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Research Article
Standardization of Long Term DNA Storage and DNA Banking Methods for Conservation of Saudi Date Palm (*Phoenix dactylifera* L.) Genetic Diversity

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Abstract

Background: Date palm (*Phoenix dactylifera* L.) has immense socio-economic significance in Saudi Arabia. Therefore, it is of great importance to survey, collect, identify and characterize all the available genetic diversity of the date palm in the Kingdom of Saudi Arabia. **Objective:** The objective of this study was to study the effect of different activities of SDDB, such as selection of famous and less famous Saudi date palm varieties, to make core collection, long term storage and maintain a well-documented date palm DNA bank. **Materials and Methods:** Date palm leaf samples were collected and preserved in buffer solution. The study standardized a genomic DNA isolation program from date palm cultivars at the research station. The date palm genomic DNA extraction was carried by a modified C-Tab method. **Results:** Saudi Date Palm DNA Bank (SDDB) was established at Date Palm Research Centre (DPRC), King Faisal University having 71 accessions from three locations inside Saudi Arabia. This study was successful in standardizing a protocol for manual isolation of date palm genomic DNA and its preservation on long term basis. The SDDB was successful in applying DNA technology for conserving date palm genetic diversity by field exploration, identification and maintaining the tissues and DNA in a well-organized date palm DNA bank. **Conclusion:** The protocol established for isolation of high purity DNA and optimization of RAPD conditions can be used for DNA isolation from different date palm varieties. This preposition can form a strong base for future molecular characterization and genetic improvement works in date palm. However, the SDDB is first of its kind and will serve the date palm research and development institutes and industry by providing consultancy for accurate genetic determination of the date palm varieties.

Key words: Date palm, DNA storage, DNA bank, genomic diversity, germplasm, conservation

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the Kingdom of Saudi Arabia, date palm occupies around 73.6% of the total cultivated area. Date palm cultivation and the related industry constitute about 13.7% of the national economy. The importance of date palm research to the Kingdom and the scientific research needs and role of Date Palm Research Center of Excellence (DPRC) in achieving the long term and short term research goals are well understood. Date palm plays an important role in food security and nutrition of the country[1]. The total land area of 2.25 million square kilometers of the country together with its geographic location led to diversity in its terrain and geological formation which in turn resulted in the diversity of flora and fauna from one part of the country to other. Besides, the arid climatic conditions prevail almost all over the country and the cultivated crops are very limited. Among the different crops naturally inhibited and adapted to the country's desert environment, date palm stands first due to its socio-economic and ecological significance. According to an estimate, there are about 400 different date palm varieties in Saudi Arabia and only a few promising are cultivated commercially. After eliminating synonyms, there is an estimated core collection of 287 date palm varieties in the kingdom. Among this core collection, only 13% constitutes the most famous varieties and 26% constitutes less famous varieties, while the remaining 61% of the available date palm cultivars in the country are found to be neglected. The progressive farmers and the local people select only high yielding and economically feasible famous varieties for cultivation[2]. This trend with large scale mono-cropping system will lead to genetic erosion of the present day available date palm germplasms in the near future. For example in Tunisia, this important subtropical fruit crop is currently in danger due to severe genetic erosion as a consequence of the predominance of the elite cultivar Deglet Nour in modern cultures[3]. This tendency led to the disappearance of many cultivars with medium and low fruit qualities.

Presently, conservation of biodiversity is a global concern and an increasing attention is focused on measuring the extent of diversity and monitoring the potential fate of these natural resources[4]. Considering the biodiversity, conservation needs globally and the United Nations (UN) celebrated 2010 as the international year of biodiversity. It is worth to quote here the words of Ahmed Djoghlaf, executive secretary of the convention on biological diversity 2010 is the international year of biodiversity and people all over the world are working to safeguard this irreplaceable natural wealth and reduce biodiversity loss. This is vital for current and future human well being. We need to do more and now is the time to act[5]. Out of the world's 34 biodiversity hot spots, three are located in West Asia[6] namely the Irano-Anatolian region, the Mediterranean forest region and the Horn of Africa region including the Arabian Peninsula and the Socotra archipelago in Yemen[7].

In the Arabian Peninsula, Saudi Arabia is one of the hot spots of date palm (*Phoenix dactylifera* L.) diversity. Owing to the region's importance as the origin of date palm and considering its immense socio-economic significance in the country, it is imperative to conserve and genetically characterize all the available diversity of the date palm in the country. The characterization and conservation of available genetic diversity is a basic necessity of traditional plant breeding and crop improvement using molecular breeding such as Marker Assisted Breeding (MAS) and recombinant DNA (rDNA) technology. It is, therefore, imperative to elaborate a strategy to evaluate the genetic diversity and to preserve the date palm germplasm. The aim of this study was to collect the genetic diversity among the date palm varieties in the Kingdom of Saudi Arabia and preserve them in Saudi Date Palm DNA Bank (SDDB).

MATERIALS AND METHODS

The study was conducted at Date Palm Research Centre (DPRC), King Faisal University during the year 2014.

Infrastructure and capacity building: A laboratory was set up to execute all the technical and research activities for the successful completion of the experiment. The devices and chemicals required for the experiment were available in the laboratory for use in the experiment.

Collection of date palm accessions: Date palm leaf samples were collected and preserved in buffer solution. The details of varieties collected and core collection maintained in Saudi Date Palm DNA Bank (SDDB) is presented in Table 1-4.

Standardization of DNA isolation protocol: Based on the previous experience and the current literature review, the study standardized a protocol for genomic deoxyribonucleic acid (DNA) isolation from date palm.

Palm genomic DNA extraction using modified C-Tab method (CTAB): The DNA extraction from date palm depends on the material used. At the initial grinding stage, liquid nitrogen was

Table 1: Date palm accessions collected from Buraidah, Al-Qasim

Accession No.	Name in english	Orchard
001	Shishi	Al-Wataniya
002	Salhiyah	Al-Wataniya
003	Swaqee	Al-Wataniya
004	Bireehee	Al-Wataniya
005	Bastaniyah	Al-Wataniya
006	Sabakah	Al-Wataniya
007	Hilaliyah	Al-Wataniya
008	Nabt Sualaiman	Al-Wataniya
009	Deglet Nour	Al-Wataniya
010	Shaqra	Al-Wataniya
011	Manoa'	Al-Wataniya
012	Nabt Ali	Al-Rahji
013	Sukary Red	Al-Rahji
014	Barhi Yellow	Al-Rahji
015	Wunanah	Al-Rahji
016	Medjoul	Al-Rahji
017	Khalas	Al-Rahji
018	Male	Al-Rahji
019	Nabt Salih	Al-Rahji
020	Sukary Yellow	Al-Rahji
021	Ruthanah	Al-Rahji
022	Maneeyee	Al-Rahji
023	Rushdiyah	Al-Rahji
024	Nabt Rajha	Al-Rahji

Table 2: Date palm accessions collected from Al-Madinah AL-Munawarah

Accession No.	Name in English	Orchard
025	Safawi	Madeena 2
026	Halayya	Madeena 2
027	Ajwa-2	Madeena 2
028	Safawi-2	Madeena 2
029	Rabeea	Madeena 2
030	Lon Musaad	Madeena 2
031	Barni Ees (Not Barni Al-Ees)	Madeena 2
032	Ruthanah Sharak	Madeena 2
033	Baid	Madeena 2
034	Hulwa	Madeena 2
035	Ruthanah 1	Madeena 1
036	Ajwa 1	Madeena 1
037	Sukari 1	Madeena 1
038	Ambar	Madeena 1

Table 3: Date palm accessions collected from Al-Ahsa

Accession No.	Name in english	Orchard
039	Um Raheem	Date Palm Center
040	Hilal	Date Palm Center
041	Barhi	Date Palm Center
042	Shahal	Date Palm Center
043	Shaishe	Date Palm Center
044	Shebebi	Date Palm Center
045	Hatmi	Date Palm Center
046	Ruzeiz	Date Palm Center
047	Ghur	Date Palm Center
048	Khenaizy	Date Palm Center
049	Wesaili	Date Palm Center
050	Khallas	Date Palm Center
051	Tanajeeb	Date Palm Center
052	Um Mejnas	Date Palm Center
053	Tayyar	Date Palm Center
054	Kasbi	Date Palm Center
055	Marzaban	Date Palm Center
056	Zamli	Date Palm Center
057	Azhabi	Date Palm Center
058	Khesab	Date Palm Center
059	Daalij	Date Palm Center
060	Khoodaj	Date Palm Center
061	Hrizi	Date Palm Center
062	Ahmar	Date Palm Center
063	Namree	Date Palm Center
064	Dinaree	Date Palm Center
065	DPC1	Date Palm Center
066	Khalas	DPRC, KFU
067	Ruziz	DPRC, KFU
068	Barhi	DPRC, KFU
069	Hilali	DPRC, KFU
070	Sukari	DPRC, KFU
071	Shishi	DPRC, KFU

Table 4: Core collection of date palm accessions maintained in SDDB

Location	Total
Buraidah, Al-Qasim	24
Madinah Munawarah	14
Al-Ahsa	33
Total	71
Duplicates	10
Core collection	61

employed to break down cell wall material and allow access to DNA while the harmful cellular enzymes and chemicals remain inactivated.

Once the tissue was sufficiently ground, it was then re-suspended in a 2.5% CTAB buffer. To purify DNA, insoluble particulates were removed by centrifugation, while the soluble proteins and other material were separated by mixing with chloroform followed by centrifugation. The isolated DNA was then precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA was then re-suspended and stored in TE buffer or sterile distilled water. This method showed reliability to provide intact genomic DNA from date palm leaf tissue. Furthermore, in order to check the quality of the extracted DNA, a sample was run on an agarose gel, stained with ethidium bromide and visualized under UV light.

Procedure of DNA isolation protocol:

- About 200 mg of fine powder of date palm tissue was converted into fine paste in approximately 500 µL of 2.5% CTAB buffer

- Transferred the CTAB plant extract mixture to 1 mL microcentrifuge tube
- Incubated the CTAB plant extract mixture for about 15 min at 55°C in a re-circulating water bath
- After incubation, the CTAB plant extract mixture was centrifuged at 12000 rpm for 5 min to spin down cell debris. Transfer the supernatant to clean microcentrifuge tubes
- About 250 µL of chloroform was added to each tube: Isoamyl alcohol (24:1) and mixed the solution thoroughly
- After mixing, the tubes were again centrifuged at 13000 rpm min^{-1}
- Transferred the upper aqueous phase only (contains the DNA) to a clean microcentrifuge tube
- About 50 µL of 7.5 M ammonium acetate was added to each tube followed by 500 µL of ice cold absolute ethanol
- Inverted the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively, the tubes can be placed for 1 h at -20°C after the addition of ethanol to precipitate the DNA
- After precipitation, the DNA was pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, the precipitate was transferred into a microcentrifuge tube containing 500 µL of ice cold 70% ethanol and slowly inverted the tube
- Alternatively, the precipitate was isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Then removed the supernatant and washed the DNA pellets twice with ice cold 70% ethanol
- After washing, the DNA was spun into pellet by centrifuging at 13000 rpm for 1 min. Then removed all the supernatant and allowed the DNA pellet to dry for about 15 min
- Re-suspended the DNA in sterile DNase free water (approximately 50-400 µL H_2O). However, the quantity of water needed to dissolve the DNA depends on how much is isolated from the sample. Around RNase A (10 µg mL^{-1}) can be added to water prior to dissolving the DNA to remove any RNA in the preparation (10 µL RNase A in 10 mL H_2O)
- After re-suspension, the DNA was incubated at 65°C for 20 min to destroy DNases stored at 4°C
- Agarose gel electrophoresis of the DNA showed the integrity of the DNA, while spectrophotometry gave an indication of the concentration and cleanliness

DNA quality confirmation:

- Prepared 1% solution of agarose by melting 1 g of agarose in 100 mL of 0.5x TBE buffer in a microwave for approximately 2 min. Allowed to cool for a couple of min, then added 2.5 µL of ethidium bromide, stir to mix
- Casted a gel using a supplied tray and comb. Allowed the gel to set for a minimum of 20 min at room temperature on a flat surface
- Loaded into separate wells, 10 µL 1 kb ladder, 5 µL sample+5 µL water+2 µL 6x loading buffer
- Ran the gel for 30 min at 100 V
- Exposed the gel to UV light and photograph (demonstration)
- Confirmed the DNA quality i.e., presence of a highly resolved high molecular weight band indicated good quality DNA while the presence of a smeared band indicated DNA degradation

DNA quantification: The DNA concentrations were determined either by running aliquots of DNA samples on 0.8% agarose gel electrophoresis or by taking the absorbance at 260 nm. The ratio between 260 and 280 nm provided an estimated purity of the DNA sample. The DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% agarose gel electrophoresis were considered as good quality DNA.

RESULTS AND DISCUSSION

Efficiency of DNA isolation protocol: The newly standardized protocol was efficient to deliver high quality DNA suitable for downstream processing. The quality was assessed by restriction digestion and PCR amplification.

Restriction digestion: The genomic DNA (1 µg) was digested overnight with 10 U of restriction enzymes *Eco*RI. The reaction was carried out in buffered condition at 37°C by following the manufacturer's instructions (Bangalore Genei, Bangalore, India). The digested DNA was electro-hosed on 0.8% agarose gel.

RAPD PCR reaction: For the optimization of RAPD reaction using DNA extracted from six date palm accessions, oligonucleotide primer OPA-04 (5'-AATCGGGCTG-3') was used for amplification to standardize the PCR conditions. The sizes

of the amplification products were estimated by comparing them to standard DNA ladder (O' Gene Ruler 1.0 kbp DNA ladder; MBI Ferment Inc., Maryland, USA).

Standardization of storage method: The date palm genomic DNA extracted was stored at three different temperatures and the integrity of DNA was checked with electrophoresis and restriction digestion reaction after 3, 6, 9, 12, 15 and 18 months interval. The DNA integrity among 6 accessions was observed and the data recorded on a five point scale.

The application of DNA technology in agricultural research has progressed rapidly over the last 20 years, especially in the area of cultivar identification and characterization[8] as well as determination of population diversity in many plant species[9,10]. However, in some plant species, the application of this powerful tool is constrained due to inefficient isolation techniques for nucleic acids. The extraction of nucleic acids was difficult in a variety of plants due to the presence of secondary metabolites that interfered with DNA isolation procedures and reactions such as DNA restriction, amplification and cloning[11].

The isolation and purification of high molecular weight DNA from plant species is encountered by many factors such as degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, the inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with subsequent enzymatic reactions[12]. A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. Similarly, different protocols for DNA extraction were successfully applied to many plant species[13-16].

High quality and more quantity of DNA was isolated successfully from date palm using the above mentioned protocol from fresh leaf tissue. The isolated DNA showed the normal spectra with A260/A280 ratios ranging between 1.6 and 1.7. Also, the agarose gel electrophoresis did not provide any evidence of protein and RNA contamination thus confirming the DNA of high molecular weight when compared to undigested λ DNA as given in Fig. 1. The yield range was 10-20 μg g^{-1} of leaf tissue. The DNA isolated from 6 accessions of date palm using this method was suitable for restriction enzyme digestion with *Eco*RI (Fig. 2) and RAPD experiments using OPA 04 as shown in Fig. 3.

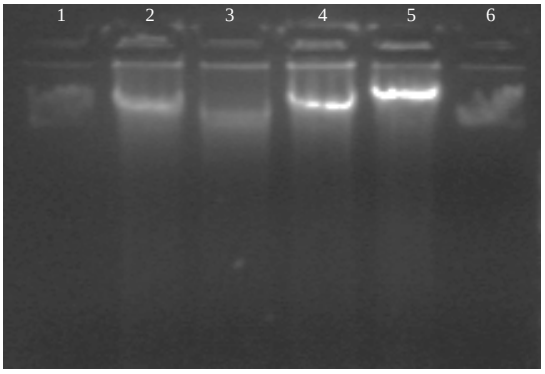

Fig. 1: Genomic DNA extracted by the currently developed method in 6 accessions of *P. dactylifera*
Lane 1-6: Khalas, Ruziz, Barhi, Hilali, Sukari and Shishi, respectively

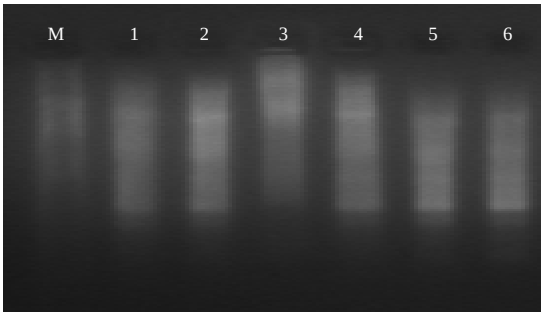

Fig. 2: Genomic DNA extracted by the currently developed method in 6 accessions of *P. dactylifera*
Lane M: λ DNA, lane 1-6: Khalas, Ruziz, Barhi, Hilali, Sukari and Shishi, respectively

The DNA stored in H_2O showed complete degradation at higher temperatures, therefore the SDDB stored DNA in buffers. Also, it was observed that repeated freezing and thawing marginally affect the DNA quality. The best practice to prevent DNA damage during storage include ensuring DNA purity, avoid exposure to nucleases, fast and optimized DNA extraction, quality control, aliquoting, low relative humidity, proper tissue age, pH and salinity in buffer and salt contents etc. Low temperature ranging between -20°C and -80°C proved useful for long term storage of date palm DNA (Fig. 4, 5). Overall, the results of this study agree with the findings of many researchers who applied modified CTAB protocol for DNA extraction from soybean, meat products, maize plants, young petals of some medicinal plants and the stem bark of Leguminosae trees[17-20].

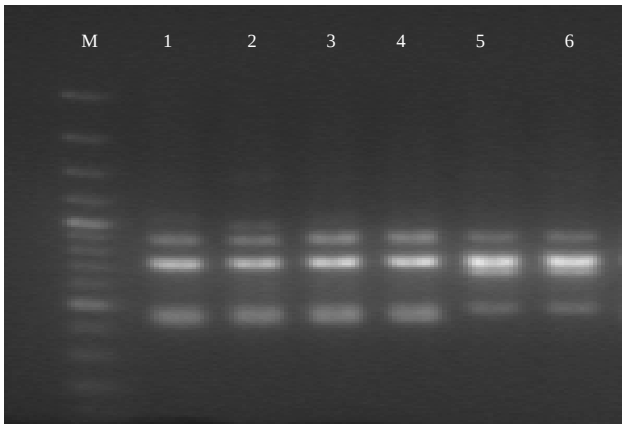

Fig. 3: RAPD banding patterns of 6 accessions of *P. dactylifera*, amplified with primer OPA 04 (5'-AATCGGGCTG-3')
Lane M: 100 bp DNA ladder, lane 1-6: Leaf DNA isolated from 6 accessions of *P. dactylifera*; Khalas, Ruziz, Barhi, Hilali, Sukari and Shishi, respectively

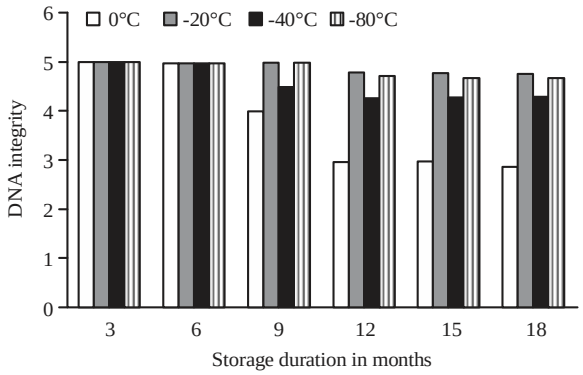

Fig. 4: Effect of storage temperature on DNA integrity

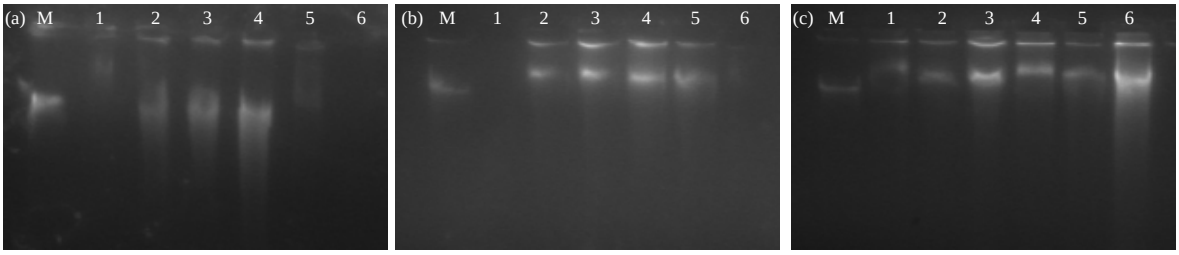

Fig. 5(a-c): DNA integrity after 12 months storage visualized under UV in 0.8% agarose gel, samples stored at (a) 0°C, (b) -20°C and (c) -80°C respectively
Lane M: λ DNA, lanes 1-6: Khalas, Ruziz, Barhi, Hilali, Sukari and Shishi, respectively

CONCLUSION

The SDDB was successful in applying DNA technology for conserving date palm genetic diversity by field exploration, identification and maintaining the tissues and DNA in a well-organized date palm DNA bank. The present protocol for isolation of high purity DNA and optimization of RAPD conditions will be used for DNA isolation from other date palm varieties also. This will form a strong base for future molecular characterization and genetic improvement works in date

palm. Finally to safeguard date palm DNA during storage based on SDDB protocol, the recommendations include preparation of variety specific samples of date palm accessions, avoid temperature fluctuations, preservation of master sample in a separate freezer, avoid repeated thawing and freezing cycles, rapid availability of ready-to-use aliquots, absence of mechanical stress and prevention of shear breakage.

SIGNIFICANT STATEMENTS

This study discovered that establishment of DNA data bank is very important to preserve and improve genetic diversity of date palm. Because it is an important fruit tree of Saudi Arabia and plays significant role in its economy. The study will help the researchers to apply SDDB that will provide consultancy for accurate genetic determination of the date palm varieties by regular field visit including new accession to the DNA bank.

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