into 20–50 μL of competent cells in a microcentrifuge. Gently mix by flicking the bottom of the tube with your finger a few times.

Source: Atia and Adawy [14]
4. Place the competent cell/DNA mixture on ice for 20–30 min.
5. Give heat shock to each transformation tube by placing into a 42 °C thermomixer for 30–60 s. Put the tubes back on ice for 2 min.
6. Add 250–500 μL SOC media (free antibiotic) and grow in 37 °C shaker-incubator for 45 min.
7. Plate some or all of the transformants onto a 10 cm LB/antibiotic/IPTG/X-Gal plate containing the appropriate antibiotic (see Note 17). Incubate plates at 37 °C overnight.
8. Screening transformants for inserts: clones that contain PCR products, in most cases, produce white colonies.

3.10 Isolation of Recombinant Plasmid DNA

1. Take a single white colony from the LB transformation plates to inoculate 5 mL LB-amp broth. Incubate the tube overnight at 37 °C with shaking on orbital shaker-incubator (150 rpm) to obtain a saturated culture.
2. Gently swirl the contents of the culture tube to resuspend the cells.
3. Label 1.5 mL tube and pipet 1000 μL cell suspension into tube, and place the tubes in a centrifuge and spin at 22,000 × g for 2 min.
4. Withdraw and discard the supernatant using a pipettor; be careful not to disturb the cell pellet. Discard the supernatant in a waste container.
5. Add 100 μL Buffer 1 to each tube and resuspend the cells by vortexing (see Note 18).
6. Add 200 μL Buffer 2 to each tube. Close the caps and mix the solutions by rapidly inverting them a few times (see Note 19). Let tubes stand on ice for 5 min.
7. Add 150 μL ice-cold Buffer 3 to each tube. Close the caps and mix the solutions by rapidly inverting them a few times. A white precipitate will form. Let tubes stand on ice for 5 min.
8. Centrifuge the tubes at 22,000 × g for 5 min.
9. Transfer the supernatants into clean 1.5 mL tubes (see Note 20). Discard the tubes with the precipitate and keep the tubes with the supernatant.
10. To each tube of supernatant, add an equal volume (about 400 μL) of isopropanol to precipitate the nucleic acids. Close the caps and mix vigorously. Let the tubes stand at room temperature for 2 min. Centrifuge at 22,000 × g for 5 min.
11. Carefully remove and discard the supernatant. Add 200 μL absolute ethanol to each tube and mix by inversion several times. Centrifuge the tubes at 22,000 × g for 2–3 min.
12. Carefully remove and discard the supernatant. Place the tubes in the fume hood without the caps for 15–20 min to dry off the last traces of ethanol.

13. After removal of ethanol (see Note 21), add 20 μL TE buffer to dissolve the pellet (see Note 22).

14. Test the quality of the purified DNA sample using agarose gel electrophoresis before sending the purified samples to any private company for Sanger sequencing.

3.11 Sanger Sequencing and Basic Local Alignment Search Tool (BLAST) Analysis

1. Sequence the purified plasmid samples with an ABI310 DNA sequencer by using synthetic primer complementary to the vector sequences flanking the multiple cloning sites (e.g., M13 forward and reverse primers).

2. Use the BLASTn and BLASTx tools to analyze your sequence data and search for their homology against the DNA and protein sequences existing in Genbank databases, respectively (http://www.ncbi.nlm.nih.gov) [14, 15] (Table 1).

4 Notes

1. For optimal results, high-quality intact DNA must be used as a starting material for AFLP. It is essential to prevent incomplete restriction digestion of genomic DNA, which may result in the false identification of polymorphisms. DNA can be isolated using the CTAB method or any other commercial plant genomic DNA purification kit, such as DNeasy Plant Mini Kit.

2. Always freshly prepare the binding solution.

3. To reduce the pungent smell of TEMED solution, store at 4 °C.

4. The unused portion of the ligation mixture may be stored at −20 °C.

5. Rubbing hard will remove too much of the Bind Silane and the gel may not adhere as well.

6. Change gloves before preparing the long glass plate to prevent cross-contamination with binding solution. If the plate becomes contaminated with Bind Silane, soak it in 10% NaOH for 30–60 min.

7. Gels thinner than 0.4 mm may give weak signals.

8. Wear a mask when weighing acrylamide. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact. Cheaper grades of acrylamide and bis-acrylamide are often contaminated with metal ions. Stock solutions of acrylamide made with these grades can easily be purified by stirring overnight with approx. 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No. 1 paper.
### Table 1
Illustration of the primer code, product length, sex linked, BLASTn (query cover, E-value, and identity), BLASTx (query cover, E-value, and identity), gene/protein hit, and KEGG ID or NCBI GeneID of differentially sequenced fragments generated from different marker systems (SCoT, CDDP, ITAP, and AFLP) distinguishing between males and females belonging to three Egyptian date palm cultivars

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Product length (bp)</th>
<th>Sex</th>
<th>BLASTn</th>
<th>BLASTx</th>
<th>Gene/protein hit</th>
<th>KEGG ID or NCBI-gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Query cover, %</td>
<td>E value</td>
<td>Identity, %</td>
<td>Query cover, %</td>
</tr>
<tr>
<td>SCoT1</td>
<td>553</td>
<td>M</td>
<td>82</td>
<td>0.0</td>
<td>99</td>
<td>82</td>
</tr>
<tr>
<td>SCoT24</td>
<td>699</td>
<td>F</td>
<td>58</td>
<td>2e−165</td>
<td>91</td>
<td>47</td>
</tr>
<tr>
<td>SCoT26–1</td>
<td>597</td>
<td>F</td>
<td>98</td>
<td>0.0</td>
<td>97</td>
<td>76</td>
</tr>
<tr>
<td>SCoT26–2</td>
<td>1245</td>
<td>F</td>
<td>50</td>
<td>0.0</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>SCoT35–1</td>
<td>1550</td>
<td>F</td>
<td>55</td>
<td>0.0</td>
<td>99</td>
<td>52</td>
</tr>
<tr>
<td>SCoT35–3</td>
<td>586</td>
<td>F</td>
<td>88</td>
<td>0.0</td>
<td>99</td>
<td>79</td>
</tr>
<tr>
<td>SCoT35–5</td>
<td>1420</td>
<td>F</td>
<td>35</td>
<td>2e−120</td>
<td>97</td>
<td>22</td>
</tr>
<tr>
<td>CDDP-4</td>
<td>195</td>
<td>M</td>
<td>40</td>
<td>8e−35</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>CDDP-6-1</td>
<td>605</td>
<td>M</td>
<td>23</td>
<td>5e−39</td>
<td>89</td>
<td>–</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Primer code</th>
<th>Product length (bp)</th>
<th>Sex linked</th>
<th><strong>BLASTn</strong></th>
<th><strong>BLASTx</strong></th>
<th>Gene/protein hit</th>
<th>KEGG ID or NCBI-gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BLASTn</td>
<td>BLASTx</td>
<td>Query cover, %</td>
<td>Query cover, %</td>
</tr>
<tr>
<td>CDDP-6-2</td>
<td>608</td>
<td>M</td>
<td>60</td>
<td>60</td>
<td>6e–26</td>
<td>68</td>
</tr>
<tr>
<td>ITAP-8/1</td>
<td>731</td>
<td>M</td>
<td>20</td>
<td>20</td>
<td>4e–16</td>
<td>80</td>
</tr>
<tr>
<td>AFLP-4/2</td>
<td>240</td>
<td>M</td>
<td>97</td>
<td>97</td>
<td>1e–109</td>
<td>99</td>
</tr>
</tbody>
</table>
9. In our laboratory, we prepare weekly fresh acrylamide solution when we cast our own gels. During storage, acrylamide and bis-acrylamide are slowly converted to acrylic acid and bis-acrylic acid. This deamination reaction is catalyzed by light and alkali.

10. It is best to prepare ammonium per sulfate fresh each time.

11. Do not exceed settings that yield a gel surface temperature of 45–50 °C. Glass plates or gel will crack as a result of excess in gel temperature during electrophoresis.

12. The gel may be stored in fix/stop solution overnight (without shaking). Save the fix/stop solution to terminate the developing reaction (step 8).

13. Lift the gel (plate) out of the wash and allow it to drain 10–20 s before the next wash.

14. The developed bands will appear fairly light. Prolonged development times result in high background. It is better to stop development early than to overdevelop the gel.

15. Gel purification is most efficient with lower percentage agarose gels, so use 0.7–0.8%. Be sure to wear proper UV protection - especially for your eyes.

16. It is important to determine the concentration of the isolated DNA before proceeding to the next step.

17. We recommend to plate 50 μL on one plate and the rest on the second plate. This gives the best chance of getting single colonies while allowing you to recover all transformants.

18. It’s very important that the cell suspension is homogenous and no clumps are visible.

19. Do not vortex since the chromosomal DNA released from the broken cells could be sheared into small fragments and contaminate your plasmid prep.

20. Be careful not to decant any of the precipitate. Discard the tubes with the precipitate and keep the tubes containing the supernatant.

21. You can check this by smelling the tube.

22. Pipet the 20 μL in and out, up the side of the tube to ensure that all of the plasmid DNA comes into contact with the TE buffer.

Acknowledgment

This work was carried out in the Molecular Genetics and Genome Mapping Laboratory (MGGM), Agricultural Genetic Engineering Research Institute (AGERI), ARC and funded by the Science and Technology Development Fund (STDF), Ministry of Scientific Research, Egypt. Contracts No. 406 and 4607.
References


Date Palm Sex Differentiation Based on Fluorescence In Situ Hybridization (FISH)

Mohamed A.M. Atia, Sami S. Adawy, and Hanaiya A. El-Itriby

Abstract

In situ hybridization (ISH) is used to visualize defined DNA sequences in cellular preparations by hybridization of complementary probe sequences. Recently, the fluorescence in situ hybridization (FISH) technique has become a powerful and useful tool for the direct detection of specific DNA fragments in the genome. Ribosomal DNA genes (45S and 5S rDNA) are commonly used as markers for the physical mapping of plant chromosomes to analyze genomic organization. Here we describe cytological-based markers to differentiate date palm gender through localization of 45S and 5S rDNA markers on date palm chromosomes using FISH.

Key words Cytogenetic marker, FISH, Molecular markers, Sex determination, rDNA

1 Introduction

Date palm is a dioecious plant (separate male and female individuals) and has a long juvenile stage up to 5–10 years, which is the major constraint for conventional breeding and genetic improvement. Usually, one male is used to pollinate 90–100 females [1]. The major focus has been on clonal propagation of females for centuries. This has led to the reduction of genetic diversity among and within date palm cultivars. Early selection of young seedlings could improve breeding programs and generate experimental male and female genetic stocks.

The development of a reliable technique for chromosome identification is critical for future advances in cytogenetics. Generally, chromosome identification is a major constraint in many plant species with small chromosomes. The identification and characterization of sex chromosomes in plants is problematic because most of them do not differ morphologically from autosomes or from one to another. Date palm has tiny and sticky chromosomes, considered as cytogenetically recalcitrant material [2–4]. Classical Giemsa
C-banding has not allowed plant cytogeneticists to differentiate between male and female individuals [5].

Fluorescence in situ hybridization (FISH) is a powerful technique enabling the visualization of nucleic acid probes on target chromosomes, nuclei, cells, and tissues [6]. Ribosomal DNA (rDNA) has highly conserved repetitive sequences in the plant genome, and the polymorphism or conservatism of their copy number and chromosomal localization are visual and comparative [7–9]. By comparing the number and distribution characteristics of rDNA sites on the chromosomes among species, interspecific phylogenetic relationships and the related mechanism of speciation and chromosomal evolution could be revealed [10, 11].

FISH of repetitive DNA sequences has been used for karyotype and genome analyses of a large number of plant species. The repetitive and tandemly organized ribosomal rDNA genes, 5S rDNA and 45S rDNA, are localized at one or more sites per chromosome set, and their characteristic positions provide useful markers for chromosome and genome identification. They have been used in several grasses such as *Triticum* and *Hordeum* [12, 13], *Oryza* [14], *Sorghum* [15], *Thinopyrum* [16], and *Festuca* [17], providing useful information about evolutionary and phylogenetic relationships between species. Recently, a FISH protocol was developed for sex determination in date palm through localization of the chromosomal positions of 5S and 45S rDNA sites using multicolor FISH analysis [18].

In this chapter, this protocol describes the FISH technique to develop a reliable sex-specific cytological-based markers for early sex differentiation in date palm. The procedures described here include material harvesting, probe labeling, slide preparations for FISH, probe hybridization to denatured metaphase chromosomes, and hybridized probe detection and visualization.

## 2 Materials

### 2.1 Plant Materials

Root tips from females of cvs. Siwy and Zagloul and from male seedling progeny of these cultivars.

### 2.2 Harvesting Root Tips

1. Ice-cold double-distilled water (ddH2O).

### 2.3 Chromosome Accumulation and Fixation

1. 2 mM 8-hydroxyquinoline solution.
2. 10× phosphate-buffered saline (PBS).
3. Fixative solution: 3:1 absolute ethanol/glacial acetic acid.
2.4 Enzymatic Digestion and Slide Preparation

1. Double-distilled water (ddH₂O).
2. Enzyme solution: 4% cellulase and 2% pectinase dissolved in 0.01 M citrate buffer.
3. 45% acetic acid solution.
4. Acetocarmine stain.

2.5 DNA Probes and Labelling

1. 10× Nick translation buffer.
2. DNase I solution.
3. DNase I dilution buffer.
4. DNA polymerase I enzyme.
5. dNTPs (A, C, and G at 0.5 mM each).
6. Biotin- or dig-labeled dUTPs.
7. 0.5 M ethylenediaminetetraacetic acid (EDTA) buffer.

2.6 Fluorescence In Situ Hybridization (FISH)

1. 100% deionized formamide.
2. 20× saline-sodium citrate (SSC) buffer.
3. Dig-labeled DNA.
4. Biotin-labeled DNA.
5. 50% dextran sulfate.
6. 2× SSC solution.
7. 70% formamide/2× SSC buffer.
8. Cold 70% ethanol.
10. 10× PBS buffer.
11. 10× Tris-NaCl-Tween (TNT) buffer.
12. 5× Tris-NaCl-blocking (TNB) buffer.
13. Alexa Fluor 488 (store at −20 °C in the dark).
15. Anti-dig-Rhodamine (store at −20 °C in the dark).

2.7 Equipment

1. Petri dishes.
2. Forceps and razor blades.
3. Microscopic glass slide and cover slips.
4. Alcohol lamp.
5. Filter paper Whatman No. 1.
6. Coplin staining jar.
7. 22 × 22 mm cover slips.
8. Fluorescent microscope system.
9. IP-Lab spectrum software.
10. Photoshop imaging software.

3 Methods

3.1 Harvesting Root Tips
1. Collect the root tips from male and female date palm trees as follows: cut off the roots tips approximately 2–3 cm distal to the root tip (see Note 1).
2. Place the tips in ice-cold ddH2O in a Petri dish.
3. Rinse the collected root tips twice (2 min each) in ddH2O, to clean the root tips of any soil debris.

3.2 Chromosome Accumulation and Fixation
1. Preparation of 2 mM 8-hydroxyquinoline solution: Dissolve 0.29 g 8-hydroxyquinoline in 1 L ddH2O pre-warmed at ~60 °C. It takes about 2–3 h to dissolve completely. Keep in the dark during dissolving and store at 4 °C in the dark.
2. Immerse the root tips in 2 mM 8-hydroxyquinoline solution for 2–4 h at 16 °C (see Note 2).
3. Preparation of PBS: Dissolve 80 g NaCl, 2 g KCl, 14.4 g Na2HPO4, and 2.4 g KH2PO4 in 800 mL ddH2O; adjust pH 7.4 with HCl; raise volume to 1000 mL with ddH2O. Autoclave or filter sterilize. Store at room temperature (RT). Dilute to a 1× concentration with ddH2O for 10 min on ice.
4. Rinse root tips in 1× PBS.
5. Place tips freshly made in 3:1 absolute ethanol/acetic acid solution.
6. Fix root tips for at least 3–4 days before preparing slides, and keep at RT for the first 2 days and later at −20 °C (see Note 3).

3.3 Enzymatic Digestion
1. Preparation of enzyme mixture: Dissolve 400 mg cellulase and 200 mg pectinase in 10 mL 0.01 M citrate buffer. Store in aliquots at −20 °C.
2. Wash the roots twice for 5 min with distilled water in a Petri dish to rinse them.
3. Excise root tips with a fine needle forceps or razor blade and transfer them in a 1.5 mL tube containing 20–50 μL enzyme mixture. Incubate enzyme mixture at 37 °C for about 1 h.
4. Carefully discard enzyme solution and wash root tips three times (5 min each) with cold ddH2O. Leave tubes on ice during rinses.
5. Discard water and add 0.5–1 mL cold fixative solution consisting of 3:1 ethanol (or methanol)/glacial acetic acid. Make fresh before use.
6. Root tips are now ready for slide preparation using one of the following methods (squash method or flame-dry method); alternatively they can be stored at 4 °C until use.

1. Optional preparation step: Stain with acetocarmine for 10–30 min, depending on the level of staining needed to visualize chromosomes.

2. Take one root tip out of staining solution. Locate the root cap. Carefully dissect the root cap away from the meristem and discard (Fig. 1a).

3. Cut a thin slice (1–2 mm) of the meristematic tissue using a razor blade and isolate it from the rest of the root material.

4. Place one drop of fresh 45% acetic acid on the small piece of root tip.

5. Cover carefully with a cover slip (see Note 4).

6. Place one edge of a double-edged razor blade under a corner of the cover slip to produce a makeshift cell spreader/squasher. Tightly press a finger down on one corner (opposite to the corner with razor blade). Do not let the cover slip move (Fig. 1b).

7. While holding one corner, use your other hand to lightly tap on the cover slip with a pointed wooden instrument. The objective is to spread out the cell debris, but not so much as to push the cell material out from under the cover slip.

8. When the spread is complete, carefully remove the razor blade (do not move cover slip) and continue to tap the cover slip to break open the protoplasts, isolating the chromosomes from the rest of the cell debris.

9. Briefly warm the slide over a small alcohol lamp (see Note 5).

10. Fold a piece of filter paper in half and press down on the cover slip to complete the squash.

11. Briefly scan the slide for the presence of chromosomes to determine the quality and quantity of spreads (see Note 6).

12. Place good slides in −20 °C freezer until ready for FISH analysis (see Note 7).

3.4 Slide Preparations

3.4.1 Slide Preparations Using Squash Method (For Digested and Undigested Samples)

1. Using a pipette with a cut tip, transfer one digested root tip to an ice-cold and clean microscopic slide. Add 20–30 μL fixation solution surrounding the specimen on the slide.

2. Using a forceps, macerate the specimen in the fixation solution. Do this quickly to avoid evaporation of the fixative solution and add a few more drops if necessary.

3. Apply additional 20 μL fixation solution to flank each side of the specimen area.
Fig. 1 Technical steps illustrate (a) the location of the root cap that is just proximal to the meristematic tissue, distal to the root stem material, (b) the way to tightly press with finger down on one corner (opposite to corner with razor blade to prevent the cover slip movement), and (c) the way to seal cover slip with a line of rubber cement.
4. Gently heat the bottom of the slide over an alcohol burner. While doing so, briefly expose the specimen and fixation solution to the flame by slightly tilting the slide into the flame. Only expose the sample to the flame long enough for the solution to ignite.

5. Pull the slide away from the flame and allow the flame to extinguish itself by burning up the rest of the fixative. **Steps 4 and 5 should take no more than about 5 s each.**

6. Let the slide dry and cool at room temperature.

7. Slides can be used immediately for FISH. Alternatively prepared slides can be stored, dry, and without a cover slip, in a slide box at RT or at 4 °C.

**3.5 DNA Probes and Labelling**

**3.5.1 Nick Translation Protocol**

1. Prior to working, set PCR machine to incubate 90 min at 15 °C. Determine concentration of DNA by using spectrophotometer or by electrophoresis. At least 1 μg plasmid/BAC DNA for 1 Nick translation reaction is required.

2. Prepare the following buffer solutions:
   
   (a) 10× Nick translation buffer: 5 mL 1 M Tris (pH 7.5), 500 μL 1 M MgCl₂, and 4.5 mL ddH₂O; filter sterilize and store in 1 mL aliquots at 20 °C.
   
   (b) DNase I dilution buffer: 10 mM HEPES (pH 7.5), 50% glycerol, 10 mM CaCl₂, and 10 mM MgCl₂.

3. Combine the following components into sterile PCR tube 0.2 mL: 1 μL of diluted DNase I, 5 μL 10× Nick translation buffer, 1 μL DNA polymerase I, 5 μL A,C,G dNTPs (0.5 mM each), 5 μL biotin- or dig-labeled dUTPs, and 1 μg DNA (plasmid), and make up the total reaction mixture volume to 50 μL with ddH₂O.

4. Mix by pipetting, centrifuge briefly, and incubate reaction in PCR machine for 90 min at 15 °C. Stop the reaction with 1 μL 0.5 M EDTA.

5. Check the Nick translation product on 1.5% agarose gel (look for smear between 100 and 600 bp). Store probe at −20 °C until use.

**3.6 Fluorescence In Situ Hybridization (FISH) Protocol**

Before starting the work, preheat the tube hot block to 90 °C. Put 70% ethanol at −20 °C freezer. Preheat slide denature block to 85 °C. Remove cover slips from the frozen slide and dehydrate with absolute ethanol.

**3.6.1 Solutions Preparation**

1. 20× SSC solution: 175.3 g NaCl, 88.2 g sodium citrate, and ddH₂O to ~800 mL. Adjust pH to 7 with a few drops of concentrated HCl. Add ddH₂O to 1 L final volume, autoclave, and store at RT.

mohan.jain@helsinki.fi
2. 50% dextran sulfate: Dissolve 2.5 g dextran sulfate in 5 mL ddH2O. Make 1 mL aliquots and store at −20 °C.

3. 2× SSC solution: Dilute 50 mL 20× SSC to a 2× concentration with ddH2O.

4. 10× PBS buffer: 80 g NaCl, 2 g KCl, 14.4 g Na2HPO4, and 2.4 g kH2PO4; dissolve in 800 mL ddH2O, adjust to pH 7.4 with HCl, and then bring to a final volume of 1000 mL with ddH2O. Autoclave or filter sterilize. Store at RT. Dilute to a 1× concentration with ddH2O.

5. 10× TNT buffer: Mix 121.13 g Tris, 87.8 g NaCl, and 5 mL of Tween-20, adjust to pH 7.5 with HCl, and bring to 1000 mL with ddH2O. Store at 4 °C.

6. 70% formamide/2× SSC: 2 mL ddH2O, 7 mL deionized formamide, and 1 mL 20× SSC. Store at 4 °C wrapped in a brown bottle.

7. 5× TNB buffer: 5 mL 1 M Tris (pH 7.5), 2.5 mL 3M NaCl, and 0.25 g blocking reagent. Adjust to 10 mL. Heat the solution to 50 °C to decrease the time to dissolve the blocking reagent.

8. Anti-dig-Rhodamine: Reconstitute 200 pg in 1 mL ddH2O. Store at −20 °C in small dark aliquots (protect from light) and avoid repeated freeze and thaw.

3.6.2 Probe Mix

1. Prepare the probe master mix as follows:
   (a) In a sterile, nuclease-free microcentrifuge tube, prepare the probe master mix by adding: 5 μL 100% deionized formamide, 1 μL 20× SSC pH 7.0, 1 μL dig-labeled DNA, 1 μL biotin-labeled DNA, and 2 μL of 50% dextran sulfate (see Note 8).
   (b) Mix well by pipetting. Centrifuge briefly and store master mix on ice until ready to probe slides (see Note 9).

2. Denature the slide as follows:
   (a) Pipette 100 μL 70% formamide/2× SSC solution directly on chromosomes.
   (b) Cover the slide with 22 × 22 mm cover slip.
   (c) Denature on heat block (80–85 °C) for about 1.5 min.
   (d) Immediately immerse in ice cold 70% ethanol about 5 min.
   (e) Again, immerse in 90% ethanol and 100% ethanol 5 min each. Air-dry on bench top.

mohan.jain@helsinki.fi
3. Denature the probe as follows:
   (a) Place probe master mix in a 90 °C hot block for about 10 min.
   (b) Briefly centrifuge (10 s).
   (c) Immerse in ice until ready to probe.

3.6.3 Hybridization and Digital Imaging

1. Probe the slide as follows:
   (a) Add 10 μL denatured probe to each hybridization spot.
   (b) Cover with a 22 × 22 mm cover slip (see Note 10) and seal with a line of rubber cement (Fig. 1c).
   (c) Incubate the slide in a moist hybridization chamber at 37 °C for 8 h.

2. Probe detection as follows:
   (a) Preheat Coplin staining jar containing 2× SSC to 42 °C.
   (b) Remove slides from moist chamber and, without moving cover slip, peel away the line of rubber cement.
   (c) Allow cover slips to fall in a 2× SSC bath.
   (d) Wash one time in 2× SSC for 5 min at RT.
   (e) Wash one more time in 2× SSC for 10 min at 42 °C and finally wash one time in 1× PBS for 5 min at RT.

3. Antibody detection as follows:
   (a) Preparation of detection master mix: Mix 20 μL 5× TNB buffer, 80 μL ddH₂O, 1 μL Rhodamine anti-dig-sheep, 1 μL Alexa Fluor 488 streptavidin in a sterile nuclease-free microcentrifuge tubes.
   (b) Before starting always calibrate the scale and number the slides being used and always protect the fluorophors from light. In a sterile, nuclease-free microcentrifuge tube, prepare the detection master mix by combining the following components: 20 μL of 5× TNB buffer, 80 μL of ddH₂O, 1 μL of Rhodamine anti-dig-sheep, and 1 μL of Alexa Fluor 488 streptavidin.
   (c) Place 100 μL of detection master mix on each slide and cover with a 22 × 40 mm cover slip. Incubate for 1 h at 37 °C in a moist chamber.
   (d) Wash three times (5 min each) in 1× TNT. Wash one time in 1× PBS for 5 min at RT.
   (e) Mount in Vectashield® with DAPI (20 μL or 1 drop).
   (f) Perform fluorescent microscopy for observation.
4. Digital imaging as follows:
   (a) Examine slides under a fluorescence microscope. Capture the chromosome and FISH signal images using CCD (charge-coupled device) camera connected to computer. Then, analyze the captured images using IP-Lab spectrum software.
   (b) While using two or more probes, capture images separately and merge them into a single image using the IP-Lab and Photoshop (Adobe system) imaging software (Fig. 2).

**Fig. 2** Fluorescence in situ hybridization of date palm cv. Zagloul (a and b: with green 45S rDNA probe) and cv. Siwy (c and d: with green 45S rDNA and red 5S rDNA probes), in metaphase chromosomes of date palm. *Arrowheads* in b and d indicate 45S rDNA sites suggested to be located on a Y chromosome. Scale bar = 5 μm. Source: Reproduced from [18]

### 4 Notes

1. Healthy actively growing roots are mostly translucent with a slight yellowing toward the tip.
2. Alternatively, root tips can be treated using any one of the following: ice water (0 °C for 24 h), colchicine (0.05% (w/v) for 3 h at 26 °C), bromonaphthalene (saturated aqueous solution of bromonaphthalene for 3 h at 26 °C), or nitrous oxide gas for 2 h.
3. Alternatively for long-term storage (longer than 1 month), the root tips can be kept in 70% ethanol at 4 or −20 °C.

4. Set down one edge and then let the cover slip fall to eliminate air bubbles.

5. Avoid reaching the boiling point of the 45% acetic acid.

6. A good slide will not only have chromosomes with good morphology, free of cell debris, but also shows many chromosomal sets.

7. Alternatively slides can be frozen by quickly immersing in liquid nitrogen or dry ice, remove cover slips, dehydrate slides using absolute ethanol, and store at 4 °C or RT.

8. Prepare the master mix based on your slide numbers, 10 μL for each slide.

9. Use two DNA probes for FISH experiments. Probe-1 is a fragment containing 45S rDNA repeat unit, and Probe-2 is a fragment of the 5S rDNA gene repeated unit. Label both probes by Nick translation with digoxigenin-11-dUTP and biotin-11-dUTP, respectively.

10. Prepare double volume of the probe master mix for each slide (22 × 22 mm). Make the exact volume, 10 μL for each slide. Use cover slip size 18 × 18 mm.

Acknowledgment

This work was carried out in the Molecular Genetics and Genome Mapping (MGGM) Laboratory at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), and Prof. Jiming Jiang laboratory at the Department of Horticulture, University of Wisconsin-Madison, Madison, WI, USA. This work was funded by the Science and Technology Development Fund (STDF), Ministry of Scientific Research, Egypt (Project No. 406 and 4607).

References


3. Al-Salih AA, Al-Najjar NR, Al-Mashdani AN (1987a) A study on the chromosome number of two specific female date palm cultivars. Date Palm J 5:134–143


mohan.jain@helsinki.fi
Part V

Genomics
Characterization and Amplification of Gene-Based Simple Sequence Repeat (SSR) Markers in Date Palm

Yongli Zhao, Manjunath Keremane, Channapatna S. Prakash, and Guohao He

Abstract
The paucity of molecular markers limits the application of genetic and genomic research in date palm (Phoenix dactylifera L.). Availability of expressed sequence tag (EST) sequences in date palm may provide a good resource for developing gene-based markers. This study characterizes a substantial fraction of transcriptome sequences containing simple sequence repeats (SSRs) from the EST sequences in date palm. The EST sequences studied are mainly homologous to those of Elaeis guineensis and Musa acuminate. A total of 911 gene-based SSR markers, characterized with functional annotations, have provided a useful basis not only for discovering candidate genes and understanding genetic basis of traits of interest but also for developing genetic and genomic tools for molecular research in date palm, such as diversity study, quantitative trait locus (QTL) mapping, and molecular breeding. The procedures of DNA extraction, polymerase chain reaction (PCR) amplification of these gene-based SSR markers, and gel electrophoresis of PCR products are described in this chapter.

Key words  Gene-based marker, EST-SSR, Polymorphism

1 Introduction
Gene sequences have been rapidly accumulated during the past two decades, because of the increase in sequencing the genomes and transcriptomes using advanced sequencing technologies at reduced cost. These gene sequence data provide an excellent resource for developing molecular markers, i.e., gene-based markers. One of the gene-based markers is simple sequence repeat (SSR) markers in which SSRs are mined within gene sequences from a given genome. Variation in SSR motifs and flanking sequences will allow us to develop either gene-targeted markers or functional markers that closely associate with genes underlying specific biochemical or physiological functions.

Date palm genome sequences and transcriptomes database are publicly available at http://qatar-weill.cornell.edu/research/
datepalmGenome/download.html, which provides a resource for identifying gene-based markers. A total of 28,889 EST sequences were used to mine SSRs with the cutoff of repeat number ≥5 for dinucleotide SSRs, ≥4 for trinucleotide SSRs, and ≥3 for tetranucleotide SSRs. Primers were designed for 4555 EST sequences containing SSRs using BatchPrimer3 [1] with the following conditions: optimum primer length of 20 nucleotides, optimum melting temperature of 50 °C, optimum product size of 150 bp, and optimum G/C content of 50% [2].

To assign putative functions to EST sequences containing SSRs, the BLASTx algorithm [3] was used to compare sequences with the nonredundant protein sequence database in the NCBI (http://www.ncbi.nlm.nih.gov) with an E-value of less than 1.0E−20. A total of 1032 sequences containing SSRs were found having homology with known proteins. Gene ontology (GO, http://geneontology.org) based annotation was performed using Blast2GO [4] to obtain annotation of EST sequences containing SSRs based on BLASTx top hits against the NCBI Nr database with an E-value cutoff 1.0E−5. The species distribution of the best match results for date palm EST sequences containing SSRs is shown in Fig. 1. Among these, most date palm sequences matched those of African oil palm (Elaeis guineensis), followed by wild banana (Musa acuminata), Indian lotus (Nelumbo nucifera),

---

**Fig. 1** Species distribution of date palm EST sequences containing SSRs

mohan.jain@helsinki.fi
grapevine \((\text{Vitis vinifera})\), etc. The functional annotations of date palm EST sequences containing SSRs were classified into three main categories: biological processes, molecular functions, and cellular components including functional 40 GO terms. The functional annotations assigned in the biological process were mainly associated with metabolic process (22.72%), cellular process (20.69%), single organism process (16.84%), biological regulation (7.90%), response to stimulus (6.22%), and localization (5.63%). Those assigned to molecular function were mainly linked to catalytic activity (44.70%) and binding (41.48%). Finally, those assigned to the cellular component were mainly related to cell (37.83%), organelle (28.97%), and membrane (18.61%) (Fig. 2). The enzyme code distribution showed that most sequences belong to enzyme code classes of oxidoreductases, transferases, and hydrolases (Fig. 3). By Blast using Blast2GO, sequences having no hit were removed and finally resulted in a total of 911 EST sequences containing SSRs with functional annotation in date palm (Table 1).

The objectives in this chapter are to describe the protocols of DNA extraction, PCR amplification of gene-based SSR markers, and gel electrophoresis, taking the marker DPG1184 linked to auxin response gene as an example for identifying polymorphism between different genotypes (Fig. 4).

### 2 Materials

#### 2.1 Plant Materials

Twelve date palm cultivars, Amir Hajj, Hilali, Khir, Ashrasi, Crane, Abada, Medjool, Black Sphinx, Bentamoda, Horra, Dayri, and Boyer #1, were selected for study from the USDA-ARS National Germplasm Repository for Citrus and Dates (Riverside CA, USA).

#### 2.2 Solutions

1. 5× TBE (Tris-Borate EDTA) stock solution pH 8.0 (1 L): 200 mL sterile distilled water, 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA, and water up to 1000 mL.

2. 1× TBE buffer: 400 mL of ddH2O and 100 mL of 5× TBE solution.

3. A 6% non-denaturing polyacrylamide gel solution: 6 mL acrylamide/bisacrylamide (29:1, 30% w/v), 6 mL 5× TBE buffer, and 18 mL water.

4. Ammonium persulfate (APS) (10%, w/v): 1 g APS per 10 mL water.

5. Ethidium bromide (10 mg/mL): 0.5 μg/mL working solution.

6. 10× loading dye: 0.8 g of bromophenol blue (40%) and 0.08 g xylene cyanol FF, final volume to 50 mL glycerol.

7. 1 kb ladder (50 ng/μL): 10 μL of 1 kb ladder (1 μg/μL) and 20 μL of 10× loading buffer and 170 μL distilled water.
Fig. 2 Gene ontology parsing annotated EST sequences containing SSRs into three main clusters: (1) biological process, (2) molecular function, and (3) cellular component.
2.3 Equipment

1. Mortar and pestle.
2. Water bath.
3. Centrifuge.
5. Nanodrop spectrophotometer.
6. Vortex machine.
7. Image system.

3 Methods

3.1 Solutions

Perpetration

1. $5 \times$ TBE (Tris-Borate EDTA) stock solution pH 8.0 (1 L): Add about 200 mL sterile distilled water to a 1 L graduated cylinder. Weigh 54 g Tris base and 27.5 g boric acid and transfer to a cylinder. Add 20 mL 0.5 M EDTA, pH 8.0, into the cylinder and stir; add water up to 1000 mL. Store at 4 $^\circ$C.

2. $1 \times$ TBE buffer: Add 400 mL of ddH$_2$O into 100 mL of $5 \times$ TBE solution.

3. A 6% non-denaturing polyacrylamide gel solution (see Note 1): Mix 6 mL acrylamide/bisacrylamide (29:1, 30% w/v) (see Note 2), 6 mL $5 \times$ TBE buffer, and 18 mL of water. The total amount (30 mL) is sufficient to make one gel (18 cm $\times$ 18 cm $\times$ 0.75 cm).

4. Ammonium persulfate (APS) (10% w/v): Dissolve 1 g APS into 8 mL of water and add water to 10 mL (see Note 3).

5. Ethidium bromide (10 mg/mL): Prepare 0.5 $\mu$g/mL working solution. Add 10 $\mu$L of 10 mg/mL ethidium bromide solution to 200 mL water.

---

Fig. 3 Enzyme code distribution of EST sequences containing SSRs

Fig. 3 Enzyme code distribution of EST sequences containing SSRs

---
Table 1
A list of selected EST sequences containing SSR from 911 EST-SSRs with functional annotation in date palm (for more EST-SSRs, see supplemental file of Zhao et al. [2])

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence description</th>
<th>Primer sequence (5′–3′)</th>
<th>Prod size</th>
<th>Motif</th>
<th>Repeat number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPG0288</td>
<td>Transport protein</td>
<td>F-TAGAGCTACCATCCAAATCATCAG R-GTTGTGGAATTATACAGAGACAGACA</td>
<td>136</td>
<td>CTTT</td>
<td>3</td>
</tr>
<tr>
<td>DPG0295</td>
<td>Protein enhanced disease resistance 2-like</td>
<td>F-TGCATATGCCTCTTGTATAGCR-AGAAGGATCGAGAAATCTCTAGG</td>
<td>152</td>
<td>TCA</td>
<td>5</td>
</tr>
<tr>
<td>DPG0301</td>
<td>Disease resistance rpp13-like protein 3</td>
<td>F-CAGCCAACTCTTCACACATAC R-GGTGCAGAGAAGAGATGACT</td>
<td>155</td>
<td>CTT</td>
<td>4</td>
</tr>
<tr>
<td>DPG0492</td>
<td>Transcription elongation factor spt5 homologue 1</td>
<td>F-AAGAGGATGAAAGGAAGGT R-GCTTCTAGGTCGAAGAACTCC</td>
<td>163</td>
<td>GAG, GAG</td>
<td>4,5</td>
</tr>
<tr>
<td>DPG0665</td>
<td>Universal stress protein family protein</td>
<td>F-CTCACCCCTCCACACATC R-GACTTTGGCTAGAGAAGCTAG</td>
<td>195</td>
<td>CAC</td>
<td>6</td>
</tr>
<tr>
<td>DPG0685</td>
<td>Swim zinc finger family protein</td>
<td>F-TGTAGGCTGATACCTCTTTTCG R-GTAAGCCAGGAGGAGATGTA</td>
<td>156</td>
<td>TG</td>
<td>5</td>
</tr>
<tr>
<td>DPG0866</td>
<td>Sucrose synthase 2-like</td>
<td>F-GATAATCGCGTTCTTCTTCTCTCT R-TGATATCGAGCAACTCCATTC</td>
<td>146</td>
<td>TG</td>
<td>5</td>
</tr>
<tr>
<td>DPG1099</td>
<td>Transcription initiation factor</td>
<td>F-AAGTCAGCTAGAAAGGTGTCGAGAGAAGACGTTGAT</td>
<td>147</td>
<td>TGA, GAT</td>
<td>5,4</td>
</tr>
<tr>
<td>DPG1115</td>
<td>Kinase-like protein tmkl1</td>
<td>F-AITTTCCATCTCCCTCTCTCTCT R-CCTTTCTACAATAACTCTCCAC</td>
<td>210</td>
<td>TTC</td>
<td>4</td>
</tr>
<tr>
<td>DPG1144</td>
<td>Sugar carrier protein c-like</td>
<td>F-CAGACAAAGGATCATCTCTTCA R-GTGAACATGTGGATGACTCTTC</td>
<td>173</td>
<td>AAG</td>
<td>4</td>
</tr>
<tr>
<td>DPG1184</td>
<td>Auxin response</td>
<td>F-CATCAAGTGCGAGTATT GT-R-CCTGTTCACAAACTAAATCTCTC</td>
<td>176</td>
<td>GCT, TGG</td>
<td>5,4</td>
</tr>
<tr>
<td>DPG1329</td>
<td>Stress-induced phosphoprotein 1</td>
<td>F-GAGCAGAGACACAGACTGAT R-ATGTCTTGGGTGATTTACCA</td>
<td>155</td>
<td>CA</td>
<td>5</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Length</td>
<td>Primer</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>DPG1697</td>
<td>Regulatory protein npr3-like</td>
<td>F-ACATGACTGAGTCTTCACCAC</td>
<td>R-AGTGCTGCTATGATTTGTGAG</td>
<td>154</td>
<td>TC</td>
</tr>
<tr>
<td>DPG1822</td>
<td>Growth-regulating factor 3</td>
<td>F-CATTTCCTCCCATCTGGTCTC</td>
<td>R-CCATATGAGCTCATTCCTACCT</td>
<td>173</td>
<td>TGG</td>
</tr>
<tr>
<td>DPG2082</td>
<td>Receptor protein kinase 1</td>
<td>F-GGCAATGACTTTATCTGGTGA</td>
<td>R-CTTCGCTCGGTTCTCTACTAT</td>
<td>153</td>
<td>GA</td>
</tr>
<tr>
<td>DPG2175</td>
<td>Disease resistance protein rga1</td>
<td>F-CTATCTTCCTTCCAGAGTGGT</td>
<td>R-TCACCATCATCATATTGTGTCATC</td>
<td>161</td>
<td>ATG</td>
</tr>
<tr>
<td>DPG2216</td>
<td>Transcriptional activator demeter-like</td>
<td>F-GATAAAAGTGGAGAAGCTCAG</td>
<td>R-GTTCAAGGGAGGTTGAACCT</td>
<td>148</td>
<td>CAG</td>
</tr>
<tr>
<td>DPG2281</td>
<td>DNA repair protein rad50</td>
<td>F-CCCGGTACTGATAACCCTA</td>
<td>R-AGATGCAAGTGATGTTAAAAAC</td>
<td>155</td>
<td>CCAT</td>
</tr>
<tr>
<td>DPG2330</td>
<td>Heat stress transcription factor b-1-like</td>
<td>F-CTCTGTTGCTCAGTAGGTTTTG</td>
<td>R-GAACGAGAACTTCCCTTCA</td>
<td>133</td>
<td>GCG</td>
</tr>
<tr>
<td>DPG2392</td>
<td>Probable aspartyl aminopeptidase</td>
<td>F-ATCTGATTGAGCGAGAAGA</td>
<td>R-GCATCCCTCTCTATCATATT</td>
<td>118</td>
<td>GAG</td>
</tr>
<tr>
<td>DPG2481</td>
<td>Zinc finger protein zat1-like</td>
<td>F-CCAAAGAAGGTTGAACTCAGA</td>
<td>R-CTTCTTCTTGAGAGGACTCTT</td>
<td>150</td>
<td>TGA</td>
</tr>
<tr>
<td>DPG2513</td>
<td>Neurofilament medium polypeptide-like</td>
<td>F-AAGAAGGAGAGAAGAGGAGGAGG</td>
<td>R-TAGACAGCAGTACAGAATT</td>
<td>170</td>
<td>CGG, GGA</td>
</tr>
<tr>
<td>DPG2530</td>
<td>Expansin-a2-like</td>
<td>F-CTCTTCTCAGTAGTACTCGCTCTCT</td>
<td>R-CCGTAGAGGTGGTTGCCATAC</td>
<td>169</td>
<td>CTT</td>
</tr>
<tr>
<td>DPG2587</td>
<td>Pre-mRNA-splicing factor cwc22 homologue</td>
<td>F-GGCCATTACAGAGAAGCTTGAGA</td>
<td>R-AGACTCTAAACGCCAGCTCAGA</td>
<td>153</td>
<td>GCA</td>
</tr>
<tr>
<td>DPG3025</td>
<td>Heat stress transcription factor a-5-like</td>
<td>F-ATCATTCCTCGAGACCCTCT</td>
<td>R-GCTCAAGATGATTGCTTTCT</td>
<td>166</td>
<td>ACG</td>
</tr>
<tr>
<td>DPG3219</td>
<td>Myb DNA-binding domain superfamily protein</td>
<td>F-TGGTCTGCTGCTTCTCTCTGT</td>
<td>R-GAGTTGGAGTTGAGTTGG</td>
<td>210</td>
<td>CCT</td>
</tr>
<tr>
<td>Primer name</td>
<td>Sequence description</td>
<td>Primer sequence (5’–3’)</td>
<td>Prod size</td>
<td>Motif</td>
<td>Repeat number</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------</td>
<td>-----------</td>
<td>-------</td>
<td>---------------</td>
</tr>
<tr>
<td>DPG3549</td>
<td>Cell wall protein rbr3</td>
<td>F-AAAGGGTCGCCGCTCGATT R-ATCTTCACAATTCCGTTTTC</td>
<td>125</td>
<td>GGAG</td>
<td>3</td>
</tr>
<tr>
<td>DPG3668</td>
<td>DNA repair and recombination protein rad54</td>
<td>F-CAGAGCTATTTCCTGGGAGAT R-GCTGAGAATGGACATGTACGA</td>
<td>169</td>
<td>TGG</td>
<td>4</td>
</tr>
<tr>
<td>DPG3742</td>
<td>Biotin-protein ligase 2-like</td>
<td>F-GAGCCTTTAGGGTTCTAGTCCT R-AAACCCCTTCTCCACCAAC</td>
<td>150</td>
<td>GAG</td>
<td>4</td>
</tr>
<tr>
<td>DPG3782</td>
<td>Myb-like protein p</td>
<td>F-ACATATACCAGAGAGGCAAC R-CAACTTCTGAGCCTTTC</td>
<td>152</td>
<td>GA</td>
<td>6</td>
</tr>
<tr>
<td>DPG3841</td>
<td>Acetolactate synthase</td>
<td>F-CGCACAGTATTACAGCTACAAG R-TTCTGAGAAACTCCCATC</td>
<td>156</td>
<td>CAG</td>
<td>4</td>
</tr>
<tr>
<td>DPG3861</td>
<td>Myb-like protein aa</td>
<td>F-CTACGTGGCTCTACTCTCCT R-TGATGAGAAACTCCCATC</td>
<td>152</td>
<td>ATG</td>
<td>4</td>
</tr>
<tr>
<td>DPG3939</td>
<td>Growth-regulating factor 1-like</td>
<td>F-AGAAAGCTGTGGGAATGTA R-AGAGGTGGTGAAGTCTGGA</td>
<td>149</td>
<td>CCA</td>
<td>7</td>
</tr>
<tr>
<td>DPG442</td>
<td>Protein suppressor of gene silencing 3 homologue</td>
<td>F-ATCCACCTCTCTCCATCTCCAGC R-GTGGGCAAGCAGGACTTCTC</td>
<td>162</td>
<td>CAG</td>
<td>6</td>
</tr>
<tr>
<td>DPG447</td>
<td>Oxalate-ligase</td>
<td>F-AGCGAATCGTTTATAAACAC R-GAGATAAATTGCGTTGAT</td>
<td>125</td>
<td>CCT</td>
<td>4</td>
</tr>
<tr>
<td>DPG4619</td>
<td>Lipid-binding protein</td>
<td>F-GATGGTGGCTGCGTGGTG R-TCGTATTGTTCAGCTACTGC</td>
<td>110</td>
<td>GGC</td>
<td>4</td>
</tr>
<tr>
<td>DPG4622</td>
<td>Disease resistance protein rga4</td>
<td>F-AATTGGCTCCCAAGAATGTC R-CTATCTTCGCCGACCTTAAAACC</td>
<td>151</td>
<td>GAT</td>
<td>5</td>
</tr>
<tr>
<td>DPG4848</td>
<td>Disease resistance protein rga3</td>
<td>F-CTCTTGCTTCTCTTCACCTTT R-AATGACCTGAGAAGGAGTGC</td>
<td>135</td>
<td>GGA</td>
<td>4</td>
</tr>
</tbody>
</table>
6. 10× loading dye: Weigh 0.8 g of bromophenol blue (40%) and 0.08 g of xylene cyanol FF. Mix with 25 mL glycerol and make the volume up to 50 mL. Aliquot to 1.5 mL tubes and store at 4 °C.

7. 1 kb ladder: Take 10 μL of 1 kb ladder (1 μg/μL) and 20 μL of 10× loading buffer and mix with 170 μL of distilled water to get final concentration (50 ng/μL).

### 3.2 DNA Extraction

The MagAttract 96 DNA Plant Core Kit (Qiagen, Inc.) is used to extract date palm DNA and follow the procedure of the kit with minor modification.

1. Each well of the deep well plates is pre-filled with 2.5 mm 8–10 zirconium beads (Glen Mills Inc.) and covered with a Capcluster (USA Scientific, Inc.).

2. About 100 mg of leaf tissue is chopped and placed into each well of a 96-deep well flat-bottom microplates (USA Scientific, Inc.), and place the microplate in lyophilizer.

3. The deep well plates with lyophilized samples are processed in a Beadbeater (Biospec, Inc.) for 2 min for three times to grind the samples and then centrifuged for 5 min at 3750 × g.

4. Carefully remove the caps from individual columns, and immediately add 500 μL buffer RLT to each well. The caps are replaced carefully and the process is repeated for the entire plate. The samples are ground again using the Beadbeater as described above.

5. Centrifuge the microplate for 5 min at 6000 × g.

6. Add 65 μL of buffer RB and 20 μL of resuspended MagAttract Suspension A (see Note 4) to each well of a new 96-well microplate or 1.5 mL microcentrifuge tube.

![Fig. 4 Polymorphism detected among 12 date palm genotypes by auxin response gene-related marker (DPG1184)](image-url)
7. Transfer 200 μL plant lysate supernatant (from step 5) into each well of the new microplate or into the microcentrifuge tube described in the previous step, and mix by pipetting up and down several times.

8. Incubate at room temperature (15–25 °C) for 5 min. Mix once during incubation.

9. Place the microplate or microcentrifuge tube on the magnet and remove the supernatant after magnetic separation.

10. Wash the pelleted MagAttract particles by adding 200 μL buffer RPW (see Note 5), resuspend the particles, place the plate or tube on the magnet, and remove the supernatant.

11. Wash the pelleted MagAttract particles by adding 200 μL ethanol (96–100%), resuspend the particles, place the plate or tube on the magnet, and remove the supernatant.

12. Repeat step 11; aspirate as much ethanol as possible.

13. Dry the MagAttract particles for 5–10 min at room temperature (15–25 °C).

14. Resuspend the MagAttract particles in 100 μL buffer AE.

15. Incubate at room temperature for 5 min.

16. Place the microplate or microcentrifuge tube on a magnet, and transfer the DNA eluates to a clean 96-well round-bottom microplate or microcentrifuge tube.

17. Measure DNA concentration using Nanodrop spectrophotometer (Thermo Scientific Co.).

### 3.3 PCR Amplification

1. Dilute DNA concentration to the working concentration of 25 ng/μL.

2. Dilute 100 μM each of forward and reverse primer stock solution to working concentration 5 μM/each by adding 95 μL water to 5 μL of 100 μM of stock solution.

3. PCR reaction mix: Add 1 μL of 25 ng/μL date palm genomic DNA, 5 μM/each of forward and reverse primers, 1× PCR buffer (20 mM Tris–HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100), 0.2 mM dNTPs, and 1 U Taq polymerase (5 U/μL) in a total volume of 20 μL.

4. PCR condition: 95 °C for 5 min for initial denaturing, followed by 35 cycles of 95 °C for 30 s, 48 °C for 30 s, 72 °C for 1.5 min, and final extension at 72 °C for 5 min using a thermal cycler.

5. PCR products are resolved in either polyacrylamide gel or agarose gel.

### 3.4 Polyacrylamide Gel Electrophoresis

1. Clean the glass plates, spacers, and combs thoroughly. Hold the plates by the edges or wear gloves, so that hand oils do not become deposited on the working surfaces of the plates. Rinse
the plates with deionized water and spray a bit of 70% ethanol on the plates, and wipe dry with Kimwipes (see Note 6).

2. Assemble the glass plates with spacers in a gel caster. Make sure that the bottom edges of the two plates are flush to avoid leakage.

3. Wear gloves (see Note 7). Add 300 μL 10% APS and 30 μL N,N,N',N'-Tetramethylethylenediamine (TEMED) into 30 mL non-denaturing polyacrylamide gel solution. Once TEMED is added, the gel will begin to polymerize, so work quickly but carefully to pour or pipette the gel solution between the plates.

4. Immediately insert the appropriate comb into the gel, being careful not to allow air bubbles to become trapped under the teeth. Clamp the comb in place with bulldog paper clips. Make sure that no gel solution is leaking from the gel mold.

5. Allow the gel solution to polymerize for 30–60 min at room temperature.

6. After polymerization is complete, if not to be immediately used, surround the comb and the top of the gel with paper towels that have been soaked in 1× TBE. Then seal the entire gel in Saran Wrap or a plastic bag and store it at 4 °C until needed.

7. When ready to proceed with electrophoresis, add the electrophoresis buffer (1× TBE) in the upper and bottom reservoirs (see Note 8), and carefully pull the combs from the polymerized gel. Use a Pasteur pipette or a syringe to flush out the wells once with 1× TBE. Mix 20 μL PCR products with 2 μL 10× loading dye and load into the wells.

8. Connect the electrodes to a power pack, turn on the power, and begin the electrophoresis run with 1–8 V/cm (see Note 9).

9. Run the gel until the loading dyes have migrated the desired distance. Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs.

10. Detach the glass plates. Lay the glass plates on the bench. Use a spacer or plastic wedge to lift a corner of the upper glass plate. Check that the gel remains attached to one plate. Pull another plate smoothly away. Remove the spacers.

11. Stain gels with ethidium bromide (20 μL of 10 mg/mL ethidium bromide in 400 mL water) (see Note 10) for 5–10 min and take a photo under UV light.

3.5 Agarose Gel Electrophoresis

1. Prepare 2% agarose gel: Weigh 2 g agarose and transfer to a 300 mL flask. Add 100 mL 1× TBE buffer and stir. Heat the slurry in a microwave oven until the agarose is completely

mohan.jain@helsinki.fi
dissolved. Cool the solution to 60 °C and add 5 μL of ethidium bromide (10 mg/mL) for every 100 mL of agarose slurry.

2. Set up the casting tray on a flat surface so that the agarose is of uniform thickness throughout the tray. Close both the ends of the casting tray with sealing tape.

3. Pour the agarose gel solution into the tray slowly without the formation of any air bubbles. Leave the tray undisturbed for about 30–45 min until the agarose solidifies completely.

4. Fill the gel tank with the electrophoresis buffer (1× TBE), and remove the sealers carefully and place the tray in the gel tank submerged in the electrophoresis buffer.

5. Allow the gel with combs for some time in the buffer, so that the removal of combs is easy without making any holes in the gel.

6. Loading the gel: PCR products are mixed with loading buffer and are loaded in the slots of the submerged gel. The maximum volume of the sample to be loaded depends on the slot size. Usually sample volume of 10–20 μL is loaded in each slot.

7. Load the size marker (1 kb ladder) along with the samples.

8. Running the gel: Run the agarose gel slowly (20–30 V/cm) at room temperature.

9. Staining the gel: Stain the gel with ethidium bromide (1 μg/mL) solution for 5–10 min.

10. Photography: Place the gel in an image system and take a picture under UV light. The DNA is lit up under UV since it has the ethidium bromide.

4 Notes

1. 6% non-denaturing polyacrylamide gels can separate DNA fragment sizes between 75 and 2000 bp and are usually run at voltages between 1 and 8 V/cm.

2. Gels can be casted with polyacrylamide solutions containing different acrylamide/bisacrylamide (cross-link) ratios, such as 19:1 and 37.5:1, rather than the 29:1 ratio recommended here.

3. Make fresh 10% APS solution before use for best performance.

4. Shake the bottle containing MagAttract Suspension A and vortex for 5 min (before the first use) or 1 min (before subsequent uses) to ensure that the magnetic particles are fully resuspended before use. Resuspension of the magnetic particles should be done very carefully since the efficiency of washing is directly related to how well the particles are resuspended. Resuspension can be performed by pipetting or by vortexing.
5. Add 125 mL isopropanol and 1 vial RNase A (1 × 220 μL) to each bottle of buffer RPW (125 mL) before use.

6. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.

7. Warning: Unpolymerized acrylamide is a neurotoxin!

8. It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

9. If electrophoresis is carried out at a higher voltage than 10 V/cm, differential heating in the center of the gel may cause bowing of the DNA bands or even melting of the strands of small DNA fragments.

10. Caution: Ethidium bromide is highly carcinogenic. Always wear gloves and protective goggles while handling it. Do not dispose it in an open environment.

References


Mitochondrial Molecular Markers for Resistance to Bayoud Disease in Date Palm

Amgad A. Saleh, Anwar H. Sharafaddin, Mahmoud H. El-Komy, Yasser E. Ibrahim, Younis K. Hamad, and Younis Y. Molan

Abstract

Bayoud disease, caused by *Fusarium oxysporum* f. sp. *albedinis*, is a very serious and destructive disease to date palm. Screening of date palm germplasm for resistance to bayoud disease is a crucial step to avoid or alleviate the disease consequences. Fortunately, it was discovered that there are two mitochondrial plasmid-like DNA molecules associated with susceptibility or resistance to bayoud disease. In this chapter, we present a fast, simple, and reliable technique to screen date palm germplasm for the presence of these mitochondrial molecular markers associated with susceptibility or resistance to bayoud.

**Key words** Bayoud, Mitochondria, Plasmid, *Fusarium oxysporum* f. sp. *albedinis*, Molecular markers

1 Introduction

Among different *Fusarium oxysporum* formae speciales causing date palm wilt, *F. oxysporum* f. sp. *albedinis* (FOA) is the most destructive one to date palm plantations [1, 2]. The date palm Fusarium wilt caused by FOA fungus is called bayoud referring to the Arabic word *abiadh* meaning white due to the whitening of the fronds of the diseased trees. Bayoud was first discovered in Morocco in 1870, and within one century, it was responsible for destroying more than 15 million trees in Morocco and Algeria [2, 3]. Moreover, there are a few reports, which need to be confirmed, claiming the existence of bayoud in Mauritania, Tunisia, and Egypt [4, 5].

Early screening of the resistance/susceptibility of date palm germplasm to bayoud is a very useful step in breeding programs for developing bayoud-resistant cultivars. Three mitochondrial plasmid-like DNA molecules, designated as U, S, and R plasmids, have been isolated from date palm [6, 7]. The U plasmid is found in all tested date palm cultivars [7]. However, the S plasmid has been isolated mainly from bayoud-susceptible cultivars, whereas the...
R plasmid has been generally found in bayoud-resistant cultivars [6–8]. The nucleotide similarity between the S and R plasmids is more than 95%, with the R plasmid lacking a DNA segment, with variable sizes according to date palm cultivars, that may be eliminated by an intra-plasmid recombination event within the S plasmid [7, 9]. It has been shown that the presence of R or S plasmids can be used as a diagnostic tool to screen bayoud-resistance and bayoud-susceptible date palm trees [8]. The absence or presence of these mitochondrial plasmids has been used successfully to evaluate resistance of date palm germplasm to bayoud disease in Morocco, Tunisia, Mauritania, and Saudi Arabia [8–11].

The main objective of this chapter is to present a fast, simple, and reliable technique to screen date palm germplasm for the presence of the mitochondrial molecular markers that are associated with susceptibility or resistance to bayoud. The early screening of the resistance/susceptibility of date palm germplasm to bayoud should be very useful in breeding programs for developing bayoud-resistant cultivars.

2 Materials

2.1 Biological and Nonbiological Materials

1. Young not-fully-opened date palm leaflets (Fig. 1; see Note 1).
2. Liquid nitrogen.

Fig. 1 Different steps of preparing date palm leaflet cuttings for DNA extraction. (a) Shows unopened date palm leaf with leaflets around it, (b) shows cleaned leaflets, (c) shows leaflet cuttings, (d–e) shows a mortar with liquid nitrogen and date palm leaflet cuttings before and after grinding, and (f) shows an Eppendorf tube containing ground date palm tissue.
3. Latex or nitrile gloves (see Note 1).
4. Eppendorf tubes (1.5 or 2 mL).
5. Mortar and pestle.
7. Microcentrifuge.
8. Thermocycler.
9. Horizontal gel electrophoresis system.
11. Gel documentation system.

2.2 Chemicals and Buffers
1. Cetyltrimethylammonium bromide (CTAB) extraction buffer: 2% CTAB, 20 mM EDTA (pH 8.0), 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, and 1% β-mercaptoethanol (see Note 2).
4. TE buffer: 10 mM Tris–HCl (pH 8) and 1 mM EDTA (pH 8).
5. 100% isopropanol.
6. Washing solution: 70% ethanol.
7. Primers: oli1 (5'-CCTTATACCAGTCGTGCTT-3') and oli2 (5'-AAGGCAGATATAATCGGA-3') to amplify the diagnostic DNA bands.
8. Agarose.
9. DNA staining dye: ethidium bromide or any other safe DNA staining dye, e.g., acridine orange, SYBR Safe, and SYBR Gold (see Note 3).
10. 10× Tris/boric acid/EDTA (TBE) buffer: 0.89 M Tris base (Trizma), 0.89 M boric acid, and 20 mM EDTA (pH 8).
11. 6× DNA loading dye: 1× TE buffer (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol.
12. DNA ladder: a collection of DNA fragments of known quantities and lengths.
13. 10× polymerase chain reaction (PCR) buffer.
14. Taq DNA polymerase 5 U/μL.
15. 25 mM MgCl2.
16. 2 mM dNTPs: 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, and 0.5 mM dTTP.
3 Methods

3.1 Tissue Grinding

1. Clean three leaflets from each tree with 96% ethanol to remove dust particles and any microorganisms (Fig. 1).
2. Cut the surface-sterilized leaflets into ca. 1 cm² small pieces using clean scissors (Fig. 1; see Note 4).
3. Grind the leaflet cutting under liquid nitrogen using a mortar and pestle (Fig. 1; see Note 5).
4. Transfer ground date palm tissue to 2-mL Eppendorf tubes and fill only 1/3 of each tube (Fig. 1; see Note 6).

3.2 DNA Extraction

1. Add 700 μL warm CTAB extraction buffer to 2-mL Eppendorf tubes containing ground tissue and vortex for 10 s (see Note 7).
2. Incubate the tubes at 65 °C in water bath for 30–60 min, and vortex the tubes every 10–15 min for few seconds (see Note 8).
3. Remove the tubes from 65 °C water bath and gently mix components by inverting the tubes few times.
4. Add 700 μL chloroform/isoamyl alcohol (24:1) to each tube, and gently invert few times (Fig. 2; see Note 9).
5. Centrifuge the tubes in a microcentrifuge at 10,000 × g for 15 min at 4 °C.
6. Transfer approximately 600 μL of upper layer to a new tube (Fig. 2; see Note 10).
7. Dispose of the tubes containing plant debris and chloroform in an appropriate place (see Note 11).
8. Precipitate DNA by adding one volume of isopropanol to the tubes and incubate the tubes at room temperature for 15–20 min or at −20 °C overnight (see Note 12).
9. Pellet DNA by centrifuging the tubes at 10,000 × g for 15 min (Fig. 2).
10. Decant the supernatant without disturbing DNA pellets and then wash them with 500 μL 70% ethanol.
11. Centrifuge the tubes at 10,000 × g for 3 min, and then carefully decant ethanol.
12. Dry DNA pellets at room temperature for 15 min.
13. Resuspend DNA pellets in 600 μL sterile double-distilled H₂O or TE buffer (see Note 13).
14. To remove RNA, add 1 μL RNase A (100 μg/μL) for each DNA preparation and incubate at 37 °C for 60 min (see Note 13).
15. Extract DNA by adding 400 μL phenol/chloroform/isoamyl alcohol (25:24:1) mixture and mix components by gentle inversion until an emulsion is formed.
16. Centrifugie at 10,000 × g for 10 min at 4 °C.
17. Transfer approximately 500 μL of the upper layer without disturbing the interphase layer to a new tube (Fig. 2; see Notes 10 and 11).

18. Add 500 μL chloroform/isoamyl alcohol (24:1) to each tube and gently invert it few times (see Note 9).

19. Centrifuge tubes at 10,000 × g for 10 min, and transfer upper layer to a new tube.
20. Precipitate DNA by adding one volume of isopropanol to the tubes and incubate the tubes at room temperature for 15–20 min or at −20 °C overnight (Fig. 2; see Note 12).

21. Pellet DNA by centrifuging the tubes at 10,000 × g for 15 min.

22. Decant the supernatant without disturbing DNA pellets and wash with 500 μL 70% ethanol.

23. Centrifuge the tubes at 10,000 × g for 3 min, and then carefully decant ethanol.

24. Dry DNA pellets at room temperature for 15 min (Fig. 2).

25. Resuspend DNA pellets in 50 μL sterile double-distilled H₂O or TE buffer and keep the tubes overnight at 4 °C (see Note 14).

26. Evaluate DNA quality and quantity by staining 1% agarose gels with ethidium bromide or acridine orange and alternatively with spectrophotometer and then adjust DNA concentration to 20 ng/μL by diluting DNA preparations with appropriate amount of sterile double-distilled H₂O or TE buffer.

### 3.3 DNA Quantification

1. Prepare 1% agarose gel by weighing 2 g agarose and putting it in a 500-mL conical flask (see Note 15).

2. Add 200 mL 0.5× TBE to the flask and microwave for 1–3 min until the solution becomes clear (see Note 16).

3. Leave the flask to cool down to 50–60 °C and then add 2 μL of 10 mg/mL ethidium bromide or 4 μL of 10 mg/mL acridine orange and swirl gently without creating air bubbles.

4. After getting a homogenous DNA dye/agarose mixture, pour it gently, without creating air bubbles, into the appropriate casting tray with the desired comb.

5. Wait approximately 30 min for the gel to solidify.

6. When gel is solidified, submerge it into the electrophoresis tank, add 0.5× TBE buffer until covering the gel, and then gently remove the comb.

7. Collect Eppendorf tubes containing DNA preparations from freezer, leave to thaw, flick gently, and then centrifuge them for few seconds.

8. Mix 2 μL of loading dye with 2 μL of each DNA preparation, add 6 μL sterile double-distilled H₂O to the 4 μL mixture, and gently mix the contents.

9. Load the first well with 5 μL DNA ladder and the following wells with 10 μL DNA/DNA loading dye mixture.

10. Set the electrophoresis to run at 1–3 V/cm (the distance is measured from negative to positive electrodes) for 15–30 min. DNAs migrate from negative to positive electrodes.
11. Turn off electric power, gently carry the gel, and examine it under UV transilluminator for visualizing separated bands, and photograph using appropriate gel imaging and analysis system, e.g., Syngene InGenius LHR system (Fig. 3).

3.4 PCR Assays

1. Set the PCR reactions as follows: for one reaction combine the following components in a 0.2-mL Eppendorf tube: 1 ng/μL genomic DNA, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 μM of each primer, and 0.05 U/μL Taq DNA polymerase (see Note 17).

2. Set the PCR program as follows: 1 cycle at 94 ºC for 5 min; 40 cycles of 94 ºC for 1 min, 56 ºC for 1 min (see Note 18), and 72 ºC for 1 min; and a terminal incubation at 72 ºC for 5 min.

3. PCR amplifications can be carried out in any programmable thermocycler such as the Techne TC-412.

4. Separate PCR products electrophoretically on 1.8% agarose gel in 0.5× TBE buffer as previously described in DNA quantification section (Fig. 4).
4 Notes

1. Collect young not-fully-opened leaflets from date palm trees, keep in plastic bags, and preserve at $-40\, ^\circ\text{C}$ until they are processed. Wear gloves during all the steps of this protocol, and it is a must to wear them at certain steps, such as handling ethidium bromide-containing solutions.

2. Autoclave the CTAB buffer without $\beta$-mercaptoethanol. After autoclaving, divide (aliquot) the CTAB buffer into 50-mL portions. Add 500 $\mu$L $\beta$-mercaptoethanol to the 50 mL CTAB buffer before ready to use.

3. Ethidium bromide is a putative mutagenic agent and should be handled carefully according to the material safety data sheet. Although alternative DNA stains such as acridine orange, SYBR Safe, and SYBR Gold are more expensive, they are much safer and can be disposed of down the sink rather than treating them as hazardous waste, so it is recommended to use them.

4. To facilitate grinding, especially for very tough date palm tissue, take young unopened leaflets and cut them into 1 cm$^2$ pieces (Fig. 1).

5. Do not leave the ground tissue to thaw during the grinding and continuously keep the tissue frozen to avoid DNA degradation by deoxyribonucleases.

6. No more than one-third of the 2.0-mL Eppendorf tubes should be filled with ground tissue to obtain high quality and quantity of DNA. Adding more ground tissue in the tubes will not enable the CTAB buffer to reach the tissue aggregates and nucleases will digest the DNA.

7. Preheat the CTAB extraction buffer at $65\, ^\circ\text{C}$ before starting DNA extraction.

8. Vortex the tubes containing the mixture of ground tissue and CTAB buffer for a few seconds, every 10–15 min, to avoid tissue aggregates, hence leading to high quality and quantity of DNA.

9. Gently invert the tubes until an emulsion is formed, and then centrifuge the tubes.

10. Pipette the aqueous upper layer without disturbing or touching the interphase layer (Fig. 2).

11. Follow the regulations for disposing of hazardous chemicals and fluids such as ethidium bromide.

12. In most cases, white threads of DNA can be seen during DNA precipitation. This is also a stop point where tubes can be kept at $-20\, ^\circ\text{C}$ for long time without affecting DNA quality or quantity (Fig. 2).
13. DNA can be used at this step, but RNA can interfere with subsequent work so it is recommended to get rid of RNA and then use phenol extraction to get high-quality DNA (Fig. 3).

14. Allow the DNA to dissolve completely in sterile double-distilled H₂O or TE buffer by incubating DNA preparations either at 37 °C for 30 min or at 4 °C overnight. Gently flick the tubes and keep them at −20 °C for long-term preservation.

15. Horizontal electrophoresis systems vary in size with different boxes and casting trays. Depending on the number of samples, use the appropriate casting tray and prepare the right gel volume that fits the casting tray. Generally, the smallest gel trays can hold 40–50 mL agarose solution. The gel wells are created by inserting a comb into the slots in the casting tray. Remember, the thicker the gel, the deeper the wells will be.

16. Be careful because eruptive boiling can occur during heating the solution in the microwave. To prevent eruptive boiling, microwave the flask containing agarose-TBE mixture for 30–45 s, stop the microwave, carefully swirl the flask, and then repeat until agarose completely dissolves and the solution becomes clear.

17. It is recommended to make a PCR master mix that contains all the components except DNA. In addition, use from 10–15% extra reactions to avoid pipetting errors. The extra reactions are also needed because negative and positive control reactions should be included. Negative control reaction has everything of PCR components except the DNA to be able to detect either any contamination in the PCR components with date palm DNA or primer dimers.

18. Use PCR gradient to obtain the optimum annealing temperature. This step needs a gradient PCR machine. Set the range of the annealing temperature between 50 and 60 °C, as empirically the annealing temperature of oli primer pair is between these two temperatures. Make a master mix of 14 reactions, each 10 μL, by adding all the PCR components in addition to date palm DNA (use three DNA preparations representing different date palm cultivars) and then divide them into 10 μL aliquots. Run the samples on the gradient PCR machine as mentioned previously.

Acknowledgment

This work was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology (KACST), Kingdom of Saudi Arabia, project number 11-AGR-1475-02.
References


mohan.jain@helsinki.fi
Analysis of Expressed Sequence Tags (EST) in Date Palm

Sulieman A. Al-Faifi, Hussein M. Migdadi, Salem S. Algamdi, Mohammad Altaf Khan, Rashid S. Al-Obeed, Megahed H. Ammar, and Jerenj Jakse

Abstract

Expressed sequence tags (EST) were generated from a normalized cDNA library of the date palm Sukkari cv. to understand the high-quality and better field performance of this well-known commercial cultivar. A total of 6943 high-quality ESTs were generated, out of them 6671 are submitted to the GenBank dbEST (LIBEST_028537). The generated ESTs were assembled into 6362 unigenes, consisting of 494 (14.4%) contigs and 5868 (84.53%) singletons. The functional annotation shows that the majority of the ESTs are associated with binding (44%), catalytic (40%), transporter (5%), and structural molecular (5%) activities. The blastx results show that 73% of unigenes are significantly similar to known plant genes and 27% are novel. The latter could be of particular interest in date palm genetic studies. Further analysis shows that some ESTs are categorized as stress/defense- and fruit development-related genes. These newly generated ESTs could significantly enhance date palm EST databases in the public domain and are available to scientists and researchers across the globe. This knowledge will facilitate the discovery of candidate genes that govern important developmental and agronomical traits in date palm. It will provide important resources for developing genetic tools, comparative genomics, and genome evolution among date palm cultivars.

Key words  Expressed sequence tags (EST), Fruit development, Stress/defense

1 Introduction

The date palm (Phoenix dactylifera L.) is one of the most important members of the Arecaceae family. It has been cultivated for more than 7000 years and is considered to be among the world’s first cultivated fruit trees [1]. Nearly 2000 date varieties are grown worldwide, and over 340 cultivars are distributed in Saudi Arabia [2] differing in color, shape, size, flavor, and ripening time.

The date palm genome contains 36 chromosomes \(2n = 36\), and its size is estimated to be between 550 Mb [3] and 658 Mb [4]. Relatively little investment has been made in furthering date palm molecular genetic research as compared to other fruit species.
This has resulted in a serious constraint on an already underdeveloped infrastructure of crop genetic and genomic tools. Although some molecular markers were developed and used, including ISSR, AFLP, and SSR markers, the overall molecular toolbox for the date palm is limited and not as efficient compared with other fruit crops.

With the advent of efficient, high-throughput, and cost-effective sequencing technologies, a significant improvement in our understanding of the genomics and biology of different plants has already been achieved. Using data generated from the Illumina GAII sequencing platform, the first draft of the date palm genome was published in 2011 [4] and the second draft in 2013 [5]. The complete chloroplast genome sequence was published in 2010 [6], followed by the complete mitochondrial genome in 2012 [7]. A transcriptomic profile of the date palm for fruit development was reported in 2012 [8], and in-depth transcriptome sequencing to build \( P. dactylifera \) gene models based on data from different tissues and several developmental stages was published in 2012 [9]. In 2014, the first genetic map of the date palm was reported [10].

The production and life span of different date palm cultivars are affected by both biotic and abiotic factors [11]. Among the abiotic factors, salinity and drought severely affect date palm cultivation, especially in the Arabian Peninsula countries, including Saudi Arabia. Functional genomics is essential for identifying the responsible genes’ encoded transcripts and understanding the mechanism behind various types of stress in date palm cultivars.

A total of 1939 genes were found to be differentially expressed between mock-treated roots and salt-stressed date palm roots. Many of these regulatory genes belong to DNA/RNA, protein, membrane, and signaling functional categories, suggesting that these genes play functional roles in tolerance to salt stress [12]. The development of a complementary DNA (cDNA) library for the date palm will facilitate genetic and breeding studies, gene discovery and allele mining, and comparative genomics.

This chapter describes analysis of the potential linkage between the transcriptome of the elite date palm Sukkari cv. and high-quality, agronomically important traits, by identifying potential ESTs that encode enzymes and proteins related to fruit development and abiotic/biotic stress.

## 2 Materials

### 2.1 Plant Materials

Female flowers, developed fruits at different stages, and leaves from a single female date palm tree, Sukkari cv.

### 2.2 Reagents

1. Liquid nitrogen.
2. Chloroform.
3. Isopropyl alcohol.
4. Absolute and 75% ethanol.
5. RNase-free water.
6. Invitrogen’s Concert Plant RNA Reagent.
7. PCR purification kit.
8. Evrogen TRIMMER-DIRECT cDNA normalization kit.
9. Clontech Creator SMART cDNA library construction kit.
10. Qiagen PCR purification kit.
11. Proteinase K.
12. QIAprep Spin Miniprep Kit.
13. Molecular biology grade EDTA.
14. Hi-Di Formamide.
17. POP-7 Polymer.
18. Capillaries (36 and 50 cm).
19. RNase A.

2.3 Buffers

1. Buffer P1: 6.06 g/L Tris base and 3.72 g/L Na₂EDTA₂H₂O.
2. Buffer P2: 8 g/L NaOH and 50 mL/L 20% SDS solution.
3. Buffer PB (binding buffer).
5. Buffer PE (wash buffer).
6. Applied Biosystems 10× Running Buffer with EDTA.
7. TE buffer: 10 mM Tris–HCl (pH 8) and 0.1 mM EDTA.
8. 4× hybridization buffer (200 mM HEPES, pH 7.5, and 2 M NaCl).
9. DSN storage buffer: 50 mM Tris–HCl (pH 8).
10. 2× DSN master buffer: 100 mM Tris–HCl (pH 8), 10 mM MgCl₂, and 2 mM DTT.
11. DSN stop solution: 5 mM EDTA.
12. 50× TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL 0.5 M EDTA (pH 8).
13. First-strand buffer: 250 mM Tris–HCl (pH 8.3), 375 mM KCl, and 30 mM MgCl₂.
14. TEN buffer: 10 mM Tris–HCl, 0.1 mM EDTA, and 25 mM NaCl.
15. Freezing buffer: 1 M K$_2$HPO$_4$, 1 M KH$_2$PO$_4$, 1 M Na-citrate, 1 M MgSO$_4$, 1 M (NH$_4$)$_2$SO$_4$, 44 mL glycerol, 10 g NaCl, 10 g tryptone, 5 g yeast extract, and water to final volume 1 L.

2.4 Culture Medium

1. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar.
2. Kanamycin.

2.5 Equipment

1. Mortar and pestle.
2. Sealing tape PCR instrument.
3. Centrifuge with microtiter plate holders capable of spinning at least 14,000 × g.
4. Petri dishes.
5. Polypropylene tubes with tight-sealing caps (15 and 50 mL RNase-free).
6. Microcentrifuge tubes (RNase-free).
8. Sterile microcentrifuge 1.5 mL tubes.
11. Base and tray or retainer for holding tubes.
12. Vortex mixer.
15. PCR Thermocycler.
16. 96-deepwell plate.

3 Methods

3.1 RNA Extraction

1. Cool RNase-free microcentrifuge tubes in dry ice before placing frozen tissue in them (see Notes 1 and 2).
2. Bake glassware at 150 °C for 4 h, soak non-disposable plastic ware in 0.5 M NaOH for 10 min, and rinse thoroughly with RNase-free water.
3. Prepare RNase-free water by drawing the water into RNase-free containers. Add diethylpyrocarbonate (DEPC) to a final concentration of 0.01% (v/v). Let it stand overnight and autoclave. Use this water to prepare RNase-free solutions.
4. Grind the tissue to powder in liquid nitrogen.

mohan.jain@helsinki.fi
5. Store all ground plant material at \(-70\, ^\circ C\). Frozen tissue must remain frozen at \(-70\, ^\circ C\) prior to extraction with Plant RNA Reagent. Accidental thawing may result in RNA degradation.

6. Add 0.5 mL cold (4 °C) Plant RNA Reagent for up to 0.1 g of frozen ground tissue. Mix by brief vortexing or flicking the bottom of the tube until the sample is thoroughly resuspended.

7. Incubate the tube for 5 min at room temperature. Lay the tube down horizontally to maximize surface area during RNA extraction.

8. Clarify the solution by centrifuging for 2 min at \(12,000 \times g\) in a microcentrifuge at room temperature. Transfer the supernatant to an RNase-free tube.

9. Add 0.1 mL 5 M NaCl to the clarified extract and tap tube to mix.

10. Add 0.3 mL chloroform. Mix thoroughly by inversion.

11. Centrifuge the sample at 4 °C for 10 min at \(12,000 \times g\) to separate the phases. Transfer the top, aqueous phase to an RNase-free tube.

12. Add to the aqueous phase an equal volume of isopropyl alcohol. Mix and let it stand at room temperature for 10 min.

13. Centrifuge the sample at 4 °C for 10 min at \(12,000 \times g\). Decant the supernatant, taking care not to lose the pellet, and add 1 mL 75% ethanol to the pellet. Pellet may be difficult to see.

14. Centrifuge at room temperature for 1 min at \(12,000 \times g\). Decant the liquid carefully, taking care not to lose the pellet. Briefly centrifuge to collect the residual liquid and remove it with a pipette.

15. Add 10–30 μL RNase-free water to dissolve the RNA.

16. Pipette the water up and down over the pellet to dissolve the RNA. If any cloudiness is observed, centrifuge the solution at room temperature for 1 min at \(12,000 \times g\) and transfer the supernatant to a fresh tube and store at \(-70\, ^\circ C\).

### 3.2 First-Strand cDNA Synthesis

1. Denature the RNA for 5 min at 68 °C in a thermocycler to remove any RNA secondary structure.

2. For each sample, combine the following reagents in separate 0.5 mL reaction tubes:
   
   (a) 1–3.5 μL RNA (1 ng to 1 μg of poly A+ RNA or 2 ng to 1 μg total RNA).
   
   (b) 1 μL 3’ SMART CDS Primer II A (12 μM).
   
   (c) \(x\) 2.5 μL deionized H₂O.
   
   (d) 4.5 μL total volume.

mohan.jain@helsinki.fi
3. Mix contents and spin the tubes briefly in a microcentrifuge. Incubate the tubes at 72 °C in a hot-lid thermocycler for 3 min, and then reduce the temperature to 42 °C for 2 min.

4. Prepare PCR master mix for all reaction tubes at room temperature by combining the following reagents in the order shown:
   (a) 2 μL 5× first-strand buffer.
   (b) 0.25 μL DTT (100 mM).
   (c) 1 μL dNTP mix (10 mM).
   (d) 1 μL SMARTer II A Oligonucleotide (12 μM).
   (e) 0.25 μL RNase inhibitor.
   (f) 1.0 μL SMARTScribe Reverse Transcriptase (100 U) (see Note 3).
   (g) 5.5 μL total volume added per reaction.

5. Aliquot 5.5 μL of the master mix into each reaction tube.

6. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.

7. Incubate the tubes at 42 °C for 1 h.

8. Terminate the reaction by heating the tubes at 70 °C for 10 min.

9. Dilute the first-strand reaction product by adding the appropriate volume of TE buffer:
   (a) Add 40 μL TE buffer if you use total RNA as the starting material.
   (b) Add 190 μL TE buffer if you use more than 0.2 μg poly A+ RNA as the starting material.
   (c) Add 90 μL TE buffer if you use less than 0.2 μg poly A+ RNA as the starting material.

### 3.3 Second-Strand cDNA Synthesis

1. Preheat the thermocycler to 95 °C.

2. Prepare the PCR master mix by combining the following reagent in a sterile 1.5 mL tube per reaction:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Sterile water</td>
<td>80 μL</td>
</tr>
<tr>
<td>(b) 10× Advantage 2 PCR Buffer</td>
<td>10 μL</td>
</tr>
<tr>
<td>(c) 50× dNTP mix</td>
<td>2 μL</td>
</tr>
<tr>
<td>(d) 5′ PCR Primer (Clontech kit)</td>
<td>4 μL</td>
</tr>
<tr>
<td>(e) 50× Advantage 2 Polymerase Mix</td>
<td>2 μL</td>
</tr>
<tr>
<td>(f) Total volume</td>
<td>98 μL</td>
</tr>
</tbody>
</table>
3. Mix well by vortexing and spin the tubes briefly in a microcentrifuge.

4. Aliquot 98 μL PCR master mix into each reaction tube.

5. Add 2 μL of the second-strand cDNA synthesis to the appropriate reaction tube and mix the contents by flicking the tube. Spin the tubes briefly in a microcentrifuge.

6. Commence thermal cycling using the following PCR program:
   (a) 95 °C for 2 min
   (b) 95 °C for 7 s
   (c) 66 °C for 30 s
   (d) 72 °C for 6 min
   (e) Repeat step (b) to step (d) for optimal cycles and finally
   (f) 72 °C for 4 min

7. Determine the optimal number of PCR cycles for the amount of poly A⁺ RNA used in the ss cDNA synthesis (see Note 4):

<table>
<thead>
<tr>
<th>Total RNA (μg)</th>
<th>poly A⁺ RNA (μg)</th>
<th>Number of PCR cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00–1.5</td>
<td>0.5–1</td>
<td>13–15</td>
</tr>
<tr>
<td>0.50–1.0</td>
<td>0.25–0.5</td>
<td>15–18</td>
</tr>
<tr>
<td>0.25–0.5</td>
<td>0.25–0.1</td>
<td>18–21</td>
</tr>
</tbody>
</table>

8. When cycling is complete, place the tubes on ice.

9. Analyze 5 μL PCR product alongside 0.1 μg of 1 kb DNA size marker on a 1.5% (w/v) agarose/EtBr gel run in 1× TAE buffer to estimate cDNA quality and concentration (Fig. 1).

10. Purify the amplified ds cDNA to remove primer excess, dNTPs, and salts using PCR purification kits.

11. Determine the concentration of the purified ds cDNA using nanodrop spectrophotometer.

12. Aliquot 1.3–1.4 μg ds cDNA into a sterile 1.5 mL tube. Store the remaining purified ds cDNA at −20 °C.

13. Optional step: Bring the volume of your sample to 50 μL by adding sterile water. The extra volume allows the subsequent ethanol precipitation to work more effectively.

14. Add 0.1 volume of 3 M sodium acetate, pH 4.8, to the reaction tube.

15. Add 2.5 volumes (sample volume + sodium acetate volume) of 100% ethanol to the reaction tube.

16. Vortex the mixture thoroughly and centrifuge the sample for 15–20 min at 12,000–14,000 × g at room temperature. Carefully remove the supernatant.
17. Gently overlay the pellets with 100 μL 80% ethanol.
18. Centrifuge the tubes for 5 min at 12,000–14,000 × g at room temperature. Carefully remove the supernatant.
19. Repeat steps 17–19.

**Fig. 1** ds cDNA produced from poly(A)+ placenta RNA using SMART protocol. Ladder is a 1 kb DNA ladder. Typical results include the following: a moderately strong smear of cDNA ranging from 0.1 to 4 kb, several bright bands corresponding to abundant transcripts, and some low-molecular-weight material. Source: Evrogen TRIMMER-DIRECT Manual, p 20
20. Air-dry the pellet for 10–15 min at room temperature. Be sure the pellet is completely dry before attempting to resuspend the samples.

21. Dissolve the pellet in sterile water to a final cDNA concentration of 100–150 ng/μL.

22. **Optional step:** Check the cDNA quality and electrophorese 1 μL cDNA solution alongside 0.1 μg of 1 kb DNA size markers on a 1.5% TAE agarose gel.

23. This ds cDNA can be stored at −20 °C for up to 3 months prior to normalization.

### 3.4 cDNA Normalization Using Duplex-Specific Nuclease (DSN)

1. For each sample to be normalized, combine the following reagents in a sterile 1.5 mL tube (see Notes 5 and 6):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume, μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) SMART-prepared ds cDNA (600–1200 ng)</td>
<td>4–12</td>
</tr>
<tr>
<td>(b) 4× hybridization buffer</td>
<td>4</td>
</tr>
<tr>
<td>(c) Sterile water</td>
<td>8</td>
</tr>
<tr>
<td>(d) Total volume</td>
<td>16</td>
</tr>
</tbody>
</table>

2. Mix contents and spin the tube briefly in a microcentrifuge.

3. Aliquot 4 μL reaction mixture into each of the appropriately labeled tube.

4. Overlay the reaction with 3 μL mineral oil and centrifuge the sample at 14,000 × g for 2 min.

5. Incubate the samples in the thermocycler at 68 °C for 5 h, and then proceed immediately to the next step.

6. During the 5-h incubation step, it is necessary to check the activity of the DSN enzyme.

7. If the normalization kit is new, dilute the DSN according to the following protocol:

   (a) Add 25 μL DSN storage buffer to the lyophilized DSN enzyme.

   (b) Mix the contents of the tube by gently flicking the tube.

   (c) Spin the tube briefly in a microcentrifuge. Avoid foaming the mixture.

   (d) Incubate the tube at room temperature for 5–7 min.

   (e) Add 25 μL glycerol to the tube and mix the contents of the tube by gently flicking the tube.

   (f) Spin the tube briefly in a microcentrifuge. Avoid foaming the mixture.

   (g) Store the DSN solution at −20 °C.
8. To check DSN activity, follow the protocol:

(a) Combine the following reagents in a sterile 1.5 mL tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>4 μL</td>
</tr>
<tr>
<td>DSN control template</td>
<td>4 μL</td>
</tr>
<tr>
<td>DSN master buffer</td>
<td>10 μL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>18 μL</strong></td>
</tr>
</tbody>
</table>

(b) Mix contents and spin the tube briefly in a microcentrifuge.

(c) Aliquot 9 μL reaction mixture into two sterile PCR tubes labeled C (control) and E (experimental).

(d) Add 1 μL DSN storage buffer into the C-tube. Mix contents and spin the tube briefly in a microcentrifuge.

(e) Add 1 μL DSN solution (generally use the lowest dilution of DSN solution, i.e., ¼ dilutions) into the E-tube. Mix contents and spin the tube briefly in a microcentrifuge.

(f) Incubate the tubes in a thermocycler at 65 °C for 10 min.

(g) Add 10 μL DSN stop solution to each tube. Mix contents and spin the tube briefly in a microcentrifuge. Place the tubes at room temperature.

(h) Electrophorese 6 μL of each reaction mixture on a 1.5% TAE agarose. The gel should look like the gel in Fig. 2.

9. 15 min before the 5-h incubation period is over, pipette enough 2× DSN master buffer into a PCR thermocycler tube to suffice for each sample with an extra 5 μL for good measure. For example, for 12 tubes, pipette 65 μL DSN master buffers into the PCR tube.

10. Place the tube into the thermocycler to preheat the DSN master buffer.

11. Make two dilutions of the DSN for the normalization, usually ½ and ¼ dilutions of DSN.

12. Pause the thermocycler and add 5 μL preheated DSN master buffer to each of the normalization tubes. Do not remove the tubes from the thermocycler except for the time needed to add the DSN master buffer.

13. Incubate the tube at 68 °C for 10 min.

14. Add the DSN enzyme. Do not remove the tubes from the thermocycler.
15. Incubate the tubes in the thermocycler at 68 °C for 25 min.
16. Add 10 μL DSN stop solution, mix contents, and spin the tube briefly in a microcentrifuge.
17. Place the tubes on ice.
18. Add 20 μL sterile water in each tube, mix contents, and spin the tube briefly in a microcentrifuge. Place the tubes on ice.
19. These normalized cDNA samples can be stored at −20 °C for up to 2 weeks.

Fig. 2 DSN activity check. L DNA ladder, 1 control, 2 unsuccessful, 3 successful. Ladder is a 1 kb DNA ladder. Source: Evrogen TRIMMER-DIRECT Manual
3.5 Amplification of Normalized cDNA

1. Preheat the thermocycler to 95 °C.

2. Prepare a PCR master mix by adding the following reagents in the following order per tube:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1× (μL)</th>
<th>4× (μL)</th>
<th>8× (μL)</th>
<th>12× (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>40.5</td>
<td>162</td>
<td>324</td>
<td>486</td>
</tr>
<tr>
<td>10× Advantage 2 PCR Buffer</td>
<td>5</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>50× dNTP mix</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Evrogen M1 primer</td>
<td>1.5</td>
<td>6</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>50× Advantage 2 polymerase mix</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Total volume</td>
<td>49</td>
<td>196</td>
<td>392</td>
<td>588</td>
</tr>
</tbody>
</table>

3. Mix well by vortexing. Mix contents and spin the tube briefly in a microcentrifuge.

4. Aliquot 1 μL of each diluted cDNA into an appropriately labeled sterile PCR tube.

5. Aliquot 49 μL PCR master mix into each of the reaction tubes.

6. Mix contents by gently flicking the tubes. Mix contents and spin the tube briefly in a microcentrifuge.

7. Commence thermal cycling with the following PCR program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>(b)</td>
<td>95 °C</td>
<td>7 s</td>
</tr>
<tr>
<td>(c)</td>
<td>66 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>(d)</td>
<td>72 °C</td>
<td>6 min</td>
</tr>
<tr>
<td>(e)</td>
<td>Repeat to step (b)</td>
<td>13 cycles</td>
</tr>
<tr>
<td>(f)</td>
<td>72 °C</td>
<td>4 min</td>
</tr>
<tr>
<td>(g)</td>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

8. Subject all tubes to 7 cycles. Label a 4-tube strip as follows: 7, 9, 11, and 13 for each control tube.

9. After 7 cycles are completed, place the experimental tubes at 4 °C and hold until the end of the experiment.

(a) Transfer 12 μL 7-cycle PCR from the control tube to the strip tube labeled 7.

(b) Run two additional PCR cycles (for a total of nine) with the remaining 38 μL PCR mix.

(c) Transfer 12 μL 9-cycle PCR from the control tube to the strip tube labeled 9.
(d) Run two additional PCR cycles (for a total of 11) with the remaining 26 µL PCR mix.

(e) Transfer 12 µL 11-cycle PCR from the control tube to the strip tube labeled 11.

(f) Run two additional PCR cycles (for a total of 13) with the remaining 14 µL PCR mix.

(g) Transfer 12 µL 13-cycle PCR from the control tube to the strip tube labeled 13.

10. Analyze 5 µL aliquots of each control PCR reaction (7, 9, 11, and 13 cycles), alongside 0.1 µg of 1 kb DNA size marker on a 1.5% (w/v) agarose/EtBr gel, run in 1× TAE buffer (Fig. 3). Store the remaining materials on ice.

11. Determine the optimal number of cycles required for amplification of the control cDNA as follows. Use $X$ to find the number of additional cycles and $N$ using the following formula: $N = X \times 7$. If the optimal number of cycles is 9, then $N = 9 - 7$ or 2.

![Fig. 3 Analysis for optimizing PCR parameters. M marker, 77 cycles, 99 cycles (optimal), 1111 cycles, 1313 cycles, 1515 cycles. From the Evrogen TRIMMER-DIRECT Manual, p 28](image-url)
12. Subject the experimental tubes to $N+9$ additional cycles (or 11 cycles from the example given in step 11).
13. When the cycling is complete, electrophorese 5 μL of each aliquot of each PCR reaction on a 1.5% TAE/agarose gel. Select the tubes with the most efficient normalization, which is illustrated in Fig. 4.
14. If the cDNA from two or more tubes appears to be well normalized, combine the contents of these tubes into a single 1.5 mL sterile tube, mix by vortexing, and spin the tubes briefly in a microcentrifuge. This amplified cDNA can be stored at $–20$ °C for 1 month.
3.6 Second Amplification of Normalized cDNA

Reamplification of normalized cDNA avoids cDNA degradation due to residual DSN activity and to prepare more cDNA for library cloning. If you plan to estimate normalization efficiency before cloning, it is essential to amplify control nonnormalized cDNA simultaneously:

1. Aliquot 2 μL normalized cDNA into a sterile 1.5 mL tube, add 20 μL sterile water, mix well by vortexing, and spin briefly in a microcentrifuge.

2. Aliquot 2 μL control cDNA into a sterile 1.5 mL tube, add 20 μL sterile water, mix well by vortexing, and spin briefly in a microcentrifuge.

3. Aliquot 2 μL diluted normalized cDNA into an appropriately labeled PCR tube.

4. Aliquot 2 μL diluted control cDNA into an appropriately labeled PCR tube.

5. Preheat the thermocycler to 95 °C.

6. Prepare a PCR master mix by adding the following reagents in the following order per tube:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1× (μL)</th>
<th>2× (μL)</th>
<th>3× (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>80</td>
<td>160</td>
<td>240</td>
</tr>
<tr>
<td>10× Advantage 2 PCR buffer</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>50× dNTP mix</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Evrogen Primer M2</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>50× Advantage 2 polymerase mix</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Total volume</td>
<td>98</td>
<td>196</td>
<td>294</td>
</tr>
</tbody>
</table>

7. Aliquot 98 μL PCR master mix into each of the reaction tubes.

8. Mix contents by gently flicking the tubes. Mix contents and spin the tube briefly in a microcentrifuge.

9. Commence thermal cycling using the following PCR program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>(b)</td>
<td>95 °C</td>
<td>7 s</td>
</tr>
<tr>
<td>(c)</td>
<td>64 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>(d)</td>
<td>72 °C</td>
<td>6 min</td>
</tr>
<tr>
<td>(e)</td>
<td>Repeat step (b) to step (d)</td>
<td>12–14 cycles</td>
</tr>
<tr>
<td>(f)</td>
<td>72 °C</td>
<td>4 min</td>
</tr>
<tr>
<td>(g)</td>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>
10. When the cycling is completed, analyze 5 μL PCR product using gel electrophoresis alongside 0.1 μg of 1 kb DNA size markers on a 1.5% agarose/EtBr gel in 1 × TAE buffer to check the PCR quality and concentration. If necessary, subject the PCR tubes to 1–2 additional PCR cycles.

11. The normalization procedure is now complete, and the amplified normalized cDNA can be stored at −20 °C for 1 month.

### 3.7 cDNA Insert Processing

#### 3.7.1 Proteinase K Digestion

1. In a sterile PCR tube, combine the 100 μL reaction from the second post-DSN amplification with 4 μL proteins from the Clontech Creator SMART kit. Mix the contents and spin the tube briefly.

2. Incubate the tube in the thermocycler at 45 °C for 30 min.

3. Purify the ds cDNA to remove both proteinase K and any remaining polymerase mix using the Qiagen PCR purification kit. Add 1 μL sodium acetate (3 M, pH 4.8) provided in the Clontech kit to the sample after the 5× volume of Buffer PB has been added in step 1.

4. Nanodrop the purified ds cDNA to ascertain the concentration of the ds cDNA.

5. Determine the amount of purified ds cDNA required to reach 2500 ng of cDNA in a 79 μL solution using the following method:

<table>
<thead>
<tr>
<th>Concentration of purified ds cDNA</th>
<th>Volume of cDNA needed for 2500 ng</th>
<th>Amount of water needed to reach 79 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.4 ng/μL</td>
<td>47.7 μL</td>
<td>31.3 μL</td>
</tr>
</tbody>
</table>

#### 3.7.2 Sfi I Digestion

1. Take the 79 μL solution of ds cDNA from step 5 above, and combine it with the following reagents from the Clontech Creator SMART kit:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds cDNA</td>
<td>79</td>
</tr>
<tr>
<td>10× Sfi I buffer</td>
<td>10</td>
</tr>
<tr>
<td>Sfi I enzyme</td>
<td>10</td>
</tr>
<tr>
<td>100× BSA</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>100</td>
</tr>
</tbody>
</table>

2. Mix well by gently flicking the tube or slow vortexing and spin down in a microcentrifuge. Incubate the tube at 50 °C for 2 h.
3.7.3 cDNA Size Fractionation

1. Before starting this procedure, prepare the following solutions:
   (a) TEN buffer.
   (b) 100% ethanol.
   (c) 7.5 M ammonium acetate $\text{C}_2\text{H}_5\text{O}_2\text{NH}_4$ ($\text{NH}_4\text{Oac}$).
   (d) 80% ethanol.

2. Attach the column to a support stand and remove the top cap first, followed by the bottom cap. Allow the 20% ethanol solution to drain completely by gravity.

3. Once the column stops draining, pipette 0.8 mL TEN buffer into the column and allow it to drain completely. If the flow rate is noticeably slower than 30–40 s, do not use the column. If the drop size from the column is not approximately 25–40 μL, do not use the column.

4. Repeat the wash step 3 more times for a total of four washes and 3.2 mL TEN buffers. Let the column drain until dry.

5. Label 20 sterile 1.5 mL tubes from 1 to 20. Place them in a rack under the bottom of the column with tube 1 directly beneath the column.

6. Add 50 μL TEN buffer to the Sfi I-digested cDNA for a total volume of 150 μL. Mix gently by pipetting. Add the entire sample to the column and let it drain into the resin bed. Collect the effluent into tube 1.

7. Move tube 2 under the column outlet and add 100 μL TEN buffer to the column. Collect the effluent into tube 2. Let the column drain completely.

8. Beginning with the next 100 μL aliquot of TEN buffer, collect single-drop fractions into individual tubes starting with tube 3. Continue to add 100 μL aliquots of TEN buffer until all 18 tubes (tubes 3–20) contain a single drop.

9. Electrophorese 6 μL of each tube on a 1.5% TAE/agarose gel at 150 V for 10 min. Collect the first 3–4 fractions in which cDNA is visible in a sterile 1.5 mL tube. These fractions will contain the largest molecular weight cDNAs.

10. Add the following reagents to the tube of pooled cDNA in this order:
   (a) Glycogen (20 μg/μL) 1 μL.
   (b) 7.5 M NH$_4$Oac 0.5 volume (0.5× volume of cDNA).
   (c) 100% ethanol 2.5 volumes (2.5× volume of cDNA + NH$_4$Oac).

11. Allow the cDNA to precipitate at −20 °C overnight.

12. Centrifuge the cDNA at 4 °C for 25 min at 14,000 × g.
13. Carefully remove the supernatant while not disturbing the pellet. Add 150 μL 70% ethanol.
14. Centrifuge the sample at 4 °C for 5 min at 14,000 × g.
15. Repeat steps 13 and 14.
16. Remove the 70% ethanol and allow the pellet to air-dry for 15–16 min to completely remove the last traces of ethanol.
17. Resuspend the pellet in 7 μL deionized water and mix gently. Allow the sample to incubate at room temperature for 10 min.

3.7.4 Ligation of ds cDNA into pDNR-LIB

1. Use 1 μg pDNR-LIB vector provided in the Clontech Creator SMART kit (10 μL), and set up the following reaction mix in a sterile PCR tube.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDNR-LIB</td>
<td>10</td>
</tr>
<tr>
<td>10× Antarctic phosphatase buffer</td>
<td>2</td>
</tr>
<tr>
<td>Antarctic phosphatase</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>13</td>
</tr>
</tbody>
</table>

2. Mix the contents by gently flicking the tube and spin the tube in a microcentrifuge.
3. Incubate the samples at 37 °C for 60 min.
4. Incubate the samples at 65 °C for 10 min to inactivate the enzyme.
5. Proceed with the ligation starting with setting up the following ligation mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (100–200 ng/μL)</td>
<td>1.5</td>
</tr>
<tr>
<td>pDNR-LIB (0.1 μg/μL)</td>
<td>1</td>
</tr>
<tr>
<td>10× ligation buffer</td>
<td>0.5</td>
</tr>
<tr>
<td>ATP (10 μM)</td>
<td>0.5</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionized water</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>5</td>
</tr>
</tbody>
</table>

6. Incubate the sample at 16 °C overnight.
7. In each sample, add 95 μL sterile DEPC-treated water and transfer the resulting 100 μL sample to a sterile 1.5-mL tube.
Add 1.5 μL glycogen. Mix with a pipette tip. Add 280 μL ice-cold 100% ethanol. Mix by gently rocking the tube. Place the tube at −80 °C for 2.5 h.

8. Centrifuge the samples at 15,000 × g for 25 min at 4 °C.

9. Carefully remove the ethanol without disturbing the pellet.

10. Air-dry the pellet for 15–16 min at room temperature to remove every last trace of ethanol.

11. Resuspend the pellet in 5 μL sterile DEPC-treated water. Place the samples on ice.

3.7.5 Transformation

1. Prepare LB medium (1 L):
   (a) Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
   (b) Adjust the medium to pH 7 using 1 N NaOH and bring volume with water up to 1 L.
   (c) Autoclave on liquid cycle for 20 min at 15 psi (1.1 kg/cm²).
   (d) Allow solution to cool to 55 °C, and add antibiotic if needed (50 μg/mL kanamycin).
   (e) Store at room temperature or 4 °C.

2. Prepare LB agar plates:
   (a) Prepare LB medium as above, but add 15 g/L agar before autoclaving. After autoclaving, cool to approx. 55 °C, add antibiotic (if needed), and pour into Petri dishes.
   (b) Let it harden, and then invert and store at 4 °C in the dark.

3. While the samples are being spun down in step 9 above, place an appropriate number of electroporation cuvettes on ice.

4. In a sterile hood, pipette 970 μL LB media into an appropriate number of 15 mL Falcon tubes. Label each tube with the sample that will be added to it and place the tubes on ice.

5. When the Falcon tubes and cuvettes have chilled for 10 min, retrieve the eletrocompetent cells from the −80 °C freezer. Allow cells to thaw on ice. Once they have thawed, these cells cannot be refrozen.

6. Add 25 μL thawed cells to the 5 μL resuspended recombinant plasmids. Mix thoroughly with a pipette tip.

7. Transfer the cell/plasmid mixture to a chilled electroporation cuvette.

8. Electroporate by discharging. The time constant for a good transformation should range between 4.6 and 5. Any reading lower or higher than this range should be treated with suspicion.
9. Using a microcapillary tip, transfer the transformants from the electroporation cuvette to the appropriate Falcon tube. Be careful not to pipette the sample overmuch. Mix the transformants into the LB by gently flicking the tube. Place the Falcon tube back on ice until all transformations are complete.

10. Incubate the transformants at 37 °C for 1 h on a shaker at 225 rpm.

3.7.6 Tittering Plasmid Libraries

1. Remove 1 μL of the library and add it to 1 mL of LB broth in a sterile 1.5 mL tube. Mix by gentle vortexing. This is Dilution A (1:1000).

2. Remove 1 μL of Dilution A and add it to 1 mL of LB broth in a sterile 1.5 mL tube. Mix by gentle vortexing. This is Dilution B (1:1000,000).

3. Add 50 μL of LB to a 1.5 mL tube labeled Dilution A. Pipette 1 μL of Dilution A into this 50 μL aliquot and spread the entire sample on a pre-warmed LB/kanamycin plate.

4. Remove 50 and 100 μL aliquots from Dilution B and spread them onto separate pre-warmed LB/LB/kanamycin plates.

5. Leave the plates at room temperature for 15–20 min to allow the inoculums to soak into the agar. Store the libraries at 4 °C.

6. Invert the plates and allow them to incubate overnight at 37 °C.

7. Count the colonies to determine the titer using the following formulas:
   (a) \( \frac{\text{Colony number Dilution A} \times 10^3 \times 10^3}{10^3} = \text{cfu/mL} \).
   (b) \( \frac{\text{Colony number Dilution B/plating volume}}{10^3} \times 10^3 \times 10^3 = \text{cfu/mL} \).

3.7.7 Quality Control

1. Label two 8-tube PCR strips 1–16 for each library produced. Pipette 50 μL of sterile water into each tube.

2. Select 16 colonies at random from the titer plates and add each colony to a separate tube. Seal the strips and vortex until the colony is no longer visible. Spin the strips briefly in a microcentrifuge.

3. Incubate the samples at 98 °C for 6–8 min in a thermocycler to lyse the cells.

4. Spin the strips at 3500 × g for 6 min to pellet the cell debris.

5. After allowing the reagents to thaw on ice, create a PCR master mix according to the table below.
Reagent | $1 \times \mu L$ | $16 \times \mu L$
--- | --- | ---
Sterile water | 4.3 | 68.8
10× Taq Polymerase Buffer | 1 | 16
10 mM dNTP mix | 0.2 | 3.2
10 μM Forward PCR Primer | 0.2 | 3.2
10 μM Reverse PCR Primer | 0.2 | 3.2
Taq DNA Polymerase | 0.1 | 1.6
Template | – | –
Total volume | 10 | 6 μL each

7. Aliquot 6 μL of the PCR master mix into each of the 16 tubes.
8. Add 4 μL of the sample plasmid DNA into the appropriate tube containing the PCR master mix. Be careful not to disturb the pelleted cell debris while doing so.
9. Seal the PCR strips containing the PCR reaction, mix by gentle vortexing, and spin briefly in a microcentrifuge. Preheat the thermocycler to 95 °C.
10. Begin thermal cycling of the samples using the following PCR program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>(b)</td>
<td>95 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>(c)</td>
<td>55 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>(d)</td>
<td>72 °C</td>
<td>4 min</td>
</tr>
<tr>
<td>(e)</td>
<td>Repeat step (b) to step (d)</td>
<td>22 cycles</td>
</tr>
<tr>
<td>(f)</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>(g)</td>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

11. When thermal cycling is complete, electrophorese the samples on a 1.5% TAE/agarose gel with a 1 kb ladder. The resulting gel should look like the one shown in Fig. 5.
12. If there is no cDNA insert present in the plasmid, this primer set will produce a 500 bp product. If the gel shows a large number of 500 bp bands (more than 4) or if it has a uniform line of 500 bp bands, the library is no good and you will have to go back to at least the ligation step if not further. If your gel looks like the one above, proceed to large-scale quality control.
3.7.8 Large-Scale Quality Control

1. Remove the libraries from the 4 °C freezer and allow them to warm to room temperature. Place the liquid LB media and the large plates at room temperature as well.

2. Label two 1.5 mL tubes 1 μL and 2 μL for each library. Add 400 μL of LB media to the tube labeled 1 μL and add 100 μL of LB to the tube labeled 2 μL.

3. Add 4 μL of the library to the tube marked 1 μL, so that there is 1 μL of library per 100 μL of LB. This solution is enough to make four colony picking plates.

4. Add 2 μL of the library to the tube marked 2 μL. This solution is enough to make one colony plate at double the concentration of the colony plates listed above in step 3.

5. Take the solution from step 3 and plate 101 μL of the solution on each of four 150 mm LB/Kan plates.

6. Take the solution from step 4 and plate the entire solution on a single 150 mm LB/Kan plate.

7. Allow the plates to sit for 10 min to allow the inoculums to soak into the plate.

8. Invert the plates and incubate them overnight at 37 °C. Restore the libraries to the 4 °C freezer.

9. If the number of colonies is satisfactory, remove the libraries from the 4 °C freezer and allow them to come to room temperature. Label two sterile 1.5 mL tubes and divide the 1 mL stock into two 500 μL stocks. Add 200 μL of 80% glycerol to each tube and mix via gentle pipetting. Freeze the tubes by placing them at –80 °C. The libraries are now safe.

10. Place two boxes of flat toothpicks (round toothpicks tend to absorb media more efficiently than flat toothpicks) into a glass beaker; cover it with aluminum foil, and autoclave. These will be needed for colony picking.

Fig. 5 Electrophoresis results of plasmid libraries on a 1.5% TAE/agarose gel with a 1 kb ladder
11. Make 1 L of freezing buffer according to the recipe below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M K₂HPO₄</td>
<td>36 mL</td>
</tr>
<tr>
<td>1 M KH₂PO₄</td>
<td>13.2 mL</td>
</tr>
<tr>
<td>1 M Na-citrate</td>
<td>1.7 mL</td>
</tr>
<tr>
<td>1 M MgSO₄</td>
<td>0.4 mL</td>
</tr>
<tr>
<td>1 M (NH₄)₂SO₄</td>
<td>6.8 mL</td>
</tr>
<tr>
<td>Glycerol (4.4% total volume)</td>
<td>44 mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>Total volume</td>
<td>1 L</td>
</tr>
</tbody>
</table>

12. Autoclave the freezing buffer and allow it to cool to room temperature.

13. For each library,

   (a) Measure out 30 mL of freezing buffer with a 50-mL Falcon tube. Add 30 µL of chloramphenicol to the freezing buffer and mix. Aliquot 60 µL of the freezing buffer/chloramphenicol mix to each well on a 96- or 192- or 384-well plate.

   (b) Using an autoclaved toothpick, pick a single colony from one of the colony picking plates and place it in well A1.

   (c) Pick colonies for the first 6 columns of the plate, mark the stopping place with a Sharpie, and then remove the toothpicks. This is to keep the toothpicks from absorbing the freezing buffer. Repeat this process until each well has been inoculated.

   (d) Place the plate at 37 °C overnight.

   (e) Wrap the colony picking plates in parafilm and store at 4 °C.

14. Produce and autoclave 1 L of 2×YT media according to the following recipe:

   (a) In 900 mL of deionized water, add 16 g tryptone, 10 g yeast extract, and 5 g NaCl.

   (b) Adjust to pH 7 with NaOH.

   (c) Adjust the volume to 1 L using deionized water.

   (d) Autoclave and allow it to cool to room temperature.
15. To produce a 96-deepwell plate, use the following steps:

(a) Measure out 150 mL of 2×YT media 50 mL at a time using a 50 mL Falcon tube. Add 50 μL of chloramphenicol to each 50 mL tube of 2×YT media. Mix well and add to a reservoir.

(b) Using a multichannel pipettor, pipette 1.4 mL of 2×YT/chloramphenicol mix into each well of the 96-deepwell plate.

(c) Take a 5 μL inoculum and inoculate the 96-deepwell plate.

(d) Incubate the 96-deepwell plate at 37 °C overnight on a shaker at 225 rpm.

(e) Seal the 96-/192-/384-well plate with a foil plate seal and store the plate at 0−80 °C.

(f) Allow the 96-deepwell plate to incubate overnight.

(g) Harvest the bacterial cells by centrifugation at 6800 × g in a conventional, tabletop microcentrifuge for 3 min at room temperature (15−25 °C).

(h) The bacterial cells can also be harvested in 15 mL centrifuge tubes at 5400 × g for 10 min at 4 °C.

(i) Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

3.7.9 Plasmid Minipreparation Using the QIAprep Spin Miniprep Kit

This protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1–5 mL overnight cultures of *E. coli* in LB medium. All protocol steps should be carried out at room temperature (15−25 °C).

1. Prepare the following buffers:

   Buffer P1: Dissolve 6.06 g Tris base and 3.72 g Na₂EDTA-2H₂O in 800 mL water. Adjust to pH 8 with HCl. Adjust the volume to 1 L with water. Add 100 mg RNase A per liter P1. Buffer P2: Dissolve 8 g NaOH pellets in 950 mL water and 50 mL 20% SDS solution. Adjust the volume to 1 L with water.

2. Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle Buffer P1 for a final concentration of 100 μg/mL. Mix and store at 2–8 °C.

3. Resuspend pellet of bacterial cells in 250 μL Buffer P1 and transfer to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved.
4. Add 250 μL Buffer P2 (see Notes 7 and 8) and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

5. Add 350 μL Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g., ≥5 mL) may require inverting up to ten times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

6. Centrifuge for 10 min at ~17,900 × g in a tabletop microcentrifuge. A compact white pellet will form.

7. Apply 800 μL supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.


9. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using end A+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α do not require this additional wash step.

10. Wash QIAprep 2.0 spin column by adding 0.75 mL Buffer PE and 14,000 × g for 30–60 s.

11. Discard the flow-through, and centrifuge at 14,000 × g for an additional 1 min to remove residual wash buffer (see Note 9).

12. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube.

13. Elute DNA, add 50 μL EB buffer (10 mM Tris–Cl, pH 8.5) or water (see Note 10) to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.

mohan.jain@helsinki.fi
3.7.10 Sequence Processing and Assembly

1. Prepare the reactions for 96-well reaction plates or microcentrifuge tubes (see Note 11). For each reaction add the following reagents to a separate tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BigDye Terminator v3.1 Terminator</td>
<td>2</td>
</tr>
<tr>
<td>BigDye Terminator v3.1 Sequencing Buffer (5x)</td>
<td>3</td>
</tr>
<tr>
<td>Mini prep plasmid DNA</td>
<td>2.5</td>
</tr>
<tr>
<td>T7 primer (10 μM stock)</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionized water</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

2. Mix well and spin briefly.
3. Place the plate in a thermocycler and set to the correct volume (20 μL).
4. Perform an initial denaturation 96 °C for 2 min.
5. Repeat the following for 30 cycles: 20 s at 96 °C, 30 s at 50 °C, and 4 min at 60 °C.
6. Spin down the contents of the tubes in a microcentrifuge.

3.7.11 Purifying Extension Products Using Ethanol/EDTA Precipitation Method

To precipitate 20 μL sequencing reactions in 96-well reaction plates (see Note 12):

1. Remove the 96-well reaction plate from the thermocycler and briefly spin.
2. Add 5 μL 125 mM EDTA to each well.
3. Add 60 μL 100% ethanol to each well.
4. Seal the plate with aluminum tape and mix by inverting four times.
5. Incubate at room temperature for 15 min.
6. Use a plate adapter and spin the plate at the maximum speed as follows: 1400–2000 × g for 45 min or 2000–3000 × g for 30 min.
7. Invert the plate and spin up to 185 × g, and then remove from the centrifuge.
8. Add 60 μL 70% ethanol to each well.
9. With the centrifuge set to 4 °C, spin at 1650 × g for 15 min.
10. Invert the plate and spin up to 185 × g for 1 min, and then remove from the centrifuge.
11. To continue, resuspend the samples in injection buffer. Cover with aluminum foil and store at 4 °C.
12. Make sure the wells are dry. You may use a Speed-Vac for 15 min to dry the plate.

13. Make sure the samples are protected from light while they are drying.

### 3.7.12 Sample Electrophoresis

1. Add 10 μL Hi-Di Formamide to each sample pellet. To prevent dye degradation, use fresh Hi-Di Formamide. Old Hi-Di Formamide or low-quality formamide can have formic acid that can contribute to the degradation of fluorescent dyes (see **Note 13**). Also, run samples on the instrument as soon as possible after resuspending them.

2. Close the wells with aluminum sealing tape.

3. Vortex thoroughly, and then centrifuge briefly.

4. Peel off the aluminum sealing tape and replace the tape with septa.

### 3.7.13 Electrophoresis Workflow

1. Create a plate record for each plate to be run. A plate record consists of the following information about the plate and all samples on the plate:
   
   (a) Plate information:
   
   - ID or barcode number (for ABI 3730/3730xl instruments only).
   - Plate name.
   - Plate type.
   - Application type.
   - Owner name.
   - Operator name.

   (b) Sample information:
   
   - Sample name.
   - Comment (optional).
   - Results group: Contains settings for file names and locations, the sequencing analysis software in use, and the auto-analysis setting.
   - Analysis protocol: Contains settings necessary for analysis and post-processing of the sequence.
   - Instrument protocol: Contains settings for running the instrument.

2. Follow the general flow to perform a run on Applied Biosystems genetic analyzer:

   (a) Prepare the instrument:
• Start the computer, the instrument, and the Data Collection Software.
• Select the capillary length, number of capillaries, and polymer type.
• Check the polymer delivery system for sufficient polymer and absence of bubbles.
• Prepare the buffer and water/waste reservoirs.

(b) Perform spatial calibration, if necessary.
(c) Perform spectral calibration, if necessary.

(d) Load the prepared samples:
• Load the prepared samples onto 96-well or 384-well plates.
• Place the tubes or the plate assembly into the instrument.

(e) Set up the run using Data Collection Software:
• Create a plate record.
• Create a results group, with file name and folder preferences.
• Create an instrument protocol, including run module and dye set configurations.
• Create an analysis protocol.
• Determine the run parameters.
• Link the plate.

(f) Start the run.

3.7.14 Sequence Processing and Assembly

1. Base-called the sequence trace files using the Phred program.
2. Eliminate low-quality bases (<Q20, 99% accuracy) from sequence ends, followed by SeqClean [13] to shorten the Poly-A/T to five continuous bases.
4. Discard the EST sequences shorter than 100 bp, and deposit greater than 100 bp into the dbEST division of GenBank.

3.7.15 Annotation and Functional Classification

1. BLAST search is done after clustering and assembly to identify similarities between the ESTs and other sequences already deposited in public databases. All of the unigene sequences are compared to GenBank nonredundant protein and nucleotide databases using either the blastx (E-value ≤ 10⁻⁶) or the blastn (E-value ≤ 10⁻⁶) program [14].

mohan.jain@helsinki.fi
2. Perform Gene Ontology (GO) annotations with BLAST2GO [15, 16] based on sequence similarity. Furthermore, carry out InterProScan and merge the results with GO annotations to improve them.

3. Finally, analyze biological pathways using the KEGG (Kyoto Encyclopedia of Genes and Genomes) [17].

4 Notes

1. Always wear disposable gloves and change them frequently, use sterile disposable plastic ware, and pipettes especially reserved for RNA work.

2. Use aerosol-resistant pipette tips to reduce sample-to-sample contamination or reagent contamination. Treat non-disposable items with RNase AWAY or similar product to remove RNase contamination.

3. Add the reverse transcriptase to the master mix just prior to use. Mix well by pipetting and spin the tube briefly in a microcentrifuge.

4. It is essential to use the minimal number of PCR cycles when performing the ds cDNA synthesis to avoid the production of unspecific PCR product. It is better to undercycle than to overcycle, since under cycling can be rectified by placing the tubes back in the thermocycler. If the tube is overcycled, the ds cDNA synthesis must be done over again.

5. Normalization using duplex-specific nuclease (DSN) is a highly efficient approach that can be applied for normalization of full-length-enriched cDNA [18, 19]. The method is based on nucleic acid hybridization kinetics [20] and unique properties of the duplex-specific nuclease (DSN) specific to the double-stranded (ds) DNA [21].

6. Before you start hybridization, make sure that the 4× hybridization buffer remains at room temperature for at least 15–20 min beforehand. If any pellet or white precipitate is present, warm the buffer at 37 °C for 10 min to dissolve the pellet or precipitate.

7. Check Buffers P2 and N3 before use for salt precipitation. Redissolve any precipitate by warming at 37 °C. Do not shake Buffer P2 vigorously. Close the bottle containing Buffer P2 immediately after use to avoid acidification of Buffer P2 from CO₂ in the air. Buffers P2, N3, and PB contain irritants. Wear gloves when handling these buffers.

8. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
9. Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. If water is used for elution, make sure that its pH is between 7 and 8.5. Elution efficiency is dependent on pH, and the maximum elution efficiency is achieved within this range. A pH < 7 can decrease yield.

11. Capillary electrophoresis using a denaturing flowable polymer has largely replaced the use of gel separation techniques due to significant gains in workflow, throughput, and ease of use.

12. While this method produces the cleanest signal, it may cause loss of small molecular weight fragments. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some sequencing results. 95% ethanol is usable, but you must make sure the final ethanol concentration for precipitation remains the same (67–71%).

13. Use Hi-Di Formamide to resuspend your purified sequencing products. Resuspension in water is not recommended because oxidative effects on terminator dyes lead to earlier dye breakdown of sequencing extension products, affecting base-calling.

Acknowledgment

The authors are grateful to the National Plan for Science and Technology (NPST) program project number 08-BIO-164-2 and to the Deanship of the Scientific Research/Agricultural Research Center at King Saud University for financial support.

References


Chapter 24

Development of Genomic Simple Sequence Repeats (SSR) by Enrichment Libraries in Date Palm

Sulieman A. Al-Faifi, Hussein M. Migdadi, Salem S. Algamdi, Mohammad Altaf Khan, Rashid S. Al-Obeed, Megahed H. Ammar, and Jerenj Jakse

Abstract

Development of highly informative markers such as simple sequence repeats (SSR) for cultivar identification and germplasm characterization and management is essential for date palms genetic studies. The present study documents the development of SSR markers and assesses genetic relationships of commonly grown date palm (*Phoenix dactylifera* L.) cultivars in different geographical regions of Saudi Arabia. A total of 93 novel simple sequence repeat (SSR) markers were screened for their ability to detect polymorphism in date palm. Around 71% of genomic SSRs are dinucleotide, 25% trinucleotide, 3% tetranucleotide, and 1% pentanucleotide motives and show 100% polymorphism. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis illustrates that cultivars trend to group according to their class of maturity, region of cultivation, and fruit color. Analysis of molecular variations (AMOVA) reveals genetic variation among and within cultivars of 27% and 73%, respectively, according to the geographical distribution of the cultivars. Developed microsatellite markers are of additional value to date palm characterization, tools which can be used by researchers in population genetics, cultivar identification, as well as genetic resource exploration and management. The cultivars tested exhibited a significant amount of genetic diversity and could be suitable for successful breeding programs. Genomic sequences generated from this study are available at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (Accession numbers. LIBGSS_039019).

Key words Heterozygosity, Microsatellite, Molecular markers, Polymorphic information content (PIC), Polymorphism, Primer

1 Introduction

Date palm (*Phoenix dactylifera* L., 2n = 36) is the most important fruit crop of arid climate region in North Africa and the Middle East, as well as in Saudi Arabia. It ranks among the top fruit trees in Saudi Arabia in terms of the number of trees, area, and production (approx. 23.7 million trees, 156,000 ha, and 992,000 mt of fruit) [1]. Saudi Arabia possesses a vast richness of date palm germplasm...
as confirmed by the great number of different cultivars and accessions, described based on the morphological characters of fruit and seeds. To manage date palm germplasm, it should also be genetically characterized, which aids in its preservation and transmits a significant genetic richness for exploitation. However, morphological traits are often variable and influenced by environmental conditions or varying with the developmental stage of the plant [2]. Molecular markers, based on polymorphisms at the DNA level, are used to assess genetic diversity in date palm.

The genome size of date palm is estimated to be approximately 658-Mbp long [3], and genomic resources available for date palms are insufficient to be used in genetic studies, assessing genetic variability, cultivar identification, and marker-assisted selection. Microsatellites or simple sequence repeats (SSR) have been widely used in many crops, due to their abundance, high degree of polymorphism, locus specificity, and reproducibility, and most of all, they are codominant in nature. These make microsatellites an attractive option for genetic studies in date palms. A total of 16 (GA)n SSR markers have been developed and used to analyze Tunisian [4] and Sudanese [5] germplasm. Furthermore, 17 microsatellite loci were developed by constructing two microsatellite-enriched libraries of date palm using (GA)n and (GT)n repeats [6]. Some 18 SSR markers and a plastid minisatellite provide new insights on the geographic origins and genetic history of the cultivated date palm and confirm the existence of at least two ancient gene pools that have contributed to the current world date palm diversity [7].

Recently, the Cornell Medical College in Qatar published an assembly of the date palm genome Khalas cv. generated by whole-genome shotgun next-generation DNA sequencing [3]. A total of 1000 microsatellite motifs across the date palm genome were analyzed [8, 10]. Some 172,075 SSR motifs were identified on the date palm genome sequence with a frequency of 450.97 SSRs per Mb. Out of these, 130,014 SSRs (75.6%) were located within the intergenic regions with a frequency of 499 SSRs per Mb [9].

This chapter describes the development of a new set of SSR markers for *Phoenix dactylifera*, defines their variability parameters, and assesses the genetic variation for some Saudi date palm cultivars.

## 2 Materials

### 2.1 Plant Material

Young fresh leaves of date palm cultivar Sukary.

### 2.2 Buffers

1. TE (Tris-EDTA) buffer (10:1 v/v): 1 M Tris–HCl, 0.5 M EDTA, and distilled H₂O.
2. 50× TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, and distilled H₂O.
3. 5× TBE buffer: 54 g Tris base, 27.5 g boric acid, 0.5 M EDTA, and (pH 8) distilled H₂O.
4. DNA extraction buffer: Tris–HCl, EDTA, NaCl, SDS, PVP, and β-mercapto-ethanol.
5. PCR reaction components: PCR buffer, 2 mM ATP, Mg++, nuclease-free water, *Taq* DNA polymerase, primers, and DNA template.
6. Fishing microsatellites formamide, SSC, SDS, NaH₂PO₄, Na₂HPO₄, and distilled H₂O.
7. 2¡¾ Rapid Ligation Buffer: Tris–HCl (pH 7.8), MgCl₂, DTT, ATP, and polyethylene glycol.
8. 10× Running buffer with EDTA (Applied Biosystems 3730 and 3730xl DNA analyzers).

### 2.3 Culture Medium

1. Agarose (1%): Agarose (1 g), TBE buffer, and acridine orange.
2. JM109 high-efficiency competent cells.
3. LB plates/carbenicillin: Agar and carbenicillin.
5. TYP broth: Bacto-tryptone, Bacto-yeast extract, NaCl, and K₂HPO₄.
6. LB medium: Bacto-tryptone, Bacto-yeast extract, and NaCl.
7. SOC medium: Bacto-tryptone, Bacto-yeast extract, NaCl, KCl, Mg²⁺ stock, and glucose.

### 2.4 Solutions and Reagents

1. Absolute and 75% ethanol.
2. Calf intestinal alkaline phosphatase (CIAP).
5. Glycerol.
6. Hi-Di formamide.
7. Liquid nitrogen.
8. Mung bean nuclease (MBN) enzyme.
9. pGEM Easy cloning kit.
10. POP-7 Polymer.
11. QIAprep Spin MiniPrep Kit.
12. Restriction enzymes *EcoRI*, *HaeIII*, *HaeII*, *MseI*, *RsaI*, *BglII*, *EcoRV*, and *HinfI* with 1× supplied buffer and BSA.

13. T4 DNA ligase.


15. SNX reverse primer.

### 2.5 Equipment

1. Applied Biosystems (ABI) 3730 and 3730xl DNA Analyzers.
2. Capillaries 36 and 50 cm (ABI).
3. Centrifuge with microtiter plate holders capable of reaching a speed of at least 14,000 × *g*.
4. Electrophoresis set: trays, combs, and power supply.
5. Gel documentation system.
6. Mortars and pestles.
7. Nuclease-free polypropylene tubes (1.5 and 2.0 mL).
8. PCR instrument.
10. 96 deep-well 2 mL plates.
11. Sterilized filters.
12. PCR tubes and plates.

### 3 Methods

#### 3.1 Reagents Preparation

1. 1 M Tris–Cl (pH 8): Dissolve 121 g Tris base in 800 mL double-distilled H₂O, and adjust pH using concentrated HCl to 8, adjust the volume to 1000 mL, and autoclave the solution.

2. 0.5 M EDTA (pH 8): Dissolve 186 g disodium salt of EDTA in 800 mL double-distilled H₂O and adjust pH to 8 using NaOH pellets, adjust the volume to 1000 mL, and autoclave the solution.

3. 5 M NaCl: Dissolve 292.2 g of NaCl in 800 mL double-distilled H₂O; adjust the volume to 1000 mL and autoclave the solution.

4. 3 M Sodium acetate pH 5.2: Dissolve 408.3 g sodium acetate 3H₂O in 800 mL double-distilled H₂O, adjust pH to 5.2 using glacial acetic acid, adjust the volume to 1000 mL, and autoclave the solution.

5. 5 M Potassium acetate pH (6.5): Dissolve 49.07 g potassium acetate in 70 mL double-distilled H₂O and adjust pH to 6.5 using glacial acetic acid, adjust the volume to 100 mL, and autoclave the solution.
6. RNase A (10 mg/mL): Dissolve 10 mg RNase A in 1 mL autoclaved double-distilled H₂O, vortexed well and boiled in water bath at 100 °C for 10–15 min, cool down, and store at −20 °C.

7. Chloroform isoamyl alcohol: Prepare freshly as 24:1 v/v.

8. TE (Tris-EDTA) buffer (10:1 v/v): Mix 1 mL 1 M Tris–HCl pH 8, 0.2 mL 0.5 M EDTA pH 8, and 98.8 mL autoclaved double-distilled H₂O.

9. 50× TAE buffer: Dissolve 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL 0.5 M EDTA, pH 8, in 800 mL H₂O and make the volume up to 1000 mL.

10. 5× TBE buffer: Dissolve 54 g Tris base and 27.5 g boric acid and add 20 mL of 0.5 M EDTA, pH 8, in 800 mL H₂O, volume made up to 1000 mL.

11. DNA extraction buffer (100 mL): as described in Table 1.

According to SDS protocol adapted from [8, 10]:

1. Grind plant sample in liquid nitrogen to a fine powder using mortar and pestle.
2. Add 200 mg fine grinded sample into 2 mL tube.
3. Add 600 μL DNA extraction buffer.
4. Incubate at 65 °C for 20–30 min.
5. Add 3 μL RNase 1 (10 mg/mL) for each sample and incubate at 37 °C for 15–30 min.
6. Add equal volume (~600 μL) of chloroform isoamyl 24:1 and centrifuge at 12,000 × g for 20 min.
7. Transfer the supernatant to new 1.5 mL tube, add (1/3 volume) about 400 μL of K-acetate (5 M), and shake vigorously; centrifuge at 12,000 × g for 20 min.

3.2 Extraction and Quantification of Genomic DNA

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>Volume used</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris–Cl (pH 8.0)</td>
<td>10 mL</td>
<td>100 mM</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>4 mL</td>
<td>20 mM</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>28 mL</td>
<td>1.4 M</td>
</tr>
<tr>
<td>SDS</td>
<td>2 g</td>
<td>2%</td>
</tr>
<tr>
<td>PVP</td>
<td>2 g</td>
<td>2%</td>
</tr>
<tr>
<td>β-Mercapto-ethanol*</td>
<td></td>
<td>0.1%</td>
</tr>
</tbody>
</table>

*Add before starting extractions
8. Transfer the supernatant to a new 1.5 mL tube and add \( \frac{1}{2} \) volume cold isopropanol, mix well, and incubate at 4 °C for 1 h (you may incubate overnight).

9. Centrifuge the samples at 12,000 \( \times g \) for 20 min at 4 °C.

10. Pour off the supernatant (you should see a pellet at the bottom of the tube).

11. Invert tubes and let them air dry for 10 min.

12. Add 300 \( \mu L \) TE and incubate at 65 °C for 20 min.

13. Centrifuge the samples at 12,000 \( \times g \) for 5 min at 4 °C.

14. Transfer the supernatant to a new 1.5 mL tube.

15. Add 30 \( \mu L \) Na-acetate (3 M) and 200 \( \mu L \) ice-cold isopropanol and mix very well.

16. Incubate at 4 °C (or over ice) for 1 h.

17. Centrifuge at 12,000 \( \times g \) for 10 min at 4 °C to pelletize the DNA.

18. Discard the solution and wash with 75% ethanol for 3 min.

19. Centrifuge at 12,000 \( \times g \) for 10 min at 4 °C.

20. Discard the solution, invert tubes, and dry for 30 min (do not over-dry the pellet).

21. Add 50–100 \( \mu L \) of TE and keep samples at 4 °C overnight (see Note 1).

22. Check quality and quantity: Weigh out and dissolve 0.8 g agarose in 100 mL 0.5\( \times \) TBE buffer. Heat the mixture until boiling. Swirl the mixture until no solid bits of agarose remain. Add 3 \( \mu L \) acridine orange (10 mg/mL) to the agarose gel and allow the agarose to cool to 50 °C.

23. Prepare electrophoresis set using proper tray and cones.

24. Load 5 \( \mu L \) DNA solutions and mix 0.2 volumes (1 \( \mu L \)) of loading dye in each well in the gel stained with acridine orange.

25. Run for 30 min at proper electrical voltage using formula of 5 V/cm distance from electrodes of the electrophoresis set.

26. Detect DNA quality under UV-light using gel documentation system.

27. Determine the DNA quantity at 260 nm using spectrophotometer available.

28. Determine the purity of DNA by the ratio of 260/280 nm. The best values ranged 1.6–1.8.

29. Standardize the DNA using TE buffer according to required concentrations.

30. Keep the DNA at -20 °C.
3.3 Cloning and Sequencing of SSR-Enriched Library

3.3.1 Restriction of Genomic DNA

1. Digest 10 μg gDNA with several combinations of restriction enzymes: EcoRI, HaeIII, HaeI, MseI, RsaI, BglII, EcoRV, and HindI using 2 U enzyme per μg of DNA with 1× supplied buffer and 1× BSA.
2. Incubate overnight at 37 °C.
3. Check digestion with 0.8% agarose gel.
4. Inactivate enzymes by heating reaction to 65 °C for 20 min.
5. The combinations of restriction enzymes for digest:
   (a) EcoRI + MseI, 20 U/μL + 10 U/μL, EcoRI buffer.
   (b) AluI + HaeIII + RsaI, 10 U/μL, 10 U/μL, 10 U/μL, NEB buffer 2.
   (c) EcoRI + EcoRV + HindI + BglII, 20 U/μL, 10 U/μL, 10 U/μL, NEB buffer 3.
   (d) Sau3AI, 4 U/μL, Sau3AI buffer.
   (e) HaeII + HaeIII + RsaI, 20 U/μL, 10 U/μL, 10 U/μL, NEB buffer 2.
   (f) PstI + MseI, 20 U/μL, 10 U/μL, NEB buffer 2.

3.3.2 End Polishing with Mung Bean Nuclease (MBN)

1. Add 1 U of MBN enzyme per μg of DNA to the inactivated restriction reaction and incubate at 30 °C for 30 min.
2. Transfer the reaction to 1.5 mL tubes.
3. Add the same volume of chloroform isoamyl alcohol to remove the enzyme.
4. Centrifuge the tubes at 12,000 × g for 10 min and transfer DNA to new tubes.
5. Precipitate DNA with 1 volume of 3 M NaAc pH 5.2 and 2.5 volumes of ethanol.
6. Wash with 70% ethanol and dissolve in 30 μL TE.

3.3.3 Dephosphorylation

1. Adjust DNA concentration to 50 ng/μL using TE buffer.
2. Use 1× of NEB3 buffer and 10 U of alkaline phosphatase (CIAP) per 5 μg DNA.
3. Incubate the reaction at 37 °C for 3 h.
4. At the end, add 5 mM EDTA and inactivate the enzyme at 75 °C for 10 min.
5. Transfer the reaction to 1.5 mL tube.
6. Add the same volume of chloroform isoamyl alcohol.
7. Transfer DNA to a new tube and precipitate with 1 volume of 3 M NaAc pH 5.2 and 2.5 volumes of ethanol.
8. Wash with 70% ethanol and dissolve in 30 μL TE.

mohan.jain@helsinki.fi
3.3.4 Preparation of Linkers

1. Combine 300 pmol SNX reverse primer (5'-GCT TCT GCT AGC AAG GCC TTA GAA AA-3') and 20 μL deionized water (dH2O) in a 0.5 mL PCR tube (see Note 2).
2. Heat in thermocycler for 5 min at 70 °C and cool immediately on ice to prevent secondary structures.
3. Add 1× ligase buffer and 10 U of T4 PK (polynucleotide kinase).
4. Mix and incubate for 1 h at 37 °C.
5. Increase temperature to 65 °C for 20 min.
6. Then add 300 pmol SNX forward primer (5'-CTA AGG CCT TGC TAG CAG AAG C-3') and 7 μL dH2O.
7. Mix and heat to 97 °C for 3 min then cool down.

3.3.5 Ligation of Linkers to CIP-Treated DNA Fragments

1. In a 20 μL volume add:
   (a) 500 ng DNA.
   (b) 60 pmol phosphorylated SNK linker.
   (c) 10× ligase buffer.
   (d) 10 U XmnI restriction enzyme (0.5 μL).
   (e) 6 Weiss U of ligase (1.5 μL).
2. Incubate the reaction at 16 °C for 30 min and then 37 °C for 10 min.
3. Inactivate enzymes at 65 °C 20 min.
4. Cool down the ligated reaction.

3.3.6 Preparation of Long Microsatellite Probes for Fishing

1. Prepare the GA, GT, ACA, and AGA probes as follows. In 50 μL combine:
   (a) Two complementary oligos (GA15 + TC10, GT15 + AC9, ACA10 + TTG6, AGA10 + TTC6), 2.5 μM of each oligo.
   (b) 1× PCR buffer.
   (c) 2.5 mM Mg++.
   (d) 10 mM dNTPs.
   (e) 2 U of Taq polymerase.
2. The cycling protocol:
   94 °C 2 min and 20 cycles:
   45 s 94 °C.
   30 s 36 °C.
   1 min 45 s 72 °C.
   Then another 20 cycles:
   45 s 94 °C.
   30 s 55 °C.
   1 min 45 s 72 °C.
3. After completing the reaction, precipitate PCR product and clean up with one round of chloroform isoamyl alcohol (24:1).

4. Ethanol precipitation with 1/10 of 3 M NaAc pH 5.2 and 2.5 volumes of ethanol (see Notes 3–6).

3.3.7 Fishing Microsatellites

1. Mix 50 mL consisting of:
   (a) 25 mL formamide.
   (b) 7.5 mL 20× SSC.
   (c) 2.5 mL 10% SDS.
   (d) 0.49 mL 1 M NaH₂PO₄.
   (e) 0.76 mL Na₂HPO₄.
   (f) dH₂O 13.75 mL.

2. Denature ligated PCR fragments (5–8 μg) for 5 min at 95 °C together with 600 pmol of SNX forward or SNX reverse primers.

3. Pipet mixture into hybridization buffer (1 mL) in 2 mL tube.

4. Pre-warm at 70 °C which contains hybridization membrane(s) for fishing.

5. Place tubes into hybridization oven (into roller bottles) at 70 °C and then lower the temperature slowly to 37 °C overnight.

3.3.8 Washing Membranes

1. Wash the membranes in 0.5 mL PCR tubes in thermocycler, 5 min each, and wash at 56 °C 5 times in 2× SSC + 0.01% SDS and three times in 0.5× SSC + 0.01% SDS.

2. After the last washing, place the membranes in 500 μL tube supplemented with 220 μL sterile dH₂O and elute catch fragments at 98 °C for 5 min.

3. Cool down the tubes and transfer the eluted fragments into new tube.

3.3.9 PCR of Eluted DNA (After Washing)

1. In a PCR tube, combine the following:
   (a) 5 μL of eluted motives.
   (b) 1× PCR buffer.
   (c) 10 μL 2 mM Mg²⁺.
   (d) 4 μL of 0.2 mM dNTPs.
   (e) 0.5 μM SNX forward primer.

2. 1.5 U Taq polymerase. Set PCR reaction program:
   3 min at 94 °C and 40 cycles:
   94 °C for 30 s.
62 °C for 1 min.
72 °C for 2 min.
Final extension at 72 °C for 8 min.

3. The PCR amplicons of ligated fragments with SNX forward primer are shown in Fig. 1.

4. Clean and precipitate PCR products using Sephadex® g-50 chromatography.

1. Clean elution of the SSR motifs is used for TA cloning using pGEM Easy cloning kit by following manufacture’s recommended protocol.

2. Prepare 2× Rapid Ligation Buffer for T4 DNA ligase as 60 Mm Tris–HCl (pH 7.8), 20 mM MgCl₂, 20 mM DTT, and 2 mM ATP 10% polyethylene glycol (MW8000, ACS grade).

3. PGEM-T Easy includes (see Notes 7–15):
   (a) 1.2 μg pGEM-T Easy Vector (50 ng/μL).
   (b) 12 μL Control Insert DNA (4 ng/μL).
   (c) 100 U T4 DNA ligase.
   (d) 200 μL 2× Rapid Ligation Buffer, T4 DNA ligase.
   (e) 1.2 mL JM109 high-efficiency competent cells (6 × 200 μL).

4. Briefly centrifuge the pGEM-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.

5. Set up ligation reactions as described in Table 2.

6. Mix the reactions by pipetting.

7. Incubate the reactions for 1 h at room temperature.

8. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4 °C.
3.3.11 Transformation Protocol and Screening Transformants for Inserts

1. IPTG stock solution (0.1 M): Add 1.2 g IPTG to 50 mL final volume of water. Filter-sterilize and store at 4 °C.

2. X-Gal (2 mL) 100 mg: Dissolve in 2 mL N,N-dimethyl-formamide a 5-bromo-4-chloro-3-indolyl-β-D-galactoside, cover with aluminum foil, and store at −20 °C.

3. LB medium (per L): Dissolve 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl in 1 L H2O; adjust pH to 7 with NaOH.

4. LB plates with carbenicillin: Add 15 g agar to 1 liter LB medium and autoclave. Allow the medium to cool to 50 °C before adding carbenicillin to the final concentration of 100 μg/mL. Pour 30–35 mL medium into 85 mm Petri dishes. Let the agar harden. Store at 4 °C for up to 1 month or at room temperature for up to 1 week.

(a) LB plates with carbenicillin/IPTG/X-Gal: Make the LB plates with carbenicillin as above; then supplement with 0.5 mM IPTG and 80 μg/mL X-Gal and pour into the plates. Alternatively, 100 μL 100 mM IPTG and 20 μL 50 mg/mL X-Gal may be spread over the surface of an LB/carbenicillin plate, allowing absorption for 30 min at 37 °C prior to use.

5. SOC medium (100 mL): Dissolve 2 g Bacto-tryptone, 0.5 g Bacto-yeast extract, 1 mL 1 M NaCl, 0.25 mL 1 M KCl, 1 mL 2 M Mg2+ stock, filter-sterilized, and 1 mL 2 M glucose and filter-sterilized. Add Bacto-tryptone, Bacto-yeast extract, NaCl, and KCl to 97 mL distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2 M Mg2+ stock and 2 M glucose, each to a final concentration of

---

Table 2
List of ligation reaction components

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Standard reaction, μL</th>
<th>Positive control, μL</th>
<th>Background control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2× Rapid Ligation Buffer, T4 DNA ligase</td>
<td>5</td>
<td>5</td>
<td>5 μL</td>
</tr>
<tr>
<td>pGEM-T Easy Vector (50 ng)</td>
<td>1</td>
<td>1</td>
<td>1 μL</td>
</tr>
<tr>
<td>PCR product</td>
<td>X*a</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control Insert DNA</td>
<td>–</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>T4 DNA ligase (3 Weiss units/μL)</td>
<td>1</td>
<td>1</td>
<td>1 μL</td>
</tr>
<tr>
<td>Nuclease-free water to a final volume of</td>
<td>10</td>
<td>10</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

*aMolar ratio of PCR product: vector may require optimization

mohan.jain@helsinki.fi
3.4 Transformation Protocol

1. Prepare two LB/carbenicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency (see Notes 16–26).

2. Equilibrate the plates to room temperature.

3. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom.

4. Add 2 μL of each ligation reaction to a sterile (17 × 100 mm) polypropylene tube or a 1.5 mL microcentrifuge tube on ice.

5. Set up another tube on ice with 0.1 ng uncut plasmid for determination of the transformation efficiency of the competent cells.

6. Remove tube(s) of frozen JM109 high-efficiency competent cells from storage and place in an ice bath until just thawed (about 5 min).

7. Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.

8. Carefully transfer 50 μL of cells into each tube prepared in step 2 (use 100 μL of cells for determination of transformation efficiency).

9. Gently flick the tubes to mix and place them on ice for 20 min.

10. Heat-shock the cells for 45–50 s in a water bath at exactly 42 °C (do not shake).

11. Immediately return the tubes to ice for 2 min.

12. Add 950 μL room temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900 μL to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).

13. Incubate for 1.5 h at 37 °C with shaking (~150 rpm).

14. Plate 100 μL of each transformation culture onto duplicate LB/carbenicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating.

15. Incubate the plates overnight (16–24 h) at 37 °C. If 100 μL is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1 × 108 cfu/μg DNA.

16. Grow positive transformed bacterial colonies in 96 deep-well 2 mL plates supplemented with carbenicillin (100 μg/mL).
17. Using a 10 μL pipette tip, select colonies from LB/carbenicillin/IPTG/X-Gal plates (look for single colonies) by scraping part of a colony from the plate and placing the tip in the corresponding well containing 2 mL LB/carbenicillin broth.

18. Be sure to laterally mix the pipette tips in each well for up to 5 s before incubating.

19. Upon incubation, be sure to leave the tips in each of the wells to assist with agitation.

20. Make sure the tips are fully immersed in the well and are touching the well bottom.

21. Tips that are not flush with the well bottom may eject during incubation or adhere to the side of the well and may not properly mix in the LB/carbenicillin. This may lead to a loss of the sample and poor/no sample growth and/or cause sample cross contamination.

22. Place the cultures at 37 °C in a shaking incubator, set at up to 250 rpm.

23. Remove the deep-well plates from the incubator and prepare a glycerol stock from the deep-well plate by manually removing 200 μL from each well of the deep-well plates, and place into each corresponding location within two additional 96 plates.

24. Centrifuge these 96 plates for 10 min at 3000 × g and remove the growth media by inverting the plate and blotting the top surface on a paper towel.

25. Resuspend the pellet in 200 μL of LB + 30% glycerol, mix 1–2 times, and follow by freezing.

26. Pelletize the cells from the growth culture remaining in the deep-well plates by centrifugation for 10 min at 3000 × g and discard the supernatant.

27. Dry the pellets by inverting the plate and blotting the plate on a paper towel for 15–30 s.


3.5 Plasmid Minipreparation Using the QIAprep Spin MiniPrep Kit

All protocol steps should be carried out at room temperature (15–25 °C).

1. Resuspend pelleted bacterial cells in 250 μL Buffer P1 and transfer to a microcentrifuge tube.

2. Suspended the bacteria completely by vortexing or pipetting up and down until no cell clumps remain. No cell clumps should be visible after resuspension of the pellet.

3. Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle.
Buffer P1 for a final concentration of 100 μg/mL. Mix and store at 2–8 °C.

4. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

5. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved.

6. Add 250 μL Buffer P2 and mix thoroughly by inverting the tube 4–6 times.

7. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear.

8. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2.

9. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

10. Add 350 μL Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.

11. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g., ≥5 mL) may require inverting up to ten times.

12. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless.

13. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

14. Centrifuge for 10 min at ~17,900 × g in a table-top microcentrifuge. A compact white pellet will form.

15. Apply 800 μL of the supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.


17. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB and centrifuging for 30–60 s.

18. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content.
19. Wash QIAprep 2.0, spin column by adding 0.75 mL buffer PE, and centrifuge for 30–60 s.

20. Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.

21. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube.

22. To elute DNA, add 50 μL Buffer EB (10 mM Tris–Cl, pH 8.5) or water to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.

Capillary electrophoresis using a denaturing flowable polymer has largely replaced the use of gel separation techniques due to significant gains in workflow, throughput, and ease of use.

3.6 Sequence Processing and Assembly

3.6.1 Preparing the Reactions for 96-Well Reaction Plates or Microcentrifuge Tubes

1. For each reaction add the following reagents to a separate tube as in Table 3.

2. Mix well and spin briefly.

3. Place the plate in a thermal cycler and set to the correct volume (20 μL).

4. Perform an initial denaturation 96 °C for 2 min.

5. Repeat the following for 30 cycles: 20 s at 96 °C, 30 s at 50 °C, and 4 min at 60 °C.

6. Spin down the contents of the tubes in a microcentrifuge.

3.6.2 Purifying Extension Products Using Ethanol/EDTA Precipitation Method

To precipitate 20 μL sequencing reactions in 96-well reaction plates:

1. Remove the 96-well reaction plate from the thermal cycler and briefly spin.

2. Add 5 μL 125 mM EDTA to each well. Make sure the EDTA reaches the bottom of the wells.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity, μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BigDye terminator v3.1 Terminator</td>
<td>2.0</td>
</tr>
<tr>
<td>BigDye Terminator v3.1 Sequencing Buffer (5×)</td>
<td>3.0</td>
</tr>
<tr>
<td>Miniprep plasmid DNA</td>
<td>2.5</td>
</tr>
<tr>
<td>T7 primer (10 μM stock)</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionized water</td>
<td>12</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>
3. Add 60 μL 100% ethanol to each well.
4. Seal the plate with aluminum tape and mix by inverting four times.
5. Incubate at room temperature for 15 min.
6. Use a plate adapter and spin the plate at the maximum speed as follows:
   - 1400–2000 × g for 45 min or 2000–3000 × g for 30 min.
7. Invert the plate and spin up to 185 × g, and then remove from the centrifuge.
8. Add 60 μL 70% ethanol to each well.
9. With the centrifuge set to 4 °C, spin at 1650 × g for 15 min.
10. Invert the plate and spin up to 185 × g for 1 min, and then remove from the centrifuge.
11. To continue, resuspend the samples in injection buffer.
12. To store, cover with aluminum foil, and store at 4 °C.

### 3.6.3 Sample Electrophoresis

1. Add 10 μL Hi-Di formamide to each sample pellet (see Notes 27–29).
2. Seal the wells with aluminum sealing tape.
3. Vortex thoroughly, and then centrifuge briefly.
4. Peel off the aluminum sealing tape and replace the tape with septa.

### 3.6.4 Electrophoresis Workflow

1. Create a plate record for each plate to be run.
2. A plate record consists of the following information about the plate and all samples on the plate:
   (a) ID or barcode number (for 3730/3730xl instruments only).
   (b) Plate name.
   (c) Plate type.
   (d) Application type.
   (e) Owner name.
   (f) Operator name.
3. For each sample:
   (a) Sample name.
   (b) Comment (optional).
   (c) Results group: Contains settings for file names and locations, the sequencing analysis software in use, and the auto-analysis setting.

mohan.jain@helsinki.fi
(d) Analysis protocol: Contains settings necessary for analysis and post processing of the sequence.
(e) Instrument protocol: Contains settings for running the instrument.

4. Prepare the instrument:
   (a) Start the computer, the instrument, and the data collection software.
   (b) Select the capillary length, number of capillaries, and polymer type.
   (c) Check the polymer delivery system for sufficient polymer and absence of bubbles.
   (d) Prepare the buffer and water/waste reservoirs.

5. Perform spatial calibration, if necessary.
6. Perform spectral calibration, if necessary.
7. Load the prepared samples into 96-well and place the tubes or the plate assembly into the instrument.
8. Set up the run using data collection software:
   (a) Create a plate record.
   (b) Create a results group, with file name and folder preferences.
   (c) Create an instrument protocol, including run module and dye set configurations.
   (d) Create an analysis protocol.
   (e) Determine the run parameters.
   (f) Link the plate.
9. Start the run.

3.6.5 Sequence Processing and Assembly

1. The sequence trace files will be base-called using the Phred program, and low-quality bases (<Q20, 99% accuracy) should be eliminated from sequence ends, followed by SeqClean [11] to shorten the Poly-A/T to five continuous bases.
2. The vector and other contaminating microbial sequences will be removed using the VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.htmL).
3. After trimming, discard the EST sequences that were shorter than 100 bp, and deposit those that are greater than 100 bp into the dbEST division of GenBank.
3.6.6 Annotation and Functional Classification

1. Carry out BLAST searches after clustering and assembly to identify similarities between the ESTs and other sequences already deposited in public databases.

2. Compare the UniGene sequences to GenBank nonredundant protein and nucleotide databases using either the blastx ($E$-value $\leq 10^{-6}$) or the blastn ($E$-value $\leq 10^{-6}$) program [12].


4. Perform Inter ProScan, and merge with the results GO annotations to improve them.

5. Perform the analysis of biological pathways using the KEGG (Kyoto Encyclopedia of Genes and Genomes) [15].

3.6.7 Bioinformatics and Primer Designing


2. Analyze sequences row data and their quality using Phred score.

3. Design specific primers flanking SSR motives using BatchPrimer3 software [16].

4. Test the microsatellite primers on selected DNA samples from date palm cultivars (Fig. 2 an example of electropherograms of four date palm samples representing four cultivars using SSR primer pairs run on the Applied Biosystems 3130xl Genetic Analyzer).

5. Perform fragment analysis with GeneMapper analysis software v3.7 Applied Biosystems (ABI).

4 Notes

1. Store DNA at $-20^\circ C$ when eluted with water, as DNA may degrade in the absence of a buffering agent. DNA can also be eluted in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8), but the EDTA may inhibit subsequent enzymatic reactions.

2. Use 0.5 mL tubes known to have low DNA-binding capacity.

3. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product.

4. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Gel and PCR Clean-Up System.

5. Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products and to improve ligation efficiency.
6. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

7. Use only the T4 DNA ligase supplied with this system to perform pGEM-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.

8. Vortex the 2× Rapid Ligation Buffer vigorously before each use.

9. 2× Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.

10. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the highest number of transformants.

Fig. 2 Electropherograms of four date palm samples representing four cultivars using four SSR primer pairs run on the Applied Biosystems 3130xl Genetic Analyzer displayed in the GeneMapper software. The y-axis is intensity of the peak measured in relative fluorescence units (rfu) and the x-axis is the number of base pairs (bp)
Fig. 2 (continued)
11. pGEM-T Easy Vectors are approximately 3 kb and are supplied at 50 ng/μL. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation:

\[
\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \left( \frac{\text{insert : vector molar ratio}}{\frac{1}{2}} \right) = \text{ng of insert.}
\]

12. Example of insert/vector ratio calculation: How much 0.5 kb PCR product should be added to a ligation in which 50 ng of 3.0 kb vector will be used if a 3:1 insert/vector molar ratio is desired:

\[
\frac{50 \text{ng vector} \times 0.5 \text{kb insert}}{3.0 \text{kb vector}} \times \left( \frac{3}{1} \right) = 25 \text{ ng insert.}
\]

13. Transformations using the pGEM-T Easy Vector Ligation Reactions: Use high-efficiency competent cells (≥1 × 10^8 cfu/μg DNA) for transformations.

14. Ligation of fragments with a single-base overhang can be inefficient, so it is essential to use cells with a transformation efficiency of 1 × 10^8 cfu/μg DNA (or higher) in order to obtain a reasonable number of colonies.

15. Use JM109 high-efficiency competent cells provided with the pGEM-T Easy Vector. Other host strains may be used, but they should be compatible with blue/white color screening and standard carbenicillin selection.

16. Selection for transformants should be done on LB/carbenicillin/IPTG/X-Gal plates. For best results, do not use plates that are more than 1 month old.

17. If a higher number of colonies are desired, the cells may be pelleted by centrifugation at 1000 × g for 10 min, resuspended in 200 μL SOC medium, and 100 μL plated on each of two plates.

18. Use of ultra-high-efficiency competent cells may result in a higher number of background colonies. Longer incubations or storage of plates at 4 °C (after 37 °C overnight incubation) may be used to facilitate blue color development. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

19. Successful cloning of an insert into the pGEM-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates.
20. Usually clones containing PCR products produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the lacZ gene.

21. The Control Insert DNA supplied with the pGEM-T Easy Systems is a 542 bp fragment from pGEM-luc Vector DNA. This sequence has been mutated to contain multiple stop codons in all six reading frames, which ensures a low background of blue colonies for the control reaction. Results obtained with the Control Insert DNA may not be representative of those achieved with your PCR product.

22. Using of larger (17 x 100 mm) polypropylene tubes (e.g., Falcon Cat. # 2059) increases transformation efficiency.

23. Colonies containing β-galactosidase activity may grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies, which are approximately 1 mm in diameter.

24. Blue color will become darker after the plate has been stored overnight at 4 °C.

25. Example of transformation efficiency calculation: After 100 μL competent cells are transformed with 0.1 ng uncut plasmid DNA, the transformation reaction is added to 900 μL SOC medium (0.1 ng DNA/mL). From that volume, a 1:10 dilution with SOC medium (0.01 ng DNA/mL) is made and 100 μL plated on two plates (0.001 ng DNA/100 μL). If 200 colonies are obtained (average of two plates), what is the transformation efficiency? 200 cfu = 2 x 105 cfu/ng = 2 x 108 cfu/μg DNA 0.001 ng.

26. Purifying extension products using ethanol/EDTA precipitation method: While this method produces the cleanest signal, it may cause loss of small molecular weight fragments. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some sequencing results. 95% ethanol is usable, but you must make sure the final ethanol concentration for precipitation remains the same (67–71%).

27. Applied Biosystems recommends using Hi-Di formamide to resuspended purified sequencing products.

28. Resuspension in water is not recommended because oxidative effects on terminator dyes lead to earlier dye breakdown of sequencing extension products, affecting base calling.

29. Use fresh Hi-Di formamide to prevent dye degradation; old Hi-Di formamide or low-quality formamide can have formic acid that can contribute to the degradation of fluorescent dyes.
Acknowledgment

The authors are grateful to the National Plan for Science and Technology (NPST) program project number 08-BIO-164-2 and to the Deanship of the Scientific Research/Agricultural Research Center at King Saud University, for financial support.

References

Chapter 25

MicroRNA Expression in Multistage Date Fruit Development

Wanfei Liu, Chengqi Xin, Jun Yu, and Hasan Awad Aljohi

Abstract

MicroRNAs (miRNAs) are small endogenous noncoding RNAs. Plant miRNAs are known to play important regulatory roles in homeostasis, stress response, and diverse developmental processes. Here, we describe the identification of conserved miRNAs in date palm (Phoenix dactylifera L.) based on transcriptomic data acquired across multistage fruit development and genome sequences, which include 238 plant conserved miRNAs and 276 novel P. dactylifera-specific miRNAs.

Key words miRNA, miRNA target, Fruit development

1 Introduction

After the first miRNA lin-4, which can repress the expression of its target gene, had been reported in 1993 [1], miRNA-mediated gene regulation became known to be deeply involved in the most important biological functions in animals and plants, such as chromatin structure, chromosome segregation, transcription, RNA processing, and RNA stability and translation [2]. The miRNAs regulate gene expression by binding to their complementary sequences, leading to either cleavage-induced degradation or translational repression of their target transcripts. Since the first discovery of miRNAs from Arabidopsis in 2002 [3], plant miRNAs from various species have been intensely studied through both experimental and computational approaches.

Generally, for miRNA identification, the combined evidence of expression, biogenesis, conservation, and structure is considered [4]. miRNAs are usually identified by the following criteria: (1) about 18–25 nt length mature miRNA produced by Dicer; (2) the precursor forms a hairpin structure, and the mature miRNA is present in one arm of the hairpin; (3) both the mature and the precursor miRNAs are usually phylogenetically conserved; (4) the precursor miRNAs should be observed when Dicer function is disturbed. Moreover, the precursor length, matching base pairs in
the precursor, free energy, bulge number and size, mismatch base number in hairpin, and other parameters like A + U content and minimal folding free energy index (MFEI) can be considered [5].

The essential step to understand miRNA regulatory function is target prediction. In animals, there are many methods like miRanda [6], RNAhybrid [7], TargetScan [8], and PicTar [9], which are mainly based on the complementation between 5’ seed of the miRNA (nucleotide positions 2–8 of the miRNA) and 3’ UTR of the target mRNA. In plants, psRNATarget [10], TargetFinder [11], and TAPIR [12] are used for target identification according to their perfect or nearly perfect complementarity between miRNAs and the target genes [13].

Recently, the genome sequence of *Phoenix dactylifera* was published [14] and facilitated miRNA identification in this economically important plant [15–17]. Here, we took a combined strategy of computational prediction and high-throughput miRNA sequencing to report the genome-wide miRNA identification and expression profiles during *P. dactylifera* fruit development. We first identified a total of 238 nonredundant conserved miRNAs based on highly conserved known plant miRNAs using the genome sequence as reference. Then, 276 novel miRNAs were identified through sequencing six small RNA libraries from different fruit developmental stages. Furthermore, the prediction of miRNA-targeted genes and functional analysis revealed that these miRNAs may participate in regulating carbon metabolism, starch and sucrose metabolism, fructose and mannose metabolism, glycolysis, and citrate cycle.

This chapter describes the detailed experimental and computational methodology in genome-wide miRNA discovery in *P. dactylifera*, which provides a proposed standard protocol for plant miRNA identification with the advantages of high-throughput sequencing technology.

## 2 Materials

### 2.1 Plant Materials

1. Fruits of date palm cultivar Khalas at different developmental stages, including 0, 15, 45, 75, 105, and 120 days after fertilization (DAF), collected from date palm trees grown in Al-Kharj (24°08′54″N, 47°18′18″E), Saudi Arabia.

### 2.2 Small RNA Libraries Construction and Sequencing

1. DEPC water.

2. 2% CTAB extraction buffer (30 mL): 3 mL Tris–HCl (1 M, pH 6), 9 mL NaCl (5 M), 3.75 mL hexadecyltrimethylammonium bromide (CTAB) (16%, w/v), 6 mL ethylenediaminetetraacetic acid (EDTA) (250 mM, pH 7), and 0.231 g dithiothreitol (DTT).
3. RNA extraction buffer: 2% CTAB, 2% polyvinylpyrrolidone (PVP), 100 mM Tris–HCl (1 M, pH 8), 25 mM EDTA, 2 M NaCl, and 1% mercaptoethanol (see Note 1).

4. Solution buffer (SSTE): 1 M NaCl, 0.5 M sodium dodecyl sulfate (SDS), 1 mM EDTA (pH 8), and 10 mM Tris–HCl (1 M, pH 8).

5. FlashPAGE Gel Loading Buffer A40 and flashPAGE Reaction Clean-Up Kit from Ambion Company.

6. QIAGEN™ MinElute PCR Purification Kit.

7. Invitrogen™ PureLink PCR Micro Kit.

8. SOLiD™ Small RNA Expression Kit from Applied Biosystems.

9. Ambion® Nuclease-free water.

10. RNase-free DNase I.

11. 10% TBE-Urea Gel.

12. ACS Reagent: chloroform/isoamyl alcohol (24:1, vol/vol) mix, LiCl, 100% ethanol, 3 M NaAC (pH 5.2).

### 2.3 Real-Time PCR Validation

1. dNTP mix (10× each), stem-loop RT primer (1 μM).

2. Invitrogen™ SuperScript® II reverse transcriptase, DTT (0.1 M), Applied Biosystems™ RNase inhibitor (20 U/μL).

3. TIANGEN™ SuperReal PreMix Plus (SYBR Green) Kit: 12.5 μL 2× SYBR green mix, 5 μL forward primer (1 μM), 5 μL reverse primer (1 μM), 2 μL cDNA, 0.5 μL 50× Rox.

### 2.4 Equipment

1. FlashPAGE™ Fractionator.

2. Agilent™ 2100 Bioanalyzer.


4. Qubit™ 2.0 fluorometric quantitation.

5. Thermo Scientific™ NanoDrop™ 2000 UV-Vis Spectrophotometer.

6. High-throughput sequencing platforms (SOLiD™ System 4.0).


### 3 Methods

#### 3.1 SOLiD Small RNA Library Construction and Sequencing (Fig. 1)

1. Collect all samples, wash with distilled water, freeze in liquid nitrogen, and store at −80 °C (Fig. 2).

mohan.jain@helsinki.fi
3.1.2 Total RNA Extraction

1. Add 20 mL RNA extraction buffer into 50 mL tube, and preheat in 65 °C water bath.

2. Add liquid nitrogen to precooling mortar, add 5 g fruit and grind until a layer of very fine dust is left, transfer to preheated RNA extraction buffer in 50 mL tube, vortex mixture thoroughly, leave at 65 °C water bath for 10–20 min, and invert several times to well mix every 3 min.

3. Add an equal volume of chloroform/isoamyl alcohol mix, invert several times to mix well, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and transfer the aqueous phase (top) to a fresh tube.

Fig. 1 The workflow of SOLiD small RNA library building

Fig. 2 The fruit of date palm cultivar Khalas at different developmental stages after fertilization
4. Repeat step 3.
5. Add 1/4 volume 10 M LiCl, invert several times to mix well, and refrigerate at 4 °C overnight (see Note 2).
6. Invert several times and aliquot to 1.5 mL tube, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and remove the supernatant.
7. Add 500 μL SSTE, vortex mixture slightly, add an equal volume of chloroform/isoamyl alcohol mix, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and transfer the aqueous phase to a fresh tube.
8. Add 2.5 volumes 100% ethanol, leave at −80 °C at least 30 min, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and remove the supernatant.
9. Add 1 mL 75% ethanol, invert several times to suspend the precipitate, centrifuge sample at 13,000 rpm for 5 min at 4 °C, and remove the supernatant.
11. Dry the precipitate at room temperature until ethanol volatilizes completely, and redissolve the pellet using 50 μL RNase-free water.
12. Quantify each sample using NanoDrop, and run 5 μL of each sample on agarose gel. Store total RNA at −80 °C for use.

3.1.3 DNase I Treatment of Total RNA

1. Transfer 10 μg total RNA into Eppendorf tube, add 8 μL 10× DNase buffer and 2 μL of DNase I, vortex mixture thoroughly, and incubate at 37 °C for 15 min.
2. Add an equal volume of chloroform/isoamyl alcohol mix, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and then transfer the aqueous phase to a new tube.
3. Add 2.5 volumes 100% ethanol, 1/10 volume 3 M NaAc, vortex mixture thoroughly, refrigerate at −80 °C at least 30 min, centrifuge sample at 13,000 rpm for 15 min at 4 °C, and remove the supernatant.
4. Add 1 mL 75% ethanol, invert several times to suspend the precipitate, centrifuge sample at 13,000 rpm for 5 min at 4 °C, and remove the supernatant.
5. Repeat step 4.
6. Dry the precipitate at room temperature until the ethanol volatilizes completely, and redissolve the pellet using 50 μL RNase-free water.
7. Quantify each sample using NanoDrop, and run 5 μL of each sample on agarose gel. Store total RNA at −80 °C for use.
3.1.4 Small RNA Fragments Isolation

1. Mix equal volumes of RNA and flashPAGE Gel Loading Buffer A40 for a maximum volume of 100 μL.
2. Heat at 95 °C for 2 min, and then place in ice.
3. Load the sample onto the upper gel surface, and close the flashPAGE Fractionator.
4. Attach the flashPAGE Fractionator to an electrophoresis power supplier. Electrophorese at 75–80 V constant voltage until the blue dye begins to exit the gel (see Note 3).
5. Open the flashPAGE Fractionator to break the circuit, and remove the flashPAGE Gel.
6. Transfer the lower running buffer, which contains the <40 nt nucleic acid fraction, to a new microtube and place on ice.
7. Concentrate the sample using the flashPAGE Reaction Clean-Up Kit or by precipitating overnight with sodium acetate and ethanol.

3.1.5 SOLiD Small RNA Library Construction and Sequencing

1. Prepare 8 μL hybridization mixture in 200 μL PCR tube on ice: 3 μL small RNA sample (1–500 ng), 3 μL hybridization solution, and 2 μL SOLiD Adaptor Mix. Then invert several times to mix well, and centrifuge sample slightly to center at the bottom of tube.
2. Place the PCR tube on the preprogrammed thermal cycler: 65 °C for 10 min, 16 °C for 5 min, and transfer the tube to ice immediately.
3. Prepare ligation buffer: add 10 μL 2× ligation buffer and 2 μL ligation enzyme mix to 8 μL hybridization mixture, mix gently, centrifuge sample slightly to center at the bottom of tube, and leave at 16 °C for 16 h.
4. Prepare reverse transcription mixture on ice: 11 μL nuclease-free water, 4 μL 10× RT buffer, 2 μL dNTP mix, and 2 μL SOLiD RT primer. Total 19 μL.
5. Mix reverse transcription mixture with ligation reaction buffer, mix gently, centrifuge sample slightly to center at the bottom of tube, leave at 70 °C for 5 min, and keep in ice immediately.
6. Add 1 μL ArrayScript™ transcriptase, vortex mixture slightly, centrifuge sample slightly to center at the bottom of tube, and keep at 42 °C for 30 min.
7. Purify cDNA production in mix as the instruction of MinElute PCR Purification Kit.
8. Separate purified cDNA production by 10% TBE-Urea Gel, select 60–80 nt region, divide into four parts, and use the middle two parts for PCR amplification and the other two parts as backup.
9. Prepare PCR reaction buffer: 76.8 μL nuclease-free water, 10 μL 10× PCR buffer, 8 μL 2.5 mM dNTP mix, 2 μL SOLiD 5’PCR primer, and 1.2 μL AmpliTaq DNA polymerase. Total 98 μL. Add PCR reaction buffer into PCR tube with cDNA production.

10. Add 2 μL SOLiD 3’PCR primer, mix thoroughly, and centrifuge sample slightly.

11. Run PCR cycling reaction: 95 °C for 5 min, followed by 15 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and then 72 °C for 5 min. Hold at 4 °C.

12. Purify PCR production as the instruction of PureLink PCR Micro Kit.

13. Check PCR production by Agilent 2100 Bioanalyzer and quantify sample using Qubit.

14. SOLiD small RNA library is used for SOLiD4 sequencing according to the manufacturer’s instructions.

3.2 miRNA Identification (Fig. 3)

3.2.1 Conserved miRNA Identification

1. Search candidate miRNA region by comparing known plant miRNA from miRBase (version 19) [18] to date palm genome sequence using BLAST (BLASTN 2.2.20) [19] with following parameters: blastall -p blastn -d database (date palm genome sequence) -i query (known plant miRNA) -v 1000 –b 1000 -e 1000 -W 7 -o outfile (see Note 4).

2. Apply region-based filter according to the alignment result and genome structure. We only keep the region satisfying the following criteria: alignment to known miRNA with no more than three mismatches, alignment length among 18–25 bp, and alignment region without overlap to protein-coding region or repeat region (see Notes 5 and 6).

3. Extract candidate miRNA sequence from 100 bp upstream to 100 bp downstream for alignment region.

4. Predict secondary structure for candidate miRNA using RNAfold [20] with following parameters: less sequence_file | RNAfold –noconv –noPS outputfile, and keep the candidate miRNA with hairpin structure (for example, Fig. 4).

5. Calculate the attribute values of candidate miRNA, including MFE (minimum free energy), matched base pairs, unmatched base pairs in mature and star region of miRNA, and maximum base bulge in the alignment region.

6. Filter candidate miRNA according to the attribute values, and only keep the region satisfying the following criteria: maximum base bulge ≤3, matched base pairs ≥16, unmatched base pairs ≤6, and no overlap between mature and star region (see Note 7).
7. Filter candidate miRNA which overlaps more than 60% with other candidate and extract hairpin sequence.

8. Calculate the attribute values for hairpin sequence of candidate miRNA, including hairpin length, match base pairs, match base percent, nucleotide A content, nucleotide U content, nucleotide G content, nucleotide C content, AU content, MFE, adjust MFE (AMFE, MFE/Length × 100), and index of MFE (MFEI, AMFE/GC content).

9. Filter candidate miRNA according to the attribute values of candidate miRNA hairpin sequence, and only keep the region satisfying the following criteria: MFEI ≤ -0.85, MFE ≤ -25 kcal/mol, hairpin sequence length ≥ 50 bp, match base pairs ≥ 16, AU content ≥ 30%, and AU content ≤ 70% (see Note 8).

3.2.2 Novel miRNA Identification

1. Filter the low-quality reads with average read quality score <20 by in-house Perl script programing language (www.perl.org).

2. Map high-quality reads to known RNA database by RNA2-MAP (RNA_pipeline_0.4.0). Known RNA database include
Fig. 4 An example of RNA second structure
date palm rRNA, tRNA, snoRNA, mRNA, and Rfam RNA families (except for miRNA).

3. Extract unmapped reads and align them to identified conserved miRNAs for expression estimation.

4. Align the remaining unmapped reads to date palm genome sequence, cluster mapped reads according to the genome position (overlap size ≥ 16 and overlap rate ≥ 80%), and identify candidate novel miRNA regions according to the clusters.

5. Repeat step 3–9 in conserved miRNA identification section (Subheading 3.2.1), and obtain the candidate novel miRNAs.

6. Filter the candidate novel miRNAs using the following criteria: mapped reads number ≥20, multi-mapped loci ≤10, and mapped read number for miRNA star ≥20 in at least one library or miRNA expressed in at least three libraries (see Note 9).

### Differential Expression Analysis

1. Normalize miRNA expression with read counts of transcript per million reads (TPM) by the following formula:

   \[
   \text{Normalized expression} = \frac{\text{actual read count}}{\text{total read count}} \times 1000000;
   \]

   miRNAs with normalized expression no less than 0.01 are considered effective expression in further analysis.

2. Identify differential expressed miRNAs with cutoffs (\(p\)-value ≤ 0.001 and fold change ≥2) by DEGseq package [21] between each two adjacent development stages.

3. Cluster the expression patterns among differentially expressed miRNA by STEM (short time-series expression miner) [22] using log normalized data option with other default parameters (see Fig. 4 in ref. 16).

### Stem-Loop Quantitative Real-Time RT-PCR Analysis

1. Extract total RNA and treat with DNase I to remove DNA contamination as the methods of Subheadings 3.1.1 and 3.1.2, respectively.

2. Add 3 µL total RNA (50 ng/µL), 1 µL dNTP mix, 1 µL stem-loop RT primer, and 4.55 µL nuclease-free water; leave at 65 °C for 5 min, and keep in ice for at least 2 min immediately.

3. Centrifuge slightly and add 3 µL 5× first-strand buffer, 2 µL DTT, 0.2 µL RNaseIN, and 0.25 µL (50 U) SuperScript II Reverse transcriptase to a final volume of 15 µL and mix well.

4. Synthesize the first-strand cDNAs: 16 °C for 30 min, followed by 60 cycles of pulsed RT at 30 °C for 30 s, 42 °C for 30 s, 50 °C for 1 s, and then 85 °C for 5 min. Store at −20 °C.

5. Prepare a 25 µL PCR reaction: 2 µL cDNA, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2× Master Mix and 0.5 µL nuclease-free water.
6. Run PCR reaction: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and then 72 °C for 10 min. Hold at 4 °C.

7. Check PCR products on 3% agarose gel (see Fig. 3 in ref. 16).

8. Prepare a 25 μL qPCR reaction using SuperReal PreMix Plus (SYBR Green) Kit.

9. Run real-time PCR cycling reaction in an ABI 7500 Sequence Detection System: incubate at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 32 s. Carry out melting curve analysis for each PCR product to avoid nonspecific amplification.

10. Calculate the fold change using the comparative Ct method (see Fig. 5 in ref. 16).

### 3.5 Target Prediction and Functional Analysis

1. Predict target genes of miRNAs by psRNATarget web server (http://plantgrn.noble.org/psRNATarget/) [10] with the default parameters (see Note 10).


### 4 Notes

1. Earlier we observed that polyvinylpyrrolidone (PVP) and mercaptoethanol can reduce the interference of quinones during RNA extraction. Therefore, we suggest to add PVP and mercaptoethanol in RNA extraction buffer. In addition, 1% mercaptoethanol should be added before the usage of RNA extraction buffer.

2. To reduce the effects of polysaccharides, add 10 M LiCl to RNA solution for overnight in order to obtain high-quality RNA.

3. When using flashPAGE Fractionator for small RNA fragment isolation, the voltage must be stable at 75–80 V during electrophoresis.

4. For conserved miRNA identification using the sequence alignment tool BLAST, we suggest adjusting the search parameters to achieve the best alignment performance. Set the expected values as 1000 (–e, default 10); set word-match size between query and database sequences as 7 (–W, default 11); and set the numbers of descriptions (–v, default 500) and alignments (–b, default 250) as 1000. In comparison to using BLAST-like alignment tool (BLAT), the results show BLAST is better.
5. To reduce the BLAST alignment error, we only keep the alignment with no more than three mismatches.

6. Some miRNAs located in intron region. For this reason, please do not remove out intron region-derived sequence during candidate miRNA filtering.

7. To make sure the proper hairpin structure of candidate miRNA, we set the filter criteria according to the structure attribute values: maximum base bulge ≤3 (remove the candidate with loop in hairpin region), matched base pairs ≥16 and unmatched base pairs ≤6 (ensure the alignment of hairpin), and no overlap between mature and star region (confirm the stem-loop structure).

8. Previous study indicated that MFE (minimal folding free energy), AMFE (adjusted MFE), MFEI (minimal folding free energy index), length of hairpin structure, A + U content, and the number of matched base pairs can be used to distinguish miRNA with other RNA [5]. Therefore, filter candidate miRNA by the following criteria: MFEI ≤−0.85, MFE ≤−25 kcal/mol, hairpin sequence length ≥50 bp, matched base pairs ≥16, AU content ≥30%, and AU content ≤70%.

9. For the novel miRNA candidates, the background expression, multiple mapping, and mapping error will increase the false-positive rate. Apply the following criteria to balance the false-positive rate: mapped loci of read ≤10, read number ≥20, read number for miRNA star ≥20 in at least one library, or miRNA expressed in at least three libraries.

10. For miRNA target prediction, several tools such as TargetFinder, miRanda, RNAhybrid, and psRNATarget are available. We found that the psRNATarget web server has the highest performance using experiment-verified data in Arabidopsis thaliana.

References

8. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human
genes are microRNA targets. Cell 120 (1):15–20

mohan.jain@helsinki.fi
Part VI

Proteomics
Chapter 26

Proteome of Abiotic Stress Tolerance in Date Palm

Haddad A. El Rabey

Abstract

This protocol describes the analysis of date palm proteome under salinity and drought stress conditions to possibly identify proteins involved in stress tolerance. Three-month-old date palm seedlings are subjected to drought (27.5 g/L polyethylene glycol 6000) and salinity stress conditions (16 g/L NaCl) for 1 month prior to leaf sample analysis. Differential in gel electrophoresis (DIGE) analysis of protein extracts identifies the sensitive proteins that respond to abiotic stress. Mass spectrometric analysis identifies the significantly changed proteins under both salt and drought stress. This chapter provides techniques for analyzing the proteome of date palm under salinity and drought stress.

Key words Proteome, Drought, Mass spectrometry, Salinity, Seedling, Stress

1 Introduction

Date palms can grow under a variety of environmental conditions such as heat, water shortage, and groundwater salinity, which provoke abiotic stresses and decrease date production [1]. Proteome analyses have identified numerous drought-responsive proteins, which are involved in redox regulation, oxidative stress response, signal transduction, protein folding, secondary metabolism, and photosynthesis [2, 3]. Date palms affected by leaf brittle disease express a manganese-stabilizing 33 kDa protein not detectable in healthy plants [4]. Proteome analysis, for example, opens the possibility to identify date palm proteins involved in transduction network regulation via posttranslational protein modification by phosphorylation/dephosphorylation under abiotic stress conditions [5]. Plants respond to stress by a modulating abundance of candidate proteins, either by upregulating expression or by synthesizing novel proteins primarily associated with plant defense systems [6]. However, the proteome of plant species changes as a response to biotic [7, 8] and abiotic [3, 9] stresses.

This protocol describes date palm proteome analysis under abiotic stress (salinity and drought). Methods include seed
germination, subjecting seedlings to drought and salinity, extraction of proteins, two-dimensional (2-D) electrophoresis analysis, differential in gel electrophoresis (DIGE) analysis, and mass spectrometry (MS) analysis.

2 Materials

2.1 Date Palm Germination

1. Date palm seeds (Sagie cultivar).
2. Sterilized Petri dishes.
3. 96% sulfuric acid.
4. 1%, v/v mercuric chloride.
5. 5% v/v calcium hypochlorite.
6. Tissue paper.
7. Growth chamber.
8. 15-cm pots containing peat moss.

2.2 Stress Experiments

1. Three-month-old date palm seedlings.
2. 27.5 g/L polyethylene glycol (PEG) 6000.
3. 16 g/L sodium chloride (NaCl).

2.3 Protein Extraction

1. Sterile mortar and pestle.
2. Liquid nitrogen.
3. Ice-cold acetone containing 0.07% (v/v) mercaptoethanol.
4. Lyophilizer.
5. Ultra deep freezer, −80 °C.
6. IEF buffer: 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 30 mM Tris pH 8.
7. Refrigerated centrifuge and Eppendorf tubes.
8. 2-D Quant Kit.

2.4 Protein Labeling and 2-D Electrophoresis

1. 2-D electrophoresis unit.
2. Fluorescent dyes.
3. Refraction-2DTM Labeling Kit.
4. Immobiline DryStrip, 24 cm, pH 4–7IPGphor 3.
5. Ettan DALTtwelve gel system.
7. 1 mM ruthenium(II)-tris(bathophenanthroline disulfonate fluorescence stain).
8. Isoelectric focusing (IEF buffer): 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 30 mM Tris pH 8.
2.5 Differential in Gel Electrophoresis (DIGE) Analysis

1. DeCyder Software v7.0.
2. The Extended Data Analysis (EDA).

2.6 Mass Spectrometry (MS) Analysis

1. Mass spectrometer.
2. Trypsin.
3. Nano-HPLC (UltiMate 3000 HPLC system).
4. AmaZon ETD MS ion trap spectrometer.
7. Reversed-phase pre-column.
8. 15-cm PepMap 100 reversed-phase C18 column, 75 μm inner diameter.
9. 2% (v/v) acetonitrile.
10. 98% (v/v) acetonitrile.
11. 0.1% (v/v) formic acid in high-purity water.
12. 0.1% (v/v) formic acid in high-purity water.
13. Compass Data Analysis v4.0—SR5 software.
14. 20% trichloroacetic acid (TCA).
15. Solution A: 4% (v/v) acetonitrile, 0.1% (v/v) formic acid in high-purity water.
16. Solution B: 96% (v/v) acetonitrile, 0.1% (v/v) formic acid in high-purity water.

3 Methods

3.1 Seed Germination

1. Scarify the seeds by soaking in 96% sulfuric acid for 5 min.
2. Wash five times with sterile distilled water.
3. Sterilize the seeds with 1% (v/v) mercuric chloride for 3 min, wash five times with sterile distilled water, and soak for 48 h in distilled water.
4. Sterilize the seeds a second time with calcium hypochlorite (5%, v/v) for 4 min, and wash four times with sterile distilled water (see Note 1).
5. Germinate the seeds between layers of wet tissue paper sheets for about 10 days until the radicals reach 1 cm, and then transfer to pots containing peat moss.
6. Irrigate daily with tap water and grow them in growth chambers under 12-h photoperiod (30 μmol/m²/s) and 30 °C until the age of 3 months.
1. Divide the 3-month-old date palm seedlings into three groups as follows:
   (a) For control, irrigate four seedlings by daily irrigation with distilled water daily for 1 month.
   (b) Subject four seedlings to drought condition by daily irrigation with 27.5 g/L PEG 6000 for 1 month.
   (c) Subject four seedlings to salt stress by daily irrigation with 16 g/L NaCl for 1 month according to a modified method of Sané et al. [10].

2. Wash the leaf samples with distilled water at the end of the stress period and freeze them in liquid nitrogen and store at −80 °C until use.

### 3.3 Protein Extraction and Quantitation

Grind four replicates of the frozen leaf materials of stressed and control plantlets to a fine powder in liquid nitrogen using a sterile mortar and pestle (see Note 2).

1. Precipitate the proteins by adding 1.8 mL ice-cold acetone containing 0.07% (v/v) mercaptoethanol.
2. Dry the raw precipitates by lyophilization and store at −80 °C for further processing.
3. Dissolve 100 mg of each of the lyophilized raw extract in 400–600 μL of IEF buffer.
4. Resolubilize the proteins overnight at room temperature.
5. Centrifuge the mixture at 4 °C for 10 min at 16,100 × g.
6. Quantify the total soluble protein in the supernatants using the 2-D Quant Kit.
7. Pool equal amounts of all single samples to get DIGE internal standard (IS).
8. Use 50 μg IS for each analytical gel and 300 μg IS for each preparative gel (necessary for protein identification by MS).

### 3.4 Protein Labeling and 2-D Electrophoresis

1. Label 50 μg of each protein sample as well as the needed amount of the internal standard with fluorescent dyes using the Refraction-2DTM Labeling Kit according to the manufacturer’s protocol.
2. Label the internal standard with G100, whereas label the single analyzed samples with G200 or G300 before mixing.
3. Perform 2-D gel electrophoresis as follows: sample mixture is separated in the first dimension according to their isoelectric point (pI) using immobilized pH gradient strips (Immobiline DryStrip, 24 cm, pH 4–7) focused by IPGphor 3 and in the second dimension according to their molecular weight by SDS-PAGE using the Ettan DALTtweelve gel system (see Note 3).
4. For preparative gels, glass plates are silanized with silane A 174 on one side prior to gel casting, and the gels are run in parallel to the analytical gels.

5. Generate the fluorescent scans of the analytical gels using Ettan DIGE Imager immediately after electrophoresis.

6. Stain the preparative gels with 1 mM ruthenium(II)-tris (bathophenanthroline disulfonate fluorescence stain) and attach the reference markers to the glass plates prior to scanning, thus enabling blind picking of the protein spots after the DIGE analysis (Fig. 1).

7. Scan the preparative gels directly after destaining in destaining buffer (40% methanol, 10% acetic acid), and store wet at 4 °C before spot cutting (Figs. 2, 3, and 4).
3.5 DIGE Analysis

1. The gel images are processed with DeCyder software v7.0.
2. Include all proteins in the internal standard in the analysis, and run on every gel along with all analyzed samples; use it for spot matching all across the gels (see Note 4).
3. Compare protein expression across multiple gels using the Biological Variation Analysis (BVA) module (see Note 5).

Fig. 2 Fluorescence scans of control (C) vs. salt stress (SS); spots shown in red had higher standardized abundance in control compared to salt stress; blue color highlighted the spots with lower abundance. Red-marked regions contained intensive spots significantly decreased in abundance in the salt stress sample; the blue one shows increased abundance (Source: [3])

Fig. 3 Fluorescence scans of control (C) vs. drought stress (DS); spots shown in red had higher standardized abundance in control compared to drought stress; blue color highlighted the spots with lower abundance. Red-marked regions contained intensive spots significantly decreased in abundance in the drought stress sample. Dashed lines indicate the regions important in salt stress but not changing significantly in drought stress (Source: [3])
4. Use the Extended Data Analysis (EDA) for multivariate analysis of protein expression data derived from the BVA module in order to perform a principal component analysis (PCA) and to identify protein spots of interest with differential expression analysis (Fig. 5).

Fig. 4 Fluorescence scans of salt stress (SS) vs. drought stress (DS); spots shown in red had higher standardized abundance in salt stress compared to drought stress; blue color highlighted the spots with lower abundance. Red- and blue-marked regions contained intensive spots significantly decreased and increased in abundance in the salt stress sample. The red regions with blue highlighted spots indicated more significant changes (decrease) for salt stress; regions without any highlighted spots indicated comparable changes for both stresses (Source: [3]).

Fig. 5 Spots chosen for picking: IS—internal standard scan shows all picked spots marked with red spot contours and yellow number boxes; spots marked with colored arrows were added to the pick from DIA. Red-marked regions correspond to the regions found in DIA for salt stress. On the scan of the preparative gel, the same spots are highlighted. The figure illustrates a good match of analytical and preparative gel (Source: [3]).
3.6 Mass Spectrometry (MS) Analysis

1. For protein identification in a single spot, fix proteins with 20% trichloroacetic acid (TCA) in the polyacrylamide gel plug, reduce, alkylate, and digest with trypsin.

2. Analyze the resulting peptides by using nano-HPLC (UltiMate 3000 HPLC system, coupled to an amaZon ETD MS ion trap spectrometer using nano-ESI spray.

3. The nano-HPLC system and the ion trap spectrometer are controlled using the Bruker Compass HyStar v3.2—SR2 software. The liquid chromatography system is supplied with reversed-phase pre-column for sample desalting and a 15-cm PepMap 100 reversed-phase C18 column, 75 μm inner diameter for peptide fractionation.

4. Separate the peptides using a 45-min linear gradient from 96% (v/v) solution A and 4% (v/v) solution B to 50% (v/v) solution A and 50% (v/v) solution B at a flow rate 300 nL/min.

5. Operate the electrospray in positive ion mode with –4000 V spray voltage and 10 psi (0.7 kg/cm²) gas pressure.

6. Set the end plate offset of the mass spectrometer to –500 V and for the acquisition the standard method Proteomics Auto MSMS Alternating Spectra CID-ETD Bruker trap Control v7.0 is used.

7. Evaluate raw data files using Compass Data Analysis v4.0—SR5 software with embedded search engine Mascot Search 2.3.01.

8. Use Swiss Prot (all species) and NCBInr (green plants) databases in the protein search using the following parameters: enzyme trypsin, up to one missed cleavage permitted; no fixed modifications and variable modifications carbamidomethyl (C), oxidation (M), and propionamide (C) are allowed, mass tolerance for both precursor ion and fragment ion ±0.3 Da (see Note 6).

4 Notes

1. For seed germination, be sure that all tools are sterilized.

2. During grinding the leaf materials in liquid nitrogen, always keep the plant materials frozen; do not let it thaw.

3. In 2-D electrophoresis, protein mixture is analyzed in the first direction according to their isoelectric focusing (IF) and in the second direction according to their molecular weight (MW).

4. In DIGE analysis, all automatically identified spots are checked manually, to confirm that they are real spots, and marked for picking.
5. In DIGE analysis, only the protein hit with highest protein score is used for further analysis.

6. When the protein is identified with one peptide only or several proteins with similar protein score are identified in a spot, the spots are excluded from the analysis.

References


Chapter 27

Electrophoresis-Based Proteomics to Study Development and Germination of Date Palm Zygotic Embryos

Besma Sghaier-Hammami, Noureddine Drira, Mouna Bahloul, and Jesús V. Jorrín-Novo

Abstract

Proteomics has become an important and powerful tool in plant biology research. To establish a proteomic reference map of date palm zygotic embryos (ZE), we separated and identified proteins from zygotic embryos during different developmental and germination phases using one, two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteins are extracted with trichloroacetic acid (TCA)/acetone-phenol and resolved by gel electrophoresis. Gel images are captured and analyzed by appropriate software and statistical packages. Quantitative or qualitative variable bands or spots are subjected to MS analysis in order to identify them and correlate differences in the protein profiles with the different stages of date palm zygotic embryo development, maturation, and germination.

Key words Mass spectrometry, Proteomics, Seed germination, Seed maturation, Zygotic embryos

1 Introduction

The date palm (Phoenix dactylifera L.) plays an important role in the stabilization of ecological systems in desert regions where it assures the protection of interplant cropping. Its fruit constitutes the principal source of income for people living in oases. In addition to being the main component of oasis agroecosystems, the date palm is used for food and fodder production and as a building material. Date palm is propagated sexually through zygotic embryogenesis. Zygotic embryogenesis is a complex process that begins with a single cell and results in the formation of mature embryos which germinate to develop into seedlings [1]. One vital factor to embryogenic development is the maturation phase, since a major change occurs during this period: a switch from a regional and cell-specific program to a storage accumulation program for postembryonic development [2]. In addition, seed germination is a crucial process in the seed plant life cycle, which determines when plants enter natural or agricultural ecosystems and is the basis for
crop production [3]. Seed tissue development and germination have been studied by using transcriptome and proteomic analysis, either independently or in combination, in legumes [4, 5], Arabidopsis thaliana [6], cereals [7, 8], and other species [9]. However, generally palms, especially date palm, are a much-neglected plant group in molecular understanding of development and propagation processes [10–15]. A deeper knowledge of proteins and genes involved in zygotic embryogenesis during the first steps of development, maturation, germination, and appearance of the radicle would be very useful for the improvement of vegetative clonal propagation techniques of elite genotypes, a challenge for this and other woody plant species [16]. Proteomics constitutes a priority approach and currently represents a fundamental discipline in the post-genomic era, as reviewed by Jorrin-Novo et al. [17].

Our aims are to understand the mechanism of date palm seed maturation and germination and provide a proteomic reference map of date palm zygotic embryos (ZE). The main objectives of this work are (a) studying of the up- and down-accumulated proteins during zygotic embryo development and germination and (b) identifying differential proteins as biomarkers. This chapter shows the workflows, methods, and techniques, as well as detailed protocols to work with different developmental and germinating stages of date palm zygotic embryos.

2 Materials

2.1 Protein Extraction, Isoelectric Focusing, and Gel Electrophoresis

1. Liquid nitrogen.

2. Trichloroacetic acid (10% w/v), acetone (80% v/v), and phenol solutions. Store at −20 °C and use directly from the freezer.

3. Ammonium acetate (0.1 M), methanol (100% and 80% v/v). Store at −20 °C and use directly from the freezer.

4. Acetone solution (80% v/v). Store at −20 °C and use directly from the freezer.

5. Phenol solution equilibrated with 10 mM Tris–HCl, pH 8. Store at 4 °C.

6. Sodium dodecyl sulfate (SDS) buffer: 0.1 M Tris–HCl, pH 8, 30% (w/v) sucrose, 2% (w/v) SDS, and 5% (v/v) β-mercaptoethanol (see Note 1). Store at 4 °C and temper prior to use.

7. Solubilization solution: 7 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonate (CHAPS), 2% (v/v) Triton-X100, and 100 mM dithiothreitol (DTT) (see Note 2). Store in 1 mL aliquots at −20 °C.

8. Protein quantitation: Bradford solution (see Note 3), protein standard: 1 mg/mL bovine serum albumin (BSA) in Milli Q water is used as a stock solution.
9. Solubilization solution diluted in Milli Q water (freshly prepared).

10. SDS lysis buffer (Laemmli buffer 2×): 62.5 mM Tris–HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol (see Note 4).

11. Rehydration buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 2% (v/v) Triton-X100, 100 mM (DTT), and bromophenol blue (3′,3″,5′,5″-tetrabromophenolsulfonphthalein) BPB 0.05% (see Note 5). Store in 1 mL aliquots at −20 °C.

12. Equilibration buffer: 50 mM Tris–HCl (pH 8.8), 6 M urea, 20% (v/v) glycerol, and 2% (w/v) SDS (see Note 6). Store at −20 °C and temper prior to use.

13. ReadyStrip™ IPG strips: Immobilized pH gradient strips of pH range 3–10 (7 cm) and pH range 5–8 (17 cm).

14. Resolving gel buffer: 1.5 M Tris–HCl (pH 8.8), 30% acrylamide/bisacrylamide (37.5:1) solution (see Note 7), sodium dodecyl sulfate solution (SDS) 10% (w/v) in water (see Note 8) and ammonium persulfate solution (APS) 10% (w/v) in water, and N,N,N′,N′-tetramethylethylenediamine (TEMED) (see Note 9).

15. Stacking gel buffer: 0.5 M Tris–HCl (pH 6.8), 30% acrylamide/bisacrylamide solution (37.5:1), SDS 10% (w/v) in water, H2O, APS 10% (w/v) in water, and TEMED.

16. Running buffer: 0.25 M Tris–HCl, 1.92 M glycine, and 10% SDS.

17. Staining solution: Colloidal Coomassie blue G-250. Store at room temperature in the dark (see Note 10).

18. Distaining solution: 0.1 M Tris–H3PO4, pH 6.5 (see Note 11), 25% (v/v) methanol (store at 4 °C), and 20% (p/v) ammonium sulfate. Store at 4 °C.

### 2.2 Protein Identification

1. Differential spots.

2. Porcine trypsin.


4. Peptide calibration standard, consisting of a combination of peptides that provides a good calibration across a typical mass range between 1000 and 3500 Da.

5. 0.1% (v/v) trifluoroacetic acid (TFA).

6. Ammonium bicarbonate.

7. Acetonitrile (ACN).

8. Trifluoroacetic acid (TFA).

9. α-Cyanohydroxycinnamic acid.
10. MALDI mass spectrometry calibration standards.
11. Matrix solution: 4.7 mg/mL α-cyano-4-hydroxycinnamic acid in 70% (v/v).

2.3 Equipment and Software

1. Airtight polyethylene bags.
2. A knife.
3. Mortar and pestle.
4. Vortex.
5. Ultrasonic homogenizer.
7. Disposable microcentrifuge tubes, 1.5 and 2 mL.
8. PROTEAN II Cell and PROTEAN Dodeca Cell.
9. PROTEAN IEF Cell system.
11. Gel shaker.
13. GS-800™ calibrated imaging densitometer.
14. Quantity One® 1-D Analysis software.
15. PD-Quest software v8.1.
16. ProGest digestion station (Genomics Solutions).
17. MALDI plates.
18. Automatic ProMs station (Genomic Solutions).
20. Proteomics Analyzer (4800 MALDI TOF/TOF™ Analyzer).
22. MASCOT search engine.
23. UV/VIS spectrophotometer.

3 Methods

3.1 Plant Material Collection and Storage

3.1.1 Seed Development Sampling

1. Collect date palm seeds of cultivar Deglet Noor after the transition stage when the endosperm has hardened, which occurs 10 weeks after pollination (WAP).
2. Group the zygotic embryos based on different developmental stages (from stage 1 to 4, see Fig. 1). These stages are identified based on the date of harvesting the seeds expressed in WAP.
3. Isolate manually the zygotic embryos from the fresh seeds (30–50 per replicate), at different developmental stages
Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | Stage 6 | Stage 7  
---|---|---|---|---|---|---  
12 WAP | 14 WAP | 17 WAP | 23 WAP | 9 DG | 12 DG | 15 DG

**Fig. 1** Date palm seeds during development, maturation, and germination. Zygotic embryos and radicle are collected from date palm kernels of the selected developmental stages (from stages 1 to 4) and germination stages (from stages 5 to 7), respectively, to perform the protein analysis. *WAP* weeks after pollination, *DG* days after germination.

(stage 1, 12 WAP; stage 2, 14 WAP; stage 3, 17 WAP; and stage 4, 23 WAP), immediately after the arrival of the seeds to the laboratory (*see Note 12*). Weigh them and store at −70 °C until protein extraction.

### 3.1.2 Seed Germination

1. Immerse the mature seeds in water and keep them in the dark at 25 ± 1 °C for germination.

2. Excise the embryos (20 per replicate, three biological replicates per sample) from germinated seeds at different time after germination (stage 5, 9 days after germination (DAG); stage 6, 12 DAG; and stage 7, 15 DAG).

3. A photograph of the germinated seeds showing samples is recorded at different time intervals (Fig. 1).

### 3.2 Protein Extraction by the TCA/Acetone-Phenol Method

1. Grind 0.5 g zygotic embryos collected from different developmental stages and from different germinated embryos (*see Subheading 3.1 and Fig. 1*) to a fine powder in liquid nitrogen using a mortar and pestle (*see Note 13*).

2. Transfer the powder to 2 mL Eppendorf tubes.

3. Fill the tubes with 10% (w/v) TCA in 80% (v/v) acetone and sonicate three times for 10 s each (50 W, amplitude 60) at 4 °C. Keep on ice for 1 min. Vortex vigorously. Use the fume hood with volatile reagents.

4. Centrifuge at 12,000 × *g* for 5 min and discard the supernatant.

5. Fill the tubes with 0.1 M ammonium acetate in 80% (v/v) methanol and mix well by vortexing.
6. Centrifuge at 12,000 × g for 5 min at 4 °C and discard the supernatant.

7. Fill the tubes with 80% (v/v) acetone and mix well by vortexing and centrifuge at 12,000 × g for 5 min at 4 °C and remove the supernatant.

8. Air-dry the pellet at room temperature to completely remove acetone. From this step, all the next steps must be performed under the hood because of the pungent smell of the phenolic solution.

9. Add 1.2 mL 1:1 phenol (pH 8)/SDS buffer. Mix well using a pipette and by vortexing. Incubate for 5 min on ice (see Note 14).

10. Centrifuge at 12,000 × g for 5 min at 4 °C and transfer the upper phenolic phase into a new 2 mL tube.

11. Fill the tube with 0.1 M ammonium acetate in 100% (v/v) methanol, mix, and allow to precipitate overnight at −20 °C.

12. Centrifuge at 12,000 × g for 5 min at 4 °C and discard the supernatant.

13. Wash the pellet with 100% (v/v) methanol and mix well by vortexing.

14. Centrifuge at 12,000 × g for 5 min at 4 °C and remove the supernatant.

15. Wash the pellet with 80% (v/v) acetone and mix well by vortexing.

16. Centrifuge at 12,000 × g for 5 min at 4 °C and discard the supernatant.

17. Air-dry the pellet to remove acetone.

18. Dissolve the pellet in 70 μL of the solubilization solution and shake for 2 h at 4 °C.

19. Quantify proteins using the Bradford method as follows:

(a) Prepare the calibration curve using several dilutions of bovine serum albumin protein, containing concentrations of 0, 1, 3, 5, 10, 15, and 20 μL BSA (1 mg/mL) into 1.5 mL tubes, and make up volume to 500 μL with Milli Q water. Add 500 μL Bradford reagent to each tube and mix well by vortexing gently. Use 1 mL Milli Q water as control. Incubate the mix at room temperature for 10 min in the dark. Measure the absorbance at 595 nm.

(b) Add 2 μL of the sample corresponding to 2–10 μg protein to 1.5 mL tubes and then adjust volume with Milli Q water up to 500 μL. Use 2 μL solubilization buffer (similar volume of the sample protein) diluted in 500 μL Milli Q water as a control. Add 500 μL Bradford reagent to
each tube, mix well, and store for 10 min in the dark. Measure with the spectrophotometer at 595 nm and compare to a standard curve prepared with BSA standards (see Note 15).

20. Store the extract at −20 °C for further analysis.

3.3 One-Dimensional Gel Electrophoresis (SDS-PAGE) on Small Gels

1. Arrange the complete electrophoresis system for small gels. Before use, clean carefully all the parts of the electrophoresis chamber, glasses, splitters, and combs. Centrifuge the samples prior to the run and remove insoluble debris, which could produce streaks in the protein lanes when stained with Coomassie blue.

2. Prepare the 12% polyacrylamide gel electrophoresis using a Mini-PROTEAN Tetra cell electrophoresis kit.

3. Prepare the resolving gel solution by mixing 6 mL 30% acrylamide, 3.75 mL Tris–HCl (pH 8.8), 150 µL SDS, 5 mL distilled water, 75 µL APS, and 7.5 µL TEMED.

4. Pour the solution into the gel cassette and cover completely the solution surface with isopropanol to obtain a flat layer on top of the resolving gel. Leave it for 20 min at room temperature.

5. When polymerization is completed, prepare the stacking gel. Mix 0.65 mL 30% acrylamide, 1.25 mL Tris–HCl (pH 6.8), 50 µL SDS, 3 mL distilled water, 25 µL APS, and 5 µL TEMED, and gently stir to obtain a uniform solution.

6. Pour the stacking gel and transfer the well-forming comb into this solution. Polymerize the gel for at least 30 min at room temperature to allow complete polymerization.

7. Remove the comb from the stacking gel, and then place the gel in the electrophoresis tank. The gel is covered with running buffer, and 15–20 µL sample is applied to the bottom of each well. Apply 8 µL molecular weight standards to one or two wells, preferably in an asymmetric position.

8. Simple method of preparing running buffer: Prepare 10× native buffers (0.25 M Tris–HCl, 1.92 M glycine). Weigh 30.3 g Tris–HCl and 144 g glycine, mix, and make it to 1 L with water. Dilute 100 mL 10× native buffer to 990 mL with water and add 10 mL 10% SDS. Care should be taken to add the SDS solution last, since it makes bubbles.

9. Connect the wires to the power supply unit and apply 80 V until the blue dye front reaches the bottom of the gel. Disconnect the electrophoresis unit from the power supply, remove the lid and discard the running buffer.

10. Remove the gel from the plates with a spatula, discard the stacking gel, and wash the separated gel with distilled water to remove traces of running buffer.

mohan.jain@helsinki.fi
11. Prepare the Coomassie Brilliant Blue G-250 staining 1 day before the staining process. Dissolve 1 g Coomassie Brilliant Blue G-250 in 200 mL methanol (solution 1) and dissolve 80 g ammonium sulfate in 22.5 mL 85% phosphoric acid (solution 2). Mix the two solutions (1 and 2) and add water to raise volume up to 1 L. Place the gel in a tray containing 200 mL staining solution.

12. Incubate overnight the gel in the staining solution. Once the gel is stained, discard the staining solution and cover the gel with 0.1 M Tris–H$_3$PO$_4$ and shake for 1–3 min.

13. Discard the staining solution and cover the gel with 25% (v/v) methanol and shake for 1 min. Remove methanol and wash the gel with 20% (w/v) ammonium sulfate for 24 h.

14. Images (Fig. 2) are digitized using a GS-800 calibrated densitometer and analyzed with Quantity One software.

**3.4 Isoelectric Focusing (IEF)**

1. Mix 500 μg proteins in 250 μL rehydration solution in 1.5 mL tube. Load the samples in each lane of the 17 cm strip holders (see Note 16).

2. Remove the protective cover from the surface of the IPG strips and slowly lower the IPG strip (gel slide down) onto the rehydration solution, without trapping air bubbles. Then cover the IPG strip with 1–2 mL mineral oil and with a plastic cover.

3. Apply a low voltage (50 V) of electric current during rehydration for 12 h at 20 °C for improving the entry of high molecular weight proteins.
4. After active rehydration, start isoelectric focusing at 20 °C using the following parameters (see Note 17):
   (a) Step 1 (linear), 250 V for 20 min.
   (b) Step 2 (linear), 10,000 V for 2.5 h.
   (c) Step 3 (rapid), a gradient of 10,000 V until an accumulated voltage of 40,000 Vh.
   (d) Step 4 (rapid), 500 V hold 99:00 h.
5. After rehydration, equilibrate the IPG strips in two steps while gently agitating on an orbital shaker at room temperature:
   (a) Equilibrate with 2% (w/v) DTT in equilibration buffer I for 10 min.
   (b) Equilibrate with 2.5% (w/v) iodoacetamide in equilibration buffer II for 10 min.
6. The second dimension is performed on 12% polyacrylamide gels using the PROTEAN Dodeca Cell. Run the gels at 150 constant volts until the dye reaches the bottom of the gel.
7. The gels are stained employing the colloidal Coomassie method. Soak the gel in a tray containing 50 mL staining solution and incubate overnight.
8. After staining of gel, discard the staining solution and cover the gel with 0.1 M Tris–H3PO4. Then, shake for 1–3 min. Discard the solution and cover the gel with 25% (v/v) methanol. Then shake for 1 min. Remove the methanol and wash the gel with 20% (w/v) ammonium sulfate for 24 h.
9. Images are digitized using a GS-800 calibrated densitometer (Fig. 3) and analyzed with PD-Quest software v8.1.
10. Select as a reference a good image with a clear and representative spot pattern and with minimum distortion, and align each of the images to the chosen reference. Select between three prominent spots for manual assignment, and use the automatic vector tool to add additional vectors.
11. After automatic spot detection and matching, check manually the spots with edition tools for correct detection.
12. Set gel groups according to the experimental design, and normalize spot volume intensity ratios for each spot by using the PD-Quest program by clicking on Analyze then Normalize.
13. List all the spots together with their normalized volume.

3.5 Protein Identification

1. Excise bands or spots from the polyacrylamide gels (see Note 18).
2. Digest gel plugs with modified porcine trypsin (sequencing grade), by using an automatic ProGest digestion station. The conditions are to detain steps for 30 min with 200 mM ammonium bicarbonate in 40% (v/v) ACN at 37 °C.
3. Wash gels twice: with 25 mM ammonium bicarbonate for 5 min and 25 mM ammonium bicarbonate in 50% (v/v) ACN for 15 min; dehydrate with 100% (v/v) ACN for 5 min and dry sample.

4. Perform gel hydration using 10 μL trypsin in 25 mM ammonium bicarbonate solution at the final concentration 12.5 ng/μL for 10 min at room temperature, and continue the digestion at 37 °C for 12 h.

5. Subsequently, digestion is stopped by adding 10 μL 0.5% TFA solution in water.

6. Purify tryptic peptides in an automatic ProMS station (Genomic Solutions) by using a resin C18 microcolumn (ZipTip, Millipore).

Fig. 3 Master gel (a) and real 2-DE gels (b and c) of zygotic embryos during maturation (b) and germination (c). The relative Mr. is given on the left, and the pI on top of the gel image. 500 μg of proteins were loaded and separated in the first dimension on an immobilized, linear, 5–8 pH gradient and in the second dimension on 12% polyacrylamide gels and stained with Coomassie Brilliant Blue. WAP weeks after pollination, DAG days after germination.
7. Elute proteins directly with a matrix solution: 5 mg/mL α-cyanohydroxycinnamic acid in 70% (v/v) ACN/0.1% (v/v) TFA, on MALDI plaque in 1 μL final volume.

8. After the cocrystallization on plaque, analyze samples by MALDI-TOF/TOF mass spectrometry to obtain the peptide mass fingerprinting (MS) in a 4800 Proteomics Analyzer. The settings are 800–4000 m/z range, with an accelerating voltage of 20 kV, in reflection mode, with delayed extraction set to on, and an elapsed time of 120 ns.

9. Spectra are internally calibrated with peptides from trypsin autolysis (M + H+ = 842.509, M + H+ = 2211.104) with an m/z precision of ±20 ppm.

10. Most abundant peptide ions are subjected to MS/MS analysis, providing information that can be used to define the peptide sequence.

11. A combined search (PMF and MS/MS) is performed with GPS ExplorerTM software v3.5 over nonredundant NCBI databases using the MASCOT search engine.

12. The database search utilized the following parameters: taxonomy restrictions to Viridiplantae, one missed cleavage site, 100 ppm mass tolerance in MS, and 0.5 Da for MS/MS data, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification.

13. The confidence in the peptide mass fingerprinting matches (p < 0.05) is based on the MOWSE score and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum.

### 3.6 Statistics

The nature of proteomic experimental conditions causes a certain number of missing spots, which alters and causes disparities between datasets.

1. To deal with this problem, we only consider consistent spots, present in the three biological replicates, thus preventing the assignment of normalized volume values to missing spots for multivariate analysis.

2. Another issue related to proteomic data is the correlation between spot normalized volume and spot variance, described in some proteomic studies [18]. This means that the higher the mean intensity of a spot, the higher the variance, this being explained by a scale phenomenon related to data acquisition. To reduce this variance-mean dependence between different spot intensities and sample sets, a cubic root transformation is applied.

3. After that, spot volumes are standardized (spot volume/Sgel spot volumes6100). Differentially expressed spots are defined after applying a one-way ANOVA test.
4. Spot values passed the Kolmogorov-Smirnov normality test. A multivariate analysis is performed over the whole set of spots showing differences. PCA is applied to the correlation matrix to reduce its dimension (SPSS v. 15 package).

5. Using un-rotated principal component (PC) scores, the relation between the different sampling times is studied by determining the spots with the highest load on the variance (Fig. 4, see Note 19).

6. Samples are clustered employing Ward’s minimum variance method over a Pearson distance-based dissimilarity matrix. Heat maps are also plotted employing PermutMatrix software v.1.9.3 [19].

---

**Fig. 4** Representation of the samples based on main principal components found after PCA. 2-D plot of main principal components (PC 1 and PC 2) of the differentially expressed spots datasets. The 2-D plots show that samples of 12 and 15 DAG were closely grouped in both plots, indicating similarity in the spot map. Seventeen WAP and mature zygotic embryo (ZE) samples were also grouped. However, ZE 9DG and ZE 14WAP were separated, showing a different protein content in the two samples. WAP weeks after pollination, DG days of germination.

mohan.jain@helsinki.fi
4 Notes

Prepare all solutions using ultrapure water and analytical grade reagents. Diligently follow all waste disposal regulations when disposing of waste materials.

1. Add the β-mercaptoethanol just before use.
2. Add the DTT just before using the solution. Store at −20 °C and use directly from the freezer.
3. Check the list of compatible chemicals and potential interfering chemicals (see Table 1 in the instruction manual of the Quick Start™ Bradford Protein Assay) typically found in the protein extraction buffer.
4. SDS lysis buffer is used for electrophoresis; SDS precipitates at 4 °C; therefore, warm it prior to use. Leave 1 aliquot at 4 °C for current use and store the remaining aliquots at −20 °C. Add the β-mercaptoethanol just before use.
5. Rehydration buffer is used for the two-dimensional electrophoresis. Add the DTT and the ampholytes just before using the solution. Store at −20 °C and use directly from the freezer.
6. Equilibration buffers are used for the two-dimensional electrophoresis. Add the 2% DTT (w/v) for the equilibration buffer I and 35 mM iodoacetamide for the equilibration buffer II just before use.
7. Acrylamide is a potent neurotoxin and should be handled with care! Wear disposable gloves when handling acrylamide solution and a mask while weighing the powder. Polyacrylamide is considered nontoxic, but polyacrylamide gels should also be handled by wearing gloves due to the possible presence of free acrylamide.
8. Wear a mask when weighing SDS powder. Store at room temperature because it precipitates at 4 °C, and prepare fresh each time. It can be stored at room temperature but we find that storing at 4 °C reduces its pungent smell.
9. APS and TEMED, being polyacrylamide polymerizing agents, need to be introduced last during resolving and stacking gel preparation. Gels should be poured immediately.
10. The Coomassie stain is photosensitive; therefore, the reaction is conducted in the dark. It can be recycled a couple of times by filtering it. When interpreting the results, it is important to realize that by using 2-DE only a minimal fraction of the total cell proteome can be visualized, and that the protein evolution pattern observed here (Fig. 3) only refers to those proteins solubilized under the experimental conditions used, with 5–8 pI, 6–120 Mr. values, and above the detection limit for

mohan.jain@helsinki.fi
Coomassie staining. After the 2-DE and the PD-Quest analysis, the results show that the number of resolved spots increased from 207 (12 WAP) to 273 (mature embryos) and then decreased during germination up to 87 (15 DAG).

11. Adjust the pH with the H₃PO₄ and store at 4 °C.

12. It is important to emphasize that ZE are difficult to access because they are minute and deeply embedded in maternal tissue and almost impossible to separate them, and that is why earlier than 12 WAP were not analyzed. At a very early developmental stage (12 WAP), embryos are excised under a light microscope.

13. Avoid keratin contamination, wear gloves and a lab coat at all times during the experiment, work in a clean and dust-free environment, do not lean over gels, tie long hair, and wear a cap or a head cloth; do not wear woolen clothes.

14. The TCA/acetone-phenol protocol provided the best results in spot focusing, resolved spots, spot intensity, unique spots detected, and reproducibility.

15. For protein extraction, the protein yield varies from 26.35 (15 DAG) to 240.85 (17 WAP) mg/g dry weight, with a decreasing tendency at early stages of development and on germination. This variation has been previously reported in other embryogenic and germination studies [20, 21] and is mainly related to the accumulation (during embryo maturation) and mobilization (during germination) of reserve proteins and biochemical reprogramming.

16. Preliminary 2-DE experiments can be carried out with the Mini-PROTEAN system, using 7 cm pH 3–10 linear gradient strips and 12% polyacrylamide gels, to examine the pI range of the concentrated proteins. In our case, the most protein spots are located in a pI range between 5 and 8. Considering this, increase the resolution for preventing protein co-migration, and perform IEF using 5–8 pH linear range strips. For the second dimension, SDS-PAGE, use 12% polyacrylamide gels (Fig. 3).

17. The condition of protein focusing must be optimized all the time wherever in use. In protocol, PROTEAN IEF cell by Bio-Rad is used. The conditioning phase involves the application of previous steps at low voltage for removing ions and other contaminants of the sample to prevent interference on protein focusing. The current should not exceed 50 μA per strip. For more information, see the 2-D Electrophoresis for Proteomics Manual by Bio-Rad.

18. Either bands or spots can manually be excised with a scalpel or excised from the polyacrylamide gel with an automatic spot
cutter In-Gel Digest. At each step, gel pieces should be covered with the appropriate solution. Do not autoclave pipette tips or solutions. Use Eppendorf Safe-Lock tubes. Protein identification is done according to the Proteomics Service protocols of the University of Córdoba. Protein identification is carried out according to the protocols of Proteomics Service of SCAI at the University of Córdoba, Spain [https://www.uco.es/servicios/scai/proteomica.html].

19. Those spots can be used to differentiate the various developmental stages. Our data show the presence of five spots showing the highest correlation (above $|0.9|$) with each PC 1 and PC 2. Out of these spots, only one spot is identified after MS analysis, corresponding to a phosphoglycerate kinase. This spot shows the highest values at the earliest embryogenic stage.

Acknowledgments

This work was supported by the Tunisian Ministry of Scientific Research, Technology and the Development of Competencies and the International Atomic Energy Agency, under TC Project RAF/5/049. We gratefully acknowledge the support from Dr. Consuelo Gomez (Proteomics Service-SCAI, Cordoba, Spain) for mass spectrometry facilities and technical assistance.

References

10. Gómez-Vidal S, Tena M, Lopez-Llorca LV, Salinas J (2008) Protein extraction from Phoenix dactylifera L. leaves, a recalcitrant material, mohan.jain@helsinki.fi
for two-dimensional electrophoresis. Electrophoresis 29:448–456
Chapter 28

Date Fruit Proteomics During Development and Ripening Stages

Claudius Marondedze

Abstract

Gel-based comparative proteomics approach is a valuable technique for studying the changes in abundance of proteins in any given system. The combination of this technique with mass spectrometry has provided immense insight into protein dynamics during fruit development and ripening. This chapter describes, informatively, the procedures for carrying out comparative proteomics analysis of date palm (*Phoenix dactylifera* L.) fruits at different developmental stages using a combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry. A comparative proteomics approach provides an overview of protein abundances during fruit maturation and insights into proteins that play key roles during fruit maturation. Moreover, 2-DE technique enables the visualization of total protein distribution and abundance in addition to providing a comparative platform following separation of complex proteins based on their molecular weight and isoelectric point. Overall, this chapter describes methodologies for extraction of proteins from a high carbohydrate-containing fruit, protein quality assessment using one-dimensional gel electrophoresis (1-DE), separation using 2-DE, comparative analysis using Delta2D v4.6, processing of spots of interest, and protein identification using mass spectrometry. This protocol is important for studies aiming at comparative proteomics to gain insights into changes of protein abundances in tissues and organs in general and date palm fruits, in particular.

Key words Fruit proteomics, Development, Ripening, Comparative proteomics, Phenol extraction, Gel electrophoresis, Mass spectrometry

1 Introduction

Two-dimensional gel electrophoresis (2-DE) is a widely used gel-based proteomics technique for resolving proteins and particularly for comparative quantitative proteomics. In this technique, proteins are separated in two dimensions based on their physicochemical properties, firstly based on their net charges or isoelectric point (pI) by isoelectric focusing (IEF) and, secondly, their molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [1]. In this way, a complex protein sample can be visualized with high resolution as when compared to
one-dimensional gel electrophoresis (1-DE) aiding better comparative analysis of samples of interest [2–4].

Recently, numerous studies have employed 2-DE technique to study changes in protein abundances in different cells, tissues, and organs including plants, and in particular fruits, for example, fruit firmness in apple (*Malus × domestica* Borkh) [5, 6] and fruit development and ripening in date palm [7], grape (*Vitis vinifera*) [8], peach (*Prunus persica*) [9], and apricot (*Prunus armeniaca*) [10]. As can be noted, comparative proteomics has been recently introduced to study protein changes occurring during fruit development and ripening. Of particular interest in this chapter is the use of this technique to gain insights into the type of proteins and their differential accumulation during date palm fruit development and ripening. Proteomics in combination with phenotypical and physiological data offers a promising approach to characterize fruit development and quality traits that are important in providing new insights into fruit biology and support efforts to improve important traits in horticulture.

The present chapter describes the procedures for the extraction of proteins from the date palm fruits at different developmental stages for optimum resolution and quantitation using 2-DE. It also elaborates 2-DE spots, comparative analysis, in-gel digestion, and mass spectrometry (MS) to identify proteins of interest and protein bioinformatics analysis of data to infer biological significance.

## 2 Materials

All solutions are prepared using analytical grade reagents and Milli-Q® “ultrapure” water (dH$_2$O) with a purity of 18.2 M Ω cm (see Note 1).

### 2.1 Plant Materials

1. Date palm cv. Barhee fruits: Fruits are collected at different developmental stages, rinsed with deionized water immediately after collection, and frozen and stored at −80 °C.

### 2.2 Protein Extraction from Date Fruits

1. 50 mL Falcon tubes.
2. PowerGen™ Model 125 homogenizer.
3. Ethylenediaminetetraacetic acid (EDTA): 0.5 M EDTA (see Note 2), filter through a 0.5 μm filter and sterilize in an autoclave.
4. Phenylmethylsulfonyl fluoride (PMSF): 100 mM PMSF in methanol solution can be stored at 4 °C.
5. Extraction buffer: 1% (w/v) polyvinylpolypyrrolidone (PVPP), 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2% (v/v) β-mercaptoethanol (see Note 3).
6. Precipitation solution: 0.1 M ammonium acetate in 100% (v/v) methanol.

7. Washing solution: 80% (v/v) ice-cold acetone in dH$_2$O.

8. Urea lysis buffer (ULB): 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% (v/v) immobilized pH gradient (IPG) buffer, pH 3–10; store the solution at –20°C.

### 2.3 Protein Quantification

1. Quick Start™ Bradford protein assay (see Note 4).

2. Quick Start™ bovine serum albumin (BSA) standard: 2 mg mL$^{-1}$ (see Note 5).


### 2.4 Quality Assessment by 1-DE

1. Resolving buffer (4×): 1.5 M Tris–HCl (pH 8.8), 0.2% (w/v) SDS (see Note 6).

2. Stacking buffer (2×): 0.5 M Tris–HCl (pH 6.8), 0.2% (w/v) SDS (see Note 6).

3. Acrylamide-Bis solution: 40% (w/v) acrylamide-Bis solution (37.5:1 acrylamide-Bis, 2.6% C), store at 4°C.

4. Ammonium persulfate (APS): 10% (w/v) APS in dH$_2$O.

5. N,N,N',N'-tetramethylethenediamine (TEMED).

6. SDS reducing buffer (2×): 200 mM Tris, 40% (v/v) glycerol, 10% (w/v) SDS, 0.02% (w/v) bromophenol blue (see Note 7).

7. Tris–glycine SDS running buffer (10×): 25 mM Tris, 1.92 M glycine, 0.1% (w/v) SDS.

8. Tris–glycine SDS running buffer (10×): Mix 100 mL of 10× running buffer stock with 900 mL dH$_2$O.

9. Colloidal Coomassie Blue (CBB) staining solution: 25% (v/v) isopropanol, 1.6% (v/v) phosphoric acid, 8% (w/v) ammonium sulfate, 0.08% (w/v) CBB G-250 (see Note 8).

10. CBB destaining solution: 10% (v/v) glacial acetic acid, 1% (v/v) glycerol.

### 2.5 Protein Separation by 2-DE

1. Isoelectric focusing (IEF) IPG buffer or ampholytes pH 3–10, linear Immobiline™ DryStrip pH range 4–7, 7 cm.

2. Immobiline™ DryStrip cover fluid.

3. Paper electrode wicks.

4. IEF rehydration buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 0.002% (w/v) bromophenol blue (see Note 9); add 0.5% (v/v) IPG buffer and 0.3% (w/v) dithiothreitol (DTT) per 1.25 mL aliquot of the rehydration buffer (see Note 10).
5. Equilibration buffer: 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue (see Note 11), and either with 2% (w/v) dithiothreitol (DTT) or 2.5% (w/v) iodoacetamide (IAA) (see Note 12).

6. Resolving buffer (4 ×): 1.5 M Tris–HCl (pH 8.8), 0.2% (w/v) SDS (see Note 6).

7. 10% (w/v) ammonium persulfate (APS) in dH2O. Prepare fresh.

8. Tris–glycine SDS running buffer (10 ×): 25 mM Tris, 1.92 M glycine, 0.1% (w/v) SDS.

9. Tris–glycine SDS running buffer (1 ×): Mix 100 mL of 10 × Tris–glycine SDS running buffer with 900 mL dH2O.

10. Sealing agarose: 0.5% (w/v) agarose and 0.002% (w/v) bromophenol blue in 1 × SDS running buffer (see Note 13).

11. Ettan™ IPGphor™ 3 isoelectric focusing system.

12. Mini-PROTEAN® Tetra cell.

2.6 Visualization of Proteins with the SYPRO Ruby Stain

1. Fixing solution: 50% (v/v) methanol, 10% (v/v) acetic acid.

2. SYPRO destaining solution: 10% (v/v) methanol, 7% (v/v) acetic acid.

3. Typhoon imaging scanner.

2.7 Comparative Analysis Using Delta2D Software

1. Delta2D v4.6 or any other gel image comparative analysis platforms.

2. Gel images saved in TIFF format.

2.8 Spot Cutting and Digestion

1. 200 μL tips with wide-open end (see Note 14).

2. P200 μL pipette.

3. 1.5 mL microcentrifuge tubes.

4. Non-UV transilluminator, the dark reader™.

5. Wash solution: 50% (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate.

6. Reducing solution: 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate.

7. Alkylation of cysteine residues: 50 mM iodoacetamide in 100 mM ammonium bicarbonate.

8. Trypsin: 6 ng μL⁻¹ trypsin in 50 mM ammonium bicarbonate.

9. Extraction solution: 50% (v/v) ACN, 5% (v/v) formic acid (FA).

10. ZipTip pipette tips with C-18 reverse-phase (ZipTip wet solution): 100% (v/v) ACN.
11. ZipTip equilibration solution: 0.1% (v/v) trifluoroacetic acid (TFA) in dH₂O.

12. ZipTip elute solution: 50% (v/v) ACN, 0.1% (v/v) TFA.

2.9 Mass Spectrometry

1. Mass spectrometry resuspension buffer (LC-MS buffer A): 0.1% (v/v) FA, 5% (v/v) ACN, prepared with HPLC grade dH₂O; sonicate the buffer for 15 min in a bath sonicator (see Note 15).

2. LC-MS buffer B: 90% (v/v) ACN, 0.1% (v/v) formic acid (FA); sonicate the buffer for 15 min in a bath sonicator (see Note 15).

3. Autosampler vials and vial closures.

4. Pre-column: 5 μm, 200 Å, 50 mm long × 0.2 mm Magic C18AQ (separation column: 3 μm, 200 Å, 150 mm long × 0.075 mm Magic C18AQ).

5. Mass spectrometer: LTQ Orbitrap Velos™ coupled to an EASY nLC system.


3 Methods

Perform all procedures at room temperature unless specified otherwise. The technical approach detailed below is summarized in Fig. 1. This extraction method is modified from [7] and some technical aspects [1] although elaborated further in this section.

3.1 Protein Extraction from Date Fruits

1. Collect fruit samples at developmental stages of interest. In this case four stages are used and these include 25 days after pollination (DAP), 70 DAP (mature fruit), 110 DAP (fruit near to ripe), and 125 DAP (Khalal or ripe stage).

2. Immediately after collection rinse with dH₂O, freeze in liquid nitrogen, and store at −20 °C until needed.

3. Before the extraction, remove the fruit skin and split open the fruit to remove the seed (see Note 16).

4. Weigh 5 g from a pool of 10 fruit samples for each biological replicate at each developmental stage (see Note 17).

5. Transfer the sample in a 50 mL Falcon tube.

6. Add 5 mL cold extraction buffer and keep sample on ice.

7. Grind sample using the PowerGen™ Model 125 homogenizer, 2–3 times of 5 sec each, until the sample is a fine soup (see Note 18).

8. Adjust volume to 15 mL and mix homogenate on a shaker for 30 min at 4 °C.
Fig. 1 Overview of the method to extract proteins and analyze and identify proteins of interest from date palm fruit.
9. Add an equal volume of saturated phenol: Tris–HCl pH 7.5 and mix homogenate for a further 30 min at 4 °C.
10. Centrifuge at 10,000 × g for 30 min at 4 °C.
11. Collect the upper phenol phase and perform extraction again twice in extraction buffer.
12. Collect the final phenol phase and precipitate proteins with five volumes of ice-cold precipitating solution overnight at −20 °C.
13. Centrifuge at 10,000 × g for 30 min at 4 °C.
14. Wash the protein pellet thoroughly, once in 100% (v/v) ice-cold methanol and subsequently three times in ice-cold 80% (v/v) acetone. Centrifuge at 10,000 × g for 10 min at 4 °C to collect proteins after each wash.
15. Air-dry the final pellet and resuspend protein pellet in urea lysis buffer.
16. Once solubilized, store proteins at −20 °C for short-term storage or −80 °C for long-term storage (see Note 19).

**3.2 Protein Quantification**

1. To estimate the concentration of proteins in the sample, mix 2 μL aliquot of the protein extract with 18 μL of ULB and 1 mL of Quick Start™ Bradford protein assay in a 1.5 mL microcentrifuge tube. In this case, the dilution factor is ten times.
2. Prepare in duplicates, mix, and pipette into separate cuvettes.
3. In parallel and in duplicate, prepare increasing concentrations of BSA from 0 to 1.5 mg mL⁻¹ according to manufacturer’s instructions and pipette each standard separately into a cuvette.
4. Allow the mix to react for a minimum of 5 min to a maximum of 60 min, as recommended by the manufacturer.
5. Measure absorbance of the sample and BSA standards at 595 nm.
6. Plot results from the BSA to build up a standard curve and deduce a trend line equation for the curve in Excel; use the linear equation to estimate the concentration of the protein sample.

**3.3 Quality Assessment by 1-DE**

1. Clean the thin and thick glass plates with 70% (v/v) ethanol and air-dry.
2. Mount the gel cassette on a gel casting stand assembly. Make sure that the thin and thick glass plates are well aligned to prevent gel solution from leaking.
3. Prepare a 12% (v/v) resolving gel by mixing 4.5 mL of dH₂O, 2.5 mL of 4 × resolving buffer, and 3 mL 40% (v/v) acrylamide stock solution in a 50 mL Falcon tube. Add 100 μL APS and 10 μL TEMED.
4. Mix by inverting slowly five times. Carefully load the gel mix between thin and thick plates and leave about 1 cm space on top of the glass plates for the stacking gel. Overlay with 1 mL of isopropanol (see Note 20).

5. Keep the excess of the gel solution in the Falcon tube to check the polymerization process. When the gel is set, two separate layers should be visible through the thin glass plate, the bottom being the gel and the top layer for the isopropanol.

6. Prepare a 4% (v/v) stacking gel by mixing 1 mL of dH$_2$O, 1.25 mL of 2 × stacking buffer, and 0.25 mL of 40% (v/v) acrylamide stock solution in a 50 mL Falcon tube. Add 25 μL of APS and 2.5 μL of TEMED.

7. Mix by inverting slowly five times. Remove the isopropanol overlay and fill the top with the stacking gel mix. Insert either a 10- or 15-well comb, depending on the number of samples to be separated. Avoid trapping any air bubbles in the well being created.

8. Use 15 μg protein sample and mix with an equal volume of 2 × reducing buffer.

9. Incubate at 95 °C for 5 min, cool down, and centrifuge at 16,800 × g for 1 min at room temperature prior to loading onto the gel.

10. When the gel has polymerized, place the gel plates into the electrode assembly module and ascertain tight clamping to avoid buffer leakages. Place the assembly into the electrophoresis tank and fill the inner chamber with 1 × SDS running buffer and the outer chamber to the recommended level, as indicated on the tank.

11. Load 7 μL of the molecular weight standard onto the first well and the samples on the subsequent wells.

12. Carry out electrophoresis at 120 V for 60 min or until the bromophenol blue dye reaches the bottom of the gel.

13. After electrophoresis, disassemble the cell electrophoresis module and unseal the plates. The gel remains on one side of the glass plates. Gently transfer the gel to a clean gel tray with CBB for staining overnight.

14. On the following day, decant the staining solution and replace with destaining solution for 2–4 h. Rinse with deionized water and visualize the gel and scan the image (Gel Doc XR molecular imager).
3.4 Protein Separation by 2-DE

3.4.1 Isoelectric Focusing: The First Dimension

2-DE protein separation is according to a previously reported study [1].

Isoelectric focusing (IEF) of IPG strips is the first dimension of the 2-DE technique [11] (see Note 21).

1. Aliquot 50 μg protein into a 1.5 mL microcentrifuge tube.
2. Adjust the volume of the protein sample to 125 μL (for a 7 cm-long IPG strip) with IEF rehydration buffer.
3. Mix well by vortexing and spin down at 16,000 x g for 5 min at room temperature.
4. Transfer the isoelectric focusing tray into the IPG box and carefully pipette out the supernatant (125 μL) avoiding taking up any pelleted debris. Spread the solution onto one of the isoelectric focusing tray channels to cover three-quarters of the strip length.
5. Using a pair of forceps, remove the protective cover slip and gently place the IPG strip, gel side facing downward into the channel containing the sample, and position the strip to cover the solution avoiding trapping air bubbles.
6. Overlay the strips with 1 mL Immobiline™ DryStrip cover fluid (commonly referred as mineral oil).
7. Close the IPG box and passively rehydrate the strip overnight. In case of passive rehydration, IPG strips are rehydrated without applying any voltage, or alternatively an active rehydration with 50 V can be applied to the IPG strips, and rehydration time varies from 1 to 99 h, and for a 7 cm strip 12 h rehydration time is optimal.
8. After rehydration, using forceps, gently pick the IPG strip from one edge, and swiftly rinse with dH2O by spraying from a squeeze bottle. Dry excess dH2O with a lint-free wipe, while avoiding disturbing the gel side.
9. Transfer the strip, gel facing up, onto the manifold plate mounted on the IPGphor machine. Place the positive end of the strip on the anode (marked +) end of the IPGphor electrode plate.
10. Place pre-damped wicks on both ends of the strip overlapping the end of the gel on the strip and overlay each strip with Immobiline™ DryStrip cover fluid.
11. Carefully and gently place the electrode assembly on top of the wicks as close as possible to the wick end on top of the gel, lock the electrode assembly, and close the lid.
12. Start isoelectric focusing under these conditions:
   (a) 500 V for 250 Vh.
   (b) 1000 V for 500 Vh.
   (c) 10,000 V for 10,000 Vh.
The run can take 2.5–3.0 h provided the sample is relatively clean from interfering substances such as salts.

13. When the run is complete, remove the wicks and gently place the IPG strip into a 15 mL Falcon tube or an IPG equilibration tray using forceps.

3.4.2 SDS Gel Electrophoresis: The Second Dimension

1. Incubate IPG strips in 5 mL equilibration buffer containing 2\% (w/v) DTT for 15 min with continuous gentle shaking to avoid damaging the strip.

2. Decant the DTT-containing equilibration buffer and replace with 5 mL equilibration buffer containing 2.5\% (w/v) IAA. Incubate for 15 min with continuous gentle shaking.

3. Rinse the strips with 1 × SDS running buffer.

4. Prepare 12\% (v/v) resolving gels as described in Subheading 3.3. Leave about 5 mm space on top of the glass plates for positioning the IPG strip and for a thin layer of molten agarose gel.

5. Prepare the molecular marker by pipetting 7 μL molecular weight standards onto a 4 × 4 mm piece of Whatman paper. Allow to air-dry.

6. Place the IPG strips onto the gels, and on one end of the strip, place the dry Whatman paper with the molecular weight standards.

7. Overlay the strips with 0.5\% (v/v) molten agarose gel.

8. Carry out electrophoresis at 50 V for 10 min and then increase to 120 V and run until the dye reaches the bottom of the gel.

3.5 Visualization of Proteins with the SYPRO Ruby Stain

All gel incubation steps are carried out at room temperature with gentle shaking at 40 rpm with the gel plate covered with aluminum foil with the exception of the fixing step.

1. Fix the gel with fixing solution for at least 1 h.

2. Discard the fixing solution and wash the gel in dH₂O twice for 15 min each.

3. Stain the gel with SYPRO Ruby total protein.

4. Stain the gel in 60 mL undiluted SYPRO Ruby stain overnight.

5. Destain the gel in SYPRO Ruby destaining solution for 30 min.

6. Rinse the gel in dH₂O twice for 5 min.

7. Scan the gel using appropriate laser and emission filter: excitation 280 nm, emission at 450/610 nm.
3.6 Comparative Analysis Using Delta2D Software

1. Import gel images into the Delta2D v2 software. Gel images of the four different developmental stages are shown in Fig. 2.
2. Align spot positions across gel images by image warping.
3. Generate a fusion gel image/proteome map by combining the images using a fusion union option.
4. Using the fusion union image as a reference for constructing the consensus spot pattern for the analysis, perform spot detection and editing.
5. Apply the consensus spot pattern from the fusion union image to all the gels.
6. Extract expression profiles or abundances.

Fig. 2 2-DE proteome maps of the date hypanthium showing expressed spot profiles. The relative molecular mass (Mwt) and isoelectric point (pI) are indicated on the left and at the top of the gels, respectively. Total soluble proteins (50 μg) from date hypanthium collected at different developmental stages are separated in the first dimension on immobilized linear pH 4–7 gradient and then on 12% acrylamide gels for the second dimension. Gels are stained with SYPRO Ruby. (a) Sample collected at 25 days after pollination (DAP), (b) 70 DAP (mature fruit), (c) 110 DAP (fruit near to ripe), and (d) 125 DAP (Khalal or ripe stage)
7. Analyze the expression profiles across developmental stages of the fruit.
8. Perform quantitative and statistical analyses to determine the significance of abundances and extract spots of interest.
9. Mark the spots on the gel images and use the image to detect the protein spots from gels for cutting.

### 3.7 Spot Cutting and Digestion

For protein digestion, either use the Tecan Freedom EVO® protein digest workstation that allows automated protein digestion and subsequent plating for matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis or manually using the procedure below:

1. Cut the spots of interest using a wide-open 200 μL tip.
2. Wash gel pieces with 200 μL wash solution and incubate at room temperature for 10 min with gentle agitation.
3. Carefully, remove solution with a pipette.
4. Wash the gel pieces two more times with 200 μL wash solution for 10 min each.
5. Dehydrate the gel pieces by adding 100% (v/v) ACN for 5 min. The gel pieces will shrink and turn into an opaque white color.
6. Remove ACN and air-dry the gel pieces for 10 min.
7. Rehydrate with 50 μL reducing solution and incubate for 1 h at 37 °C.
8. Cool the gel pieces to room temperature, remove the reducing solution and add 50 μL alkylating solution, and mix and incubate for 45 min in the dark at room temperature.
9. Remove the alkylating solution and wash the gel pieces with 100 μL of 100 mM ammonium bicarbonate for 10 min with continuous gentle vortexing.
10. Remove the supernatant and wash twice with 50% (v/v) ACN and 50% (v/v) 50 mM ammonium bicarbonate mix for 10 min, each with continuous gentle vortexing.
11. Dehydrate the gel pieces with 100 μL of ACN. The gel pieces should shrink and turn opaque white.
12. Pipette out the acetonitrile and air-dry the gel pieces at room temperature for 10 min.
13. Rehydrate the gel with 25 μL of 6 ng μL⁻¹ trypsin solution.
14. Digest overnight at 37 °C.
15. Spin down briefly and add 25 μL extraction solution to gel pieces and agitate gently by vortexing at lowest setting for 30 min.
16. Spin down the sample by brief centrifugation.
17. Transfer the supernatant (containing tryptic peptides) to a clean microcentrifuge tube.
18. Re-extract the gel pieces with an additional 25 μL solution.
19. Spin down the sample and transfer supernatant to tube from step 17.
20. Dry the pooled extracted peptides using a SpeedVac to near dryness. Do not apply heat.
21. Resuspend the extracted peptides in 20 μL 0.1% (v/v) TFA and vortex for 10 min.
22. Purify peptides with ZipTip pipette tips.
23. Wet the ZipTip with 100% (v/v) ACN 3 times and wash 3 times with ZipTip equilibration solution.
24. Draw in and out several times the sample solution and wash the ZipTip twice with ZipTip equilibration solution.
25. Elute with 5 μL ZipTip elute solution by drawing in and out three times and finally dispense the eluent into a clean microcentrifuge tube.
26. Repeat once steps 23–25 and combine both extracts.
27. Dry the sample using a SpeedVac.
28. Purified peptides can be stored in −80 °C until analysis by mass spectrometry for protein identification.

3.8 Identification of Peptides by MS and Database Search

1. Resuspend the peptides in MS resuspension buffer. Vortex well to ensure complete resuspension and centrifuge at 16,100 × g for 5 min (see Note 22).
2. Pipette out the peptide solution into an autosampler vial. Spin down briefly the vial to remove any air bubbles. Load the vials in the nLC system connected to the MS and set up the run program.
3. Inject ~1 μg or appropriate volume of peptide mixtures.
4. In this case peptides are loaded onto pre-column then separation column (for dimensions, see Subheading 2.8) using a mass spectrometer, LTQ Orbitrap Velos, with an Advance Captive-Spray source and operated in positive mode. Apply spray voltage of 1500 V. The mobile phases consist of LC-MS buffer A [0.1% (v/v) FA and 5% (v/v) ACN] and LC-MS buffer B [0.1% (v/v) FA and 90% (v/v) ACN]. In addition, apply a three-step gradient of 0–40% B for 20 min, then 40–90% B for 5 min, and finally 90% B for 20 min with a flow of 500 nL min⁻¹ over 45 min for peptide elution. Data are acquired between m/z 350 and m/z 1600 and the normalized collision-induced dissociation at 35.0 V. The top 10 precursor ions are selected in the MS scan by Orbitrap with resolution $r = 60,000$ for fragmentation in the linear ion trap using collision-induced
dissociation. Data are recorded using Xcalibur™ software version 2.1 (see Note 23).

5. Convert .raw files into .mgf using Proteome Discoverer software, and perform identification against the date palm database uploaded in MASCOT.

6. For MASCOT search incorporate the following parameters: trypsin as the proteolytic enzyme, one missed cleavage; carboxyamidomethyl cysteine as fixed modifications; and oxidation of methionine as variable modification.

7. Apply filtering to the resulting peptides to show the list of proteins identified with less than 1% false discovery rate. In addition, consider positive identifications only when at least two peptides are matched, and their MOWSE score is >32 for MASCOT.

### 3.9 Functional Annotation (Systems Analysis) of Identified Proteins

1. Group proteins into two categories, one with those increasing in abundance and the other with proteins decreasing in abundance during maturation.

2. Upload the sequences of the protein list into Blast2GO (B2G) software [12] (see Note 24).


### 4 Notes

1. Store all reagents and solutions at room temperature unless stated otherwise. Please note that it is important to follow safety regulations for all chemicals and the proper biological waste handling and disposal. The material safety data sheets (MSDS) and risk assessment should be read before using any chemicals.

2. If you use solid NaOH pellets, you will need about 18–20 g NaOH. Add the last of the NaOH slowly to avoid overshooting the pH. You may wish to switch from solid NaOH to a solution toward the end, for more precise control. The EDTA will slowly go into solution as the pH of the solution nears 8.0.

3. Add 2% (v/v) β-mercaptoethanol and 1 mM PMSF just before extraction.

4. Allow the Quick Start 1 × dye reagent to settle to room temperature before use.

5. A stock of 2 mg mL⁻¹ BSA can be prepared by dissolving 2 mg BSA into 1 mL ULB. The Quick Start™ Bradford protein assay is compatible with urea and thiourea present in ULB.
6. The buffer needs to be brought to room temperature prior to adjusting the pH.

7. At the time of use, add either 100 μL 2-mercaptoethanol (in fume hood) or 10 mg dithiothreitol (DTT) per 1 mL aliquot of the thawed SDS reducing buffer to act as the reductant.

8. Filter CBB staining solution to avoid interaction of unresolved CBB with the gel.

9. Mix thoroughly until fully dissolved on a stirring plate set to 30 °C. Dissolving the buffer components takes about 2 h.

10. Store the buffer at −20 °C and only add IPG buffer and DTT into the aliquot of the thawed rehydration buffer prior to use and vortex well.

11. The addition of bromophenol blue will not interfere with the migration of proteins during IEF. It is only added to help tracking of the electrophoresis.

12. DTT and IAA should be added separately to the equilibration buffer aliquot just prior to use.

13. Use low-melting agarose to ensure rapid melting of the gel at time of use.

14. Widen the tip end of a 200 μL tip by cutting using a pair of scissors or blade.

15. Sonication of the buffers prior to LC-MS is essential to limit risk of pressure problems on the LC system. Sonication will break down particles and degas the solution.

16. The fruits are skinned using a sharp scalpel blade making sure to remove the outer skin layer. The seed is then removed together with the inner layers leaving the hypanthium. However, if one is interested in analyzing the whole fruit, then just remove the seed. This procedure needs to be done fast enough to prevent the fruit from thawing. Wear thick gloves to prevent fingers from freezing during the skinning process.

17. Mixing different fruits of the same age/stage for each biological replicate is important to obtain an informative representative proteome for each developmental stage and takes into consideration natural biological variations likely to exist between fruits within the same tree and from different trees.

18. If the soup is not fine enough, continue grinding making sure that the sample is always on ice and does not heat up.

19. For long-term storage, it is recommended to store proteins at −80 °C.

20. The overlay prevents inhibition of acrylamide polymerization, which can be caused by chemical interaction with atmospheric oxygen, and in addition helps to level the resolving gel solution.
Fig. 3 The number of proteins enriched in each biological process. The black bars represent the number of proteins that are consistently changing in abundances during development and ripening of the date palm fruits in each of the biological processes. *Maroon bars* represent the number of proteins changing in abundance at mid fruit development (MD; 70 days after pollination) stage that are enriched in each of the biological processes. *Blue bars* represent the number of proteins changing in abundance at near to ripe (NTR; 110 days after pollination) stage that are enriched in each of the biological processes all changing in abundance during fruit maturation. *Purple bars* represent the number of proteins changing in abundance at ripe (125 days after pollination) stage that are enriched in each of the biological processes.
21. Proteins migrate along the strip based on their charge until they reach their isoelectric point (pI) at which point they are referred to as completely focused. It is therefore important to note the optimal running conditions to prevent precipitation and aggregation of proteins and to accomplish reproducible resolution across samples.

22. The centrifugation step after resolubilization of the purified peptides ensures that any precipitates are pelleted down to avoid blockage of the column in the LC-MS.

23. The mass spectrometry run procedure and data acquisition steps described here have been reported previously [13].

24. Functional annotation is an important part of data analysis that provides a systems view of interpreting data. Here we infer function based on enriched ontology terms. The list of proteins identified in the comparative proteomics experiment is submitted into B2G software in order to acquire information mainly about functional annotation based on Gene Ontology. The B2G uses Blast searches to find similar sequences, and the program extracts the GO terms associated with each of the obtained protein hits and returns as well an evaluated annotation for the query sequences. The information obtained includes biological processes, molecular function, cellular localization of proteins, and KEGG maps associated with the proteome input data. As an example, see Fig. 3 showing the number of proteins increasing and decreasing in abundance during fruit maturation that are enriched in various biological processes.

Acknowledgments

This project was supported by the date proteome initiative grant from King Abdulaziz City for Science and Technology (KACST). I am grateful for the support of Dr. L. Thomas and Ms. R. Feret for revising this book chapter.

References


mohan.jain@helsinki.fi
INDEX

A

Abscisic acid (ABA) ................................................. 50, 52, 53, 57, 58, 62, 64, 68, 69
Acclimatization ......................................................... 21, 22
Accumulated proteins .................................................. 366
Acetocarmine ................................................................. 3, 4, 7, 8, 247, 249
Acetonitrile (ACN) .................................................... 357, 367, 384, 392
Activated charcoal (AC) ............................................. 17, 19, 52, 63, 92, 93
Adventitious ................................................................. 40, 46, 63
Agricultural Genetic Engineering Research Institute (AGERI) ................................................................. 243
Alexa Fluor ................................................................. 253
Algeria ................................................................. 154, 273
Alginate ................................................................. 27, 29, 32, 36, 45, 47, 71, 72, 74–77
Alginate beads ................................................................. 46, 77
Alkaline phosphatase (CIAP) ....................................... 148, 317, 321
Allele frequency .......................................................... 119–121
Ammonium persulfate (APS) ...................................... 127, 129, 131, 139, 141, 145, 224, 230, 234, 261, 367, 383, 384
Amplicon ................................................................. 105, 108, 201, 219, 237, 238, 324
Amplified products .................................................... 100, 108, 149, 186, 204
Analysis of polymorphism ........................................... 87, 105, 120, 126, 144, 149, 150, 267
Ancymidol ................................................................. 62, 68
Annotation ................................................................. 260, 264, 310, 311, 331–332, 397
Anther dehiscence ...................................................... 5
Antibiotic ................................................................. 74, 75, 92, 99, 232, 258, 301
Antibody detection ....................................................... 253
Anti-dig-Rhodamine .................................................... 247, 252
Antioxidant ............................................................... 51, 68
Antioxidant solution ................................................... 16, 50, 53, 56, 66, 72, 92, 94
Aqueous solution ....................................................... 53, 107, 174, 179, 254
Arbitrary primers ....................................................... 151, 154, 210
Artificial seeds ........................................................... 72
Aseptic ................................................................. 65, 77, 83

B

Bacterial transformation ............................................. 228, 229, 232, 238
Banding ................................................................. 85, 87, 120, 186
Banding profile ........................................................ 162, 163, 205
Barhee cv. ............................................................... 46, 115, 122, 149, 155, 174, 178, 191, 192, 382
Bayoud ................................................................. 277, 279, 281
Bead ................................................................. 28, 46, 76, 77
Bentamoda cv. .......................................................... 126, 261
Benzyladenine (BA) .................................................... 50, 52, 53, 62, 64, 6-Benzylaminopurine (BAP) ................. 82, 84
BigDye Terminator .................................................... 308
Binary data matrix .................................................... 161, 162
Binding solution ....................................................... 127, 131, 139, 212, 219, 224, 230, 233, 240
Biodiversity ............................................................ 105, 125
Index

C

Calcium chloride (CaCl₂) .............. 3, 72, 74, 75, 77, 93
Calcium ........................................ 17, 19, 21–23, 49–55, 57, 58, 65, 84, 92
Callus induction ................................ 17, 19, 21, 22, 65, 84
Candidate miRNA ......................... 345, 346, 350
Capsule ............................................. 4, 5, 7, 12, 77
Carbenicillin ...................................... 317, 325–327, 335
Cell culture ........................................ 64, 77, 81
Cell suspension .................................. 16, 25, 26, 239, 243, 307, 328
Cellulase ............................................ 247, 248
Cereals .............................................. 366
Cetyl trimethylammonium bromide (CTAB) .......... 82, 88, 114, 116, 139, 155, 156, 163, 186, 200, 210, 213, 220, 240, 275–277, 280, 340
Characterization ................................ 92, 144, 154, 245, 271
Chloroform ....................................... 92, 82, 85, 94, 96, 98, 106, 107, 110, 114, 118, 157, 158, 200, 202, 205, 210, 213, 275–277, 284, 287, 319, 321, 323, 341, 343
Chloroform/isooamyl alcohol (24:1) v/v .............. 82, 85, 106, 107, 114, 157, 166, 210, 213, 275, 276, 341, 343
Chromosome ...................................... 199, 209, 210, 245, 246, 248, 249, 252, 254, 255, 283, 339
Chromosome fixation ........................................ 246, 248
Cluster analysis ....................................... 122, 126, 137, 138, 178, 182, 195
Colchicine ......................................... 254
Commercial production .......................... 199
Comparative analysis ............................. 382, 391
Competent cells .................................... 216, 238, 317, 324, 326, 335, 336
Condensation ....................................... 108
Conservation ....................................... 15–21, 23, 24, 49–55, 57, 58, 61, 62, 64, 65, 67, 68, 71, 105, 125, 144, 339
Conserved miRNA ................................ 340, 345, 346, 348, 349
Coomassie brilliant blue ......................... 372, 374
Coomassie stain .................................... 377
Cotyledonal somatic embryos ................... 77
Cryodamages ..................................... 47
Cryopreservation ................................... 3, 4, 6, 15, 16, 20, 25–36, 39–47, 49, 62
Cryoprotectant .................................... 16
CTAB buffer ...................................... 106, 200, 202, 210, 277, 280
CTAB method ..................................... 107, 108, 139, 156, 201, 240
Cytogenetic markers .............................. 245

D

D cryo-plate ........................................ 26, 33–34, 36
Dark .................................................... 7, 20, 32, 33, 36, 45, 50, 53, 65, 75, 76, 94, 97, 109, 119, 146, 148, 189, 205, 234, 247, 248, 252, 301, 367, 369, 377, 384, 392
Data analysis ........................................ 126, 137, 228, 229, 231, 237, 346, 357, 361, 397

mohan.jain@helsinki.fi
Date palm cultivation ...................................................... 284
Date palm fruits .......................................................... 212, 282, 286
Days after germination (DAG) ........................................ 369, 374, 376
Decamer ........................................................................... 107, 154, 187, 211
Deglet Noor cv ............................................................. 40
Dehydrated pollen ........................................................... 6
Dehydration ................................................................. 16, 21, 35, 58, 68
2-D electrophoresis (2-DE) ............................................. 356, 358, 359, 361, 374, 378, 379, 383, 389, 391
Delta2D ............................................................................ 384, 391
Dendrogram ..................................................................... 122, 137, 138, 140, 161–163, 166, 171, 195
Deoxynucleotide triphosphates ........................................ 214
Deyrophosphorylation ................................................... 321, 355
Desiccation ........................................................................ 77
Desiccator ........................................................................ 3, 4, 6, 7
Developer solution .......................................................... 172, 132, 212, 221, 225, 230, 235
Developmental stage ........................................................ 209, 228, 284, 316, 340, 342, 368, 369, 378, 379, 382, 385, 391, 392, 395
6-Diamidino-2-phenylindole (DAPI) ................................. 247, 253, 277, 279, 290
Diaminoethane tetraacetic acid (EDTA) ...................... 186
2,4-Dichlorophenoxyacetic acid (2,4-D) ......................... 17, 19, 26, 27, 30, 41, 50, 52–54, 62, 64, 67, 68, 72, 74, 82, 92, 93
Differential expression .................................................. 348, 361
Differential in gel electrophoresis (DIGE) analysis ........ 356, 357, 359, 363
Differential proteins ......................................................... 366
Digital imaging ............................................................... 253, 254
Dig-labeled DNA ............................................................. 247, 252
Dimethyl sulfoxide (DMSO) ........................................... 16, 20, 41
Dioecious ........................................................................... 3, 16, 61, 81, 199, 209, 227, 245
Disease ............................................................................ 113, 264, 273, 277, 279, 281, 355
Disinfect ............................................................................... 20, 75
Disinfectant ....................................................................... 50, 53, 94
DNA .............................................................................. 81, 92, 105, 144, 153, 173, 185, 200, 210, 228, 246, 267, 274, 284, 289–291, 293, 295, 298, 300, 303, 306–308, 311, 316, 345
DNA amplification ......................................................... 131, 190, 192, 222, 234
DNA-based markers ..................................................... 82, 153
DNA extraction buffer ................................................... 106, 317, 319
DNA fingerprinting ........................................................ 85, 105, 125, 173, 191
DNA isolation ............................................................... 107, 108, 110, 126, 163, 186, 200, 206, 210, 237
DNA markers .............................................................. 82, 109, 114, 133, 153, 185, 192
DNA pellet ........................................................................ 96, 99, 108, 118, 157, 167, 176, 214, 276, 277
DNA probes ................................................................. 247, 251, 255
DNA quantification ......................................................... 100, 157, 167, 214, 277, 279
DNA sequencing ............................................................ 97, 98, 100, 316
Dominant marker ........................................................... 106, 154, 173
Droplet-vitrification (DV) ................................................ 25, 40, 45, 46
Drought stress (DS) ........................................................... 360, 361
ds cDNA ................................................................. 289–291, 298, 300, 311
Duplex-specific nuclease (DSN) ...................................... 285, 290, 293, 297, 311

E

Early sex identification .................................................. 199–206, 227
Early stage ....................................................................... 114, 199, 209, 210, 378
Egypt ................................................................................. 149, 154, 155, 273
Electrode buffer ............................................................. 145
Electroporation ............................................................... 301
Electrocompetent cells ................................................... 301
Embryo germination ..................................................... 52, 77, 84
Embryogenic callus ....................................................... 26, 55, 63–65, 67, 84
Embryonic culture .......................................................... 20, 22, 23
Encapsulated somatic embryos ...................................... 71, 76
Encapsulation ............................................................... 34, 40, 45–47, 71–75, 77
End polishing ................................................................. 321
Enzymatic digestion ....................................................... 246–248
Ethylene glycol ............................................................... 20, 41
Ethylendiaminetetraacetic acid (EDTA) ......................... 106, 107, 144, 247, 340
Euclidean similarity index ............................................... 119
Evaluation ...................................................................... 81–88, 144, 210
Evaluation of clonal fidelity ........................................... 81–88
Evaporation ................................................................. 108, 139, 153
Ex vitro .............................................................. 82, 109, 114, 133, 153, 185, 192

Index
Hayany cv.......................................................... 126, 133, 174, 178, 228, 238
Hazardous chemicals ............................................... 280
Hexadecytrhimethylammonium bromide (CTAB) ....... 340
Hi-Di formamide .................................................... 285, 309, 312, 317, 330, 336
High-throughput sequencing ................................... 340, 341
Homomorphic .......................................................... 209
Hormone stock solution ........................................... 17, 19, 20, 26, 50, 52, 72, 74, 92
Hybridization .......................................................... 185, 245–253, 255, 285, 290, 311, 323, 344
Hybridization membrane(s) ....................................... 323
Hypercry tissues .......................................................... 68
Hyperhydricity .......................................................... 57, 58, 68

I
Ice crystal ........................................................................ 16, 40
Image Lab™ Software ...................................................... 129, 134–137, 235–237
In vitro...................................................................... 3, 7, 9, 15–21, 23, 24, 39–46, 49–55, 57, 58, 62, 71, 72, 81, 82, 92, 186
In vitro conservation ..................................................... 15–21, 23, 24, 49–55, 57, 58, 68
In vitro culture ............................................................... 25, 26, 40, 46, 62–65
In vitro germination ......................................................... 3, 7, 9
In vitro propagation ......................................................... 39, 62, 81
In vitro storage ............................................................... 9, 15, 71
India ........................................................................... 82, 106, 109, 200
Indole-3-acetic acid (IAA) ............................................ 82, 84, 384, 390, 395
Indole-3-butyric acid (IBA) ............................................ 41, 82, 84
Induction medium .......................................................... 17, 19, 21, 22, 52, 65, 72, 74
In-gel digestion ............................................................. 382
Initiation medium .......................................................... 17, 19, 20, 63, 65, 72, 74, 75, 92, 94
International exchange ................................................... 15
Inter-simple sequence repeat (ISSR) ......................... 82, 105–111, 151, 171, 173–182, 186, 194, 210, 284
Intron-targeted amplified polymorphism (ITAP) ....... 126, 128, 133–135, 228, 231, 236–238, 241
IPTG ........................................................................ 211, 217, 220, 232, 238, 317, 325, 326, 335
Iran ............................................................................. 155
Iraq .......................................................................... 114, 115, 155
Isoamyl alcohol ............................................................ 82, 85, 98, 106, 107, 275, 341, 343
Isoclectric focusing (IEF) ............................................. 356, 361, 366, 367, 372, 373, 381, 383, 384, 389
Isogenic lines ............................................................... 82
2-Isopentenyladenine (2iP) ........................................ 17, 19, 50, 52, 53, 62, 64, 72, 74, 92, 93
Isozyme electrophoresis ............................................. 145
Isozymes .................................................................... 144, 145, 148, 210
ISSR-PCR analysis ...................................................... 149

K
Kanamycin ................................................................. 217, 286, 301, 302
Khadrawy cv .............................................................. 115, 122, 155, 164, 200, 210, 223, 224
Khalas cv ................................................................. 155, 164, 316, 340, 342

L
Large-scale propagation ................................................ 26
Late cotyledonary embryo ............................................. 77
LB medium ............................................................... 216, 217, 286, 301, 306, 317, 325
Legumes ....................................................................... 366
Ligation ...................................................................... 126, 130, 139, 216, 228, 229, 232, 238, 240, 300, 303, 322, 324–326, 332, 335, 344
Linkers .......................................................................... 212, 321–322
Loading solution (LS) .................................................. 27, 32–33, 35, 41, 44, 45
Long-term conservation .............................................. 15, 25, 26
Long-term storage ........................................................ 3, 5, 7, 35, 40, 71, 110, 255, 387, 395
Low temperature ......................................................... 4, 7, 15, 22, 40, 50, 55, 57, 62

M
Major allele frequency .................................................. 119–121
Male specific marker ...................................................... 199–206
Marker-assisted selection (MAS) ................................ 105, 143, 228, 316
PCR products ..................................................87, 97, 100,
119, 131, 134–136, 141, 149, 160–161, 170,
181, 215, 216, 229, 234–239, 268–270, 279,
289, 298, 311, 323–325, 332, 335, 356, 345, 349
PCR reaction ......................................................... 85,132,
134–136, 159, 168, 169, 181, 190, 192, 195,
203, 204, 214, 235–237, 268, 279, 295, 296,
303, 317, 323, 332, 345, 348, 349
PCR-based molecular marker ......................................105
Pectinase ..........................................................247, 248
Pellet ..................................................................324, 325, 333, 335, 336
Phenol ..................................................110, 180, 186,
210, 275, 277, 366, 369–371, 378, 387
Phenol-chloroform ............................................... 163
Phenol extraction ................................................... 220, 277, 281
Phoenix dactylifera ................................................ 39,49,
61, 81, 91, 108, 111, 113, 125, 143, 153, 186,
227, 283, 284, 315, 316, 340, 365
Photoperiod ........................................................ 7, 14, 32,
34, 36, 43, 55, 65, 75, 77, 357
Phylogenetic diagram ............................................ 119
Phylogenetic relationships ...................................... 106,
126, 153–171, 186, 246
Phylogenetic tree .................................................. 138, 178, 182, 195
Plant recovery ..................................................... 67
Plasmid ..................................................107, 211, 217,
218, 228, 229, 232, 239, 240, 243, 251, 273,
274, 279, 301, 302, 304
Plasmid mini-preparation .................................. 306, 327, 328
Plasmid-like DNAs ................................................ 273
Pollen ..........................................................3–12, 199
Pollen encapsulation ............................................. 5
Pollen germination ................................................3, 4, 6, 7, 9, 11
Pollen storage .................................................. 3–5, 7
Pollen tube ...................................................... 4, 7, 9, 11, 12
Pollen viability .................................................. 3, 4, 6–7, 9
Pollen viability test ................................................ 4, 6, 7, 9
Pollination ...................................................... 3, 4, 385, 391, 396
Poly A+ RNA ....................................................... 287, 288
Polyacrylamide gel ............................................... 127, 129,
136, 141, 145, 147, 154, 169, 181, 219–220,
Polyembryonic mass (PEM) .................................. 27, 34
Polyethylene glycol (PEG) .................................. 58,
63, 317, 324, 356, 358
Polymerase chain reaction (PCR) ......................... 85, 92,
105, 173, 185, 200–201, 251, 261, 279, 285, 341
Polymorphism ...................................................... 82, 105,
106, 122, 137, 144, 171, 186, 246, 261, 316
Polymorphism information content (PIC)............ 119, 120
Polymorphisms .................................................. 87, 192,
194, 200, 240, 316
Polyvinylpyrrolidone (PVP) .................................. 88,
106, 148, 155, 166, 317, 319, 341, 349
Population structure ........................................... 113, 114, 125
Potassium acetate .................................................. 175,
186–189, 211, 232, 318
Pre-acclimatization ............................................. 63, 67
Preparative gel ................................................... 358, 359, 361
Preservation ..................................................... 16, 40, 50,
57, 67, 72, 125, 281, 316
Primer ...................................................... 83, 94, 105,
174, 186, 201, 229, 268, 281, 287, 332, 341
Primer designing ................................................ 218, 332
Principal coordinate analysis (PCA) ...................... 96, 119,
122, 376
Probe .......................................................... 185, 246, 251–254
Probe detection .................................................. 246, 253
Probes .......................................................... 246, 247, 251,
254, 255, 322, 323
Pro-embryonic mass (PEM) .................................. 36
Proliferation ..................................................... 40
Proliferation medium ......................................... 17, 19, 20,
22, 27, 30, 32, 33, 36, 52
Propagules ....................................................... 71
Propanoic acid ..................................................... 20
Protein .......................................................... 100, 175, 186,
240, 260, 284, 332, 345, 355, 366
Protein assay ..................................................... 377, 383, 387, 394
Protein electrophoresis ........................................ 144, 147, 171
Protein extract .................................................. 147, 387
Protein extraction .............................................. 147, 356,
358, 366, 367, 369–371, 378, 382, 385
protein extraction buffer .................................... 377
Protein identification .......................................... 358, 361,
367, 373, 379, 393
Protein labeling .................................................. 356, 358, 359
Protein markers .................................................. 372
Protein profile .................................................... 372
Protein quantitation .......................................... 366
Protein spots ...................................................... 359, 361, 378, 392
Proteinase K .................................................... 186–189, 285, 298
Proteins .......................................................... 58, 381
Proteome ......................................................... 355–361, 363,
377, 391, 394, 395, 397
Proteomics ....................................................... 361, 365–379,
381–397
Protocorm .........................................................
Pyridoxine ........................................................ 18, 28, 42,
51, 63, 73, 83, 93

mohan.jain@helsinki.fi
Index

R

Radicle .........................................................366, 369
RAPD-PCR analysis ...........................................148
RAPD PCR products .............................................135
RAPD Primer Screening ......................................85, 87
Recovery .......................................................284, 358, 365
Recovery medium ..............................................17, 23, 35
Recovery solution (RS) ....................................21, 41, 44–46
Regeneration ................................................21, 24, 52, 54, 56, 72, 74, 76
Regeneration .....................................................21, 27, 30, 32, 36, 39–41, 46, 49, 52, 57, 68, 75, 77, 81
Resistance .......................................................110, 113, 264, 266, 277, 279, 281
Restriction digestion ........................................126, 130, 217–218, 228, 232, 240
Restriction enzyme ............................................318, 321, 322
Restriction fragment length polymorphism (RFLP) ....82, 186, 210, 228
Reverse transcriptase .........................................288, 311, 341, 348
Rewarming ....................................................21, 23, 35
Rhizomes .........................................................71
Ribosomal DNA (rDNA) ....................................99, 246, 254, 255
Rifampicin .....................................................74, 75
RITA bioreactor ................................................40, 41, 43
RNA ..............................................................110, 176, 202, 281, 285, 339
RNA extraction ..............................................286, 287, 341–342, 349
RNase solution A ...........................................187, 188
Rooting ............................................................17, 21, 46, 52
Rooting medium (RoM) .....................................17, 19, 21, 41, 52, 55, 63, 64, 67

S

Salt stress (SS) ..................................................284, 358, 360, 361
Samany cv ....................................................126, 228, 238
Sanger sequencing .........................................228, 229, 240
Saudi Arabia ...................................................154, 155, 274, 283, 284, 315, 340
Screening ......................................................85, 87, 209, 210, 239, 273, 274, 325, 335
SDS-PAGE Electrophoresis ................................144, 147
Seed germination .............................................356, 357, 361, 365, 366
Seedling .....................................................126, 173, 191, 199, 210, 212, 213, 218, 222, 224, 225, 228, 245, 246, 356, 358, 365
Senescence ....................................................22
Sensitivity .....................................................106, 109, 119
Sequence-characterized amplified region (SCAR) ....186, 199–206, 210, 211, 215–219, 222, 223
Sequencing Gel ..............................................130–131, 233
Sex differentiation ..........................................245–253, 255
Sex identification ..........................................199–206, 210
Sex-specific marker ........................................126, 227–243, 246
Shoot bud .....................................................17, 20–23, 50, 71
Shoot tip .......................................................16, 17, 20, 25, 49–55, 57, 58, 65, 66, 68, 72, 75, 83, 94, 95
SigmaCote® solution ....................................131, 219, 224, 233
Silver solution ...............................................212, 220
Silver staining ..............................................131, 220, 234
Siwy cv .......................................................126, 144, 149, 246, 254
Slow-growth storage .....................................50
Small RNA ...................................................341, 343–344, 349
Small RNA library .........................................340–345
SNX forward primer ......................................322–324
SNX reverse primer ......................................318, 322, 323
Sodium acetate ............................................82, 85, 157, 167, 174, 176, 187, 188, 190, 202, 205, 289, 298, 318, 344
Sodium alginate .............................................27, 29, 32, 34, 36, 45, 72, 74–77
Sodium carbonate ........................................127, 132, 212, 230, 234
Sodium chloride .........................................82, 200, 356
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)........144, 358, 370, 372, 378, 381
Sodium hypochlorite .......................................16, 20, 23, 50, 62, 72, 92
Sodium thiosulfate .......................................127, 132, 212, 230, 238
SOLID .................................................................341–345
Solution ..............................................................................290
Somaclonal variation .............................................24, 39, 40, 81, 82, 186
Somatic embryo induction (SEI) .................52, 55, 74, 84
Somatic embryogenesis ........................................26, 49, 58, 72, 84
Sorbitol ...............................................................15, 20, 21, 50, 57, 62
Spain .................................................................................379
Squash method .............................................................249
Streptavidin .................................................................247
Storage .................................................................3–7, 9, 11, 12, 15, 16, 21–23, 30, 31, 35, 36, 45, 49, 50, 57, 62, 71, 77, 238, 243, 255, 326, 335, 365, 368–369, 387, 395
T
TA-cloning vector ......................................................211, 215
Taq polymerase ..................................................108, 149, 158, 159, 168, 190, 214, 268, 303, 322, 323
Target gene ..............................................................339, 340, 349
Target prediction .................................................340, 349, 350
T4 DNA ligase .....................................................216, 219, 268, 286, 288, 292, 293, 308, 328, 344
Thiamine .................................................................18, 28, 42, 51, 63, 73, 83, 93
Tissue culture ......................................................15–21, 23, 24, 26, 30–32, 39, 49, 50, 53, 62, 72, 82, 84, 87, 88, 91–94, 96–98, 100, 191, 192
Total RNA ......................................................287–289, 341–343, 348
T7 primer .................................................................308, 329
Transformant ......................................................243, 302, 324, 333, 335
Transformation ......................................................216, 239, 301, 325–327, 335, 336, 374
Transport .................................................................30, 71, 148, 264
Tris borate EDTA ..................................................83, 88
Tris(hydroxymethyl)aminomethane (Tris) .......144, 186
Tunisia .................................................................114, 154, 155, 273, 274
Two-dimensional gel electrophoresis
(2-DE) .................................................................356, 377, 381
U
Ultrasound .................................................................368
Unipolar .................................................................71
Unweight pair group method with arithmetic
mean (UPGMA) .....................................................137, 141, 162, 171, 195
Urea solution ......................................................127, 131, 230, 233, 234
USA .................................................................227, 261, 267
UV lamp .................................................................109, 167, 181
UV-transilluminator ................................................201, 203, 279

mohan.jain@helsinki.fi
Index

V
Viability ........................................................................................................... 3–7, 9, 11, 12, 22, 56, 67, 69, 77
Vitrification .............................................................. 16, 21, 23, 25, 26, 40, 44–47
Vitrification solution (VS) .................................................. 16, 20, 21, 23, 41, 44

W
Weeks after pollination (WAP) .......................................... 368, 369, 374, 376, 378
Whitening of the fronds .................................................. 273

X
X-gal ................................................................. 211, 217, 232, 238, 317, 325, 326, 335

Y
Y chromosome ........................................................... 254
Yield ........................................................................ 106, 110, 113, 141, 154, 180, 186, 199, 203, 243, 378

Z
Zagloul cv. ......................... 72, 126, 133, 228, 246, 254
Zygotic embryo (ZE) ............. 369, 372, 374, 376, 379