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Molecular variability and genetic relationships of date palm (*Phoenix dactylifera* L.) cultivars based on inter-primer binding site (iPBS) markers

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Abstract

Date palm (*Phoenix dactylifera* L.) is an important fruit crop in many arid areas and understanding the relatedness among genotypes is important for effective date palm improvement. Inter-primer binding site (iPBS) markers were used to assess the molecular variation and genetic diversity of 54 and 12 date palm (*Phoenix dactylifera* L.) genotypes collected from Australia and Iraq, respectively. The main objectives were to survey genetic diversity and to determine varietal differences among the collected date palm germplasm. The PCR of five iPBS primers (dominant markers) selected from an initial 60 produced a total of 111 bands ranging from 180 to 3500 bp. The PIC value for these five primers ranged from 0.2135 to 0.3289 with a mean value 0.2816. The mean expected heterozygosity (0.218), mean unbiased expected heterozygosity (0.229) and Shannon's information index (0.33) indicated a high level of inbreeding among the accessions tested. Ordination and cluster analysis showed that the genetic relationships among all accessions could be separated into geographic origin; specifically Iraqi female cultivars, exotic female cultivars collected in Australia and male accessions also collected in Australia, with a few exceptions. Date palm accessions collected in Australia and Iraq are highly divergent and the abundant genetic diversity observed provides a beginning platform for date palm improvement in Australia. The iPBS PCR-based genome fingerprinting technology used in this study is low-cost and effectively differentiated accessions of date palm and their related species.

Keywords: Date Palm (*Phoenix dactylifera* L.), Genetic diversity, Germplasm, inter-primer binding site (iPBS) markers, Molecular markers, Plant breeding
Abbreviations: AFLP_amplified fragment length polymorphism, FAO_food and agriculture organization of the United Nations, iPBS_inter-primer binding site, ISSR_inter-simple sequence repeat, ITS_internal transcribed spacer, PCA_principal component analysis, RAPD_random amplified polymorphic DNA, RFLP_Restriction fragment length polymorphisms, SSR_simple sequence repeat, UPGMA_unweighted pair group method with arithmetic mean.

Introduction

The date palm (*Phoenix dactylifera* L.) is a monocotyledonous and dioecious perennial species belonging to the family Arecaceae (Coryphoideae) (Allen, 1910). There are 14 species in the genus *Phoenix*. The species *P. theophrastii*, and *P. sylvestris* are very closely related to date palm (*P. dactylifera*) (Barrow, 1998; Rivera et al., 2008). Dates are one of the world's oldest cultivated fruits and it was domesticated in Mesopotamia (now Iraq) more than 5000 years ago (Barrow, 1998, 1999; Hamza et al., 2011; Wrigley, 1995). The date palm (*Phoenix dactylifera*) has long been an integral part of desert culture and provides food, building material, shade, fuel and income for the local people. It is an important fruit crop mostly grown in the arid regions of Africa, the Middle East and South Asia (Elmeer et al., 2011; Khan and Bi, 2012) and has high tolerance to environmental constraints including drought, high temperature and salinity (Elsafi, 2012; Elshibli and Korpelainen, 2010).

Trade in dates is steadily rising and demand is increasing on all five inhabited continents. In 2012, the harvested area of date palms was estimated as 1.11 million hectares in over 40 countries with an annual production of 7.5 million tonnes (FAOSTAT, 2012). The tolerance of date palm to high temperature, drought and salinity led to the introduction of this crop to other regions including eastern China, northern India, USA, South Africa and Australia (Arabnezhad et al., 2012; Zaid and de Wet, 2002). The number of date palm cultivars around the world is thought to be as high as 5,000 (Jaradat and Zaid, 2004). Based on morphological characterization, more than 220 clonally propagated cultivars exist in Morocco (Toutain, 1972), 140 in Tunisia, 450 in Saudi Arabia, 400 in Sudan, 1,000 in Algeria (Al-Khalifah and Askari, 2003), over 300 in Pakistan and more than 200 in the Sultanate of Oman (Al-Yahyai and Al-Khanjari, 2008).

The introduction of date palms in Australia commenced in the 1890s through the random distribution of date seeds by cameleers. These early introductions led to well-established populations of date palms around isolated springs and waterholes on outback transportation routes (McColl, 1992; Petherbridge, 1980). Despite the long heritage of successful date palm establishment in Australia, the annual consumption of 5,000 to 7,000 tonnes of dates is satisfied almost exclusively by imports (Reilly et al., 2010). Petherbridge (1980) found that many areas in Australia were suitable for date palm cultivation and the Australian government has encouraged investigation of date production in central Australia, Western Australia, South Australia and in the Cunnamulla-Eulo areas of western Queensland (Kenna and Mansfield, 1997; McColl, 1992; Reilly et al., 2010).

All commercial cultivars in the Australian date industry are female and there is no effective method of producing male palms of these cultivars. Therefore, farmers use mixed pollen to pollinate the female flowers in commercial production (Kenna and Mansfield, 1997). Mixed pollen leads to variable fruit quality and quantity through metaxenia (Ahmad and Ali, 1960; Shafique et al., 2011). Male genotypes with desirable qualities are in high demand for the hand pollination of female trees for Australian date industry (Kenna and Mansfield, 1997). Consequently, a thorough genetic analysis of both female and male germplasm will allow date producers to optimize crossing combinations thus increasing productivity and quality.

The existence of intra-cultivar variation could potentially cause confusion in cultivar nomenclature, preservation and utilization of the collected germplasm (Trifi et al., 2000; Zaid and de Wet, 2002). Introduction of new genotypes from neighbouring countries, together with traditional hand pollination systems for production, can generate recombinant genotypes in different geographical locations. Therefore, an understanding of date palm genetic diversity at the regional level will provide a basis for genetic improvement and the maintenance of date palm germplasm (Akkak et al., 2009; Arabnezhad et al., 2012).

The morphological markers of date palm are primarily based on a few characteristics of the fruit (shape, weight, colour, skin aspect, consistency and texture); the leaves, spines and pedigree information. However, genetic diversity analysis among closely related cultivars using morphological characters alone is often unreliable and influenced by the environment. An alternative is to use isoenzyme, however, this method is limited by the number of informative data and gives no direct assessment of DNA genomic variation (Akbari et al., 2012; Khanam et al., 2012; Mehmood et al., 2014; Sedra et al., 1993; Sedra et al., 1996).

The genome size of date palm (*Phoenix dactylifera* L., 2n = 36) is estimated to be approximately 658-Mbp (Al-Dous et al., 2011). Restriction fragment length polymorphisms (RFLP) were evaluated for date palm cultivar identification (Corniquel and Mercier, 1994) but the technique was considered laborious and unsuitable for studying large numbers of samples. Other molecular markers including random amplified polymorphic DNA (RAPD) (Sedra et al., 1998; Soliman et al., 2003; Williams et al., 1990), inter simple sequence repeats (ISSR) (Hamza et al., 2013; Hamza et al., 2012), amplified fragment length polymorphism (AFLP) (Al-Khalifah and Askari, 2003; Cao and Chao, 2002; El-Assar et al., 2005; Elassar et al., 2003) and simple sequence repeat (SSR) (Ahmed and Al-Qaradawi, 2009; Akkak et al., 2009; Al-Ruqaishi et al., 2008; Arabnezhad et al., 2012; Billotte et al., 2004; Elmeer et al., 2011; Elsafi, 2012; Elshibli and Korpelainen, 2008; Hamza et al., 2013; Hamza et al., 2011; Khierallah et al., 2011; Pintaud et al., 2010; Zehdi et al., 2004) have been used for measuring genetic diversity of date palm germplasm collected from a variety of countries. Other researchers have used a combination of these marker systems to study the genetic diversity of date palm germplasm (Abdulla and Gamal, 2010; Adawy et al., 2005; Elshibli and Korpelainen, 2009; Hussein et al., 2005; Rivera et al., 2008; Saker et al., 2006; Saker et al., 2000).

Recently, the inter-primer binding site (iPBS) markers were developed as an alternative method to exploring genetic diversity and relationships in plants (Alzohairy et al., 2014; Kalendar et al., 2010; Kalendar et al., 2011; Smykal et al., 2011). This marker system was recently used to DNA fingerprint in apricot (*Prunus armeniaca*) (Baranek et al., 2012), grapes (*Vitis vinifera* L.) (Guo et al., 2014), guava (*Psidium guajava* L.) (Mehmood et al., 2013; Mehmood et al., 2015) and cordyline (*Cordyline hybida*) (Luo et al., 2015). The current study investigated the genetic relationships of 54 Australian collections and 12 Iraqi genotypes from the Diyala district of Iraq, using the new iPBS technique.

Results

DNA polymorphism for 5 iPBS primers

For primer screening, a total of 60 iPBS primers were initially screened for PCR amplification using the cultivar Medjool, and 58 primers generated PCR products with a varied number of bands. Five primers (2271, 2074, 2276, 2374, 2380) were selected for iPBS PCR amplification because of the large number of polymorphic bands they generated (Table 2). The sizes of reproducible and scorable bands ranged from 180 to 3500 bp. The iPBS fingerprinting pattern of the 66 accessions from primer 2276 are shown in Fig. 2. The number of unique banding patterns among the 66 accessions validated the use of iPBS markers for the identification of date palm DNA. Furthermore, the five primers used amplified a total of 111 scorable bands indicating a high degree of genetic variability. The information from these five primers, including the number of bands and mean PIC values, is included in Table 2. Primer 2374 produced the highest number of bands (25) and primer 2380 generated the lowest (19). Primer 2276 had the highest PIC value (0.3289) whereas primer 2380 had the lowest (0.2135). These results indicate that the iPBS marker system used in this study can reveal a wide range of genomic DNA diversity in date palm (*Phoenix dactylifera* L.) and its related species.

Heterozygosity and diversity of species

Summary statistics for each of the four groups of accessions (AF: Australian female, AM: Australian male, IR: Iraqi female and SP: related species) covering the number of different alleles, number of effective alleles, Shannon's information index, expected heterozygosity, unbiased expected heterozygosity and percentage of polymorphic loci are listed in Table 3. Expected heterozygosity values (He) ranged from 0.172 (IR) to 0.245 (SP), with an average of 0.218, whereas unbiased expected heterozygosity (uHe) ranged from 0.180 (IR) to 0.272 (SP) with an average of 0.229. The Shannon's information index among the four groups ranged from 0.261(IR) to 0.374 (SP), with an average of 0.330. Nei's (1972) pairwise population matrix of the genetic identity among all the four groups is listed in Table 4. The highest genetic identity (0.952) exists between Australian female and the Australian male groups while the lowest genetic identity (0.821) was found between the Iraqi female accessions and the related species accessions.

Principal component analysis for the five iPBS markers

The principal component analysis as presented in a spatial representation of the relative genetic distances among the individual accessions revealed four distinct groups (Fig. 3). The plane of the first three PCoA axes accounted for 31.11% of the total variation (first axis = 14.36%, second = 9.21%, third = 7.54%). Most of the Australian female accessions

Table 1. Sixty six date palm (*Phoenix dactylifera*) accessions used in iPBS analysis.

Accession	Cultivar name	Collection Site	Genus	Species	Male/Female
1	Barhi	South Australia	*Phoenix*	*dactylifera*	Female
2	Medjool	South Australia	*P.*	*dactylifera*	Female
3	Khadrawy	South Australia	*P.*	*dactylifera*	Female
4	Sultana	South Australia	*P.*	*dactylifera*	Female
5	Lulu	South Australia	*P.*	*dactylifera*	Female
6	Fard	South Australia	*P.*	*dactylifera*	Female
7	Kalas	South Australia	*P.*	*dactylifera*	Female
8	Iraqi	South Australia	*P.*	*dactylifera*	Female
9	Nemeish	South Australia	*P.*	*dactylifera*	Female
10	Dary	South Australia	*P.*	*dactylifera*	Female
11	Saga	South Australia	*P.*	*dactylifera*	Female
12	*P. reclinata*	RBGS	*P.*	*reclinata*	Female
13	Hallawi	NT, Australia	*P.*	*dactylifera*	Female
14	Baskary	NT, Australia	*P.*	*dactylifera*	Female
15	Basturami	NT, Australia	*P.*	*dactylifera*	Female
16	Kalas oman	NT, Australia	*P.*	*dactylifera*	Female
17	Hayani	NT, Australia	*P.*	*dactylifera*	Female
18	Deglat noor	NT, Australia	*P.*	*dactylifera*	Female
19	Zahdi	NT, Australia	*P.*	*dactylifera*	Female
20	Thoree	NT, Australia	*P.*	*dactylifera*	Female
21	B.s	NT, Australia	*P.*	*dactylifera*	Female
22	B.f	NT, Australia	*P.*	*dactylifera*	Female
23	*P. theophrasti*	RBGS	*P.*	*theophrasti*	Female
24	*P. theophrasti*	RBGS	*P.*	*theophrasti*	Female
25	*P. canarienis*	Sydney, Australia	*P.*	*canarienis*	Female
26	*P. roebelenii*	Sydney, Australia	*P.*	*roebelenii*	Female
27	Fard	South Australia	*P.*	*dactylifera*	Male
28	Jarvis	South Australia	*P.*	*dactylifera*	Male
29	16-c	NT, Australia	*P.*	*dactylifera*	Male
30	16-D	NT, Australia	*P.*	*dactylifera*	Male
31	16-B	NT, Australia	*P.*	*dactylifera*	Male
32	15-35	NT, Australia	*P.*	*dactylifera*	Male
33	A-male	NT, Australia	*P.*	*dactylifera*	Male
34	15-42	NT, Australia	*P.*	*dactylifera*	Male
35	15-41	NT, Australia	*P.*	*dactylifera*	Male
36	6-5	NT, Australia	*P.*	*dactylifera*	Male
37	10-1	NT, Australia	*P.*	*dactylifera*	Male
38	10-46	NT, Australia	*P.*	*dactylifera*	Male
39	12-7	NT, Australia	*P.*	*dactylifera*	Male
40	12-12	NT, Australia	*P.*	*dactylifera*	Male
41	15-28	NT, Australia	*P.*	*dactylifera*	Male
42	16-23	NT, Australia	*P.*	*dactylifera*	Male
43	16-27	NT, Australia	*P.*	*dactylifera*	Male
44	17-10	NT, Australia	*P.*	*dactylifera*	Male
45	17-15	NT, Australia	*P.*	*dactylifera*	Male
46	17-35	NT, Australia	*P.*	*dactylifera*	Male
47	18-2	NT, Australia	*P.*	*dactylifera*	Male
48	18-4	NT, Australia	*P.*	*dactylifera*	Male
49	18-7	NT, Australia	*P.*	*dactylifera*	Male
50	2-3	NT, Australia	*P.*	*dactylifera*	Male
51	9-20	NT, Australia	*P.*	*dactylifera*	Male
52	2-5	NT, Australia	*P.*	*dactylifera*	Male
53	17-4	NT, Australia	*P.*	*dactylifera*	Male
54	BG 3	RBGS	*P.*	*dactylifera*	Male
55	Ostaomran	Iraq	*P.*	*dactylifera*	Female
56	Barhi	Iraq	*P.*	*dactylifera*	Female
57	Ashrasi	Iraq	*P.*	*dactylifera*	Female
58	Barben	Iraq	*P.*	*dactylifera*	Female
59	Breem	Iraq	*P.*	*dactylifera*	Female
60	Tabrzal	Iraq	*P.*	*dactylifera*	Female
61	Kstawi	Iraq	*P.*	*dactylifera*	Female
62	Khadrawi	Iraq	*P.*	*dactylifera*	Female
63	Zahdi	Iraq	*P.*	*dactylifera*	Female
64	Abdooly	Iraq	*P.*	*dactylifera*	Female
65	Shwithi	Iraq	*P.*	*dactylifera*	Female
66	Maktoom	Iraq	*P.*	*dactylifera*	Female

RBGS: Royal Botanical Garden, Sydney. NT: Northern Territory. Accessions 55 to 66 are collected from Diyala district in Iraq.

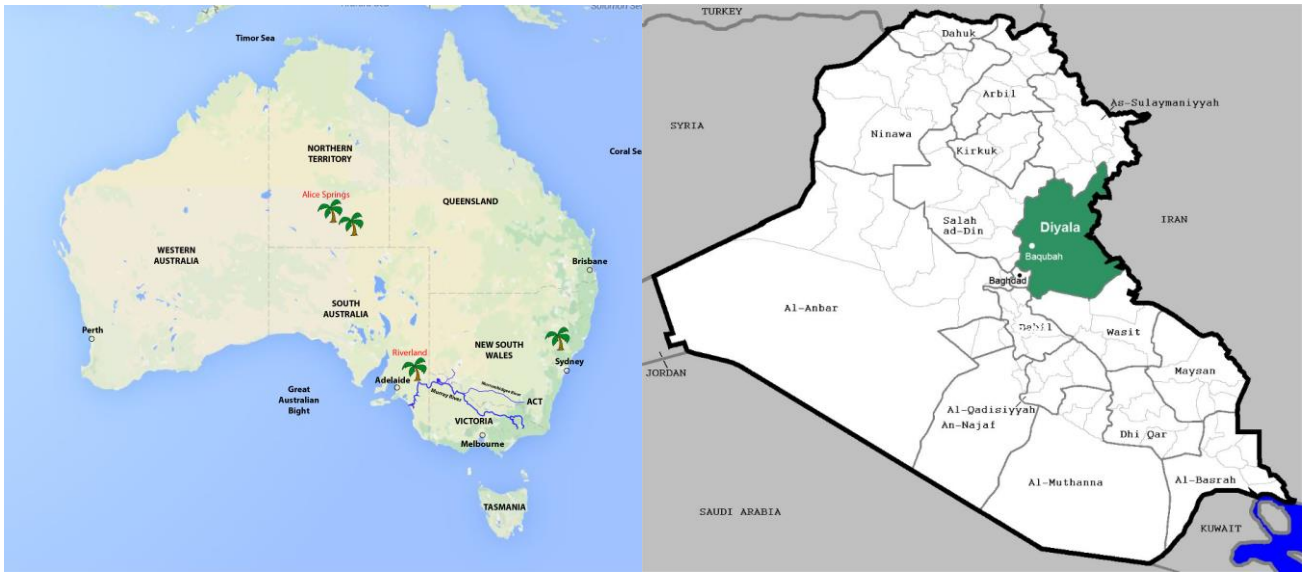

Fig 1. Map of Australia and Iraq showing the collection sites of date palm accessions. Collection sites on the Australian map are represented by date tree symbols.

except for BG3 were clustered in the first group and distributed to the right of the plane. The second group, including five accessions of the related species, was also clustered as a small group at the far right axis. A third group clustered in the lower centre of the left plane and included all the Australian male accessions except Fard, Jarvis, 16-C, 16-D and 15-35. All 12 Iraqi female cultivars were separated into a fourth group on the top left plane.

Dendrogram generated from the five iPBS markers

The dendrogram produced using the un-weighted pair group method with arithmetic mean (UPGMA), placed the 66 accessions into six major clusters (Figure 4) which supports the results of the principal component analysis. The first cluster was comprised of three related species accessions - *P. roebelenii*, *P. canariensis*, and *P. reclinata*. The second cluster contained two Australian female cultivars (Hallawi, Baskary) and two Australian male accessions (16-D, 15-35). The third cluster included two Iraqi female cultivars (Shwithi, Maktoom). The fourth cluster comprised 10 Iraqi female cultivars and two Australian male accessions (17-4, BG3 male). The fifth cluster included 20 Australian male accessions. The sixth cluster comprised two accessions of *P. theophrasti*, 19 Australian female cultivars and four Australian male accessions (Fard, Jarvis, 16-C and 16-B). Overall, the generated dendrogram has six main clusters but it can be noted that some clusters contain a number of sub-groups.

Discussion

Investigation of the genetic diversity and relationships in *Phoenix* germplasm is important for genetic improvement, conservation, management and utilization of date palms. Accurate identification of accessions in a germplasm collection is an important first step in crop improvement. Molecular marker systems such as the iPBS can more accurately differentiate germpalsm than other methods based on morphological traits or isoenzymes because they are objective, reproducible and independent of environmental influences. iPBS markers have recently been used to differentiate grapes (*Vitis vinifera* L) (Guo et al., 2014) and guava (*Psidium guajava*) (Mehmood et al., 2015) germplasm. In our study, polymorphic iPBS markers distinguished a range of *Phoenix dactylifera* accessions including their related species. This produced valuable information on the genetic relationship amongst these accessions.

The iPBS system separated our accessions into groups according to their genetic constitution and species categorization. For example, the iPBS marker system distinguished three wild species (*P. canariensis*, *P. reclinata* and *P. roebelenii*) from the *P. dactylifera* and *P. theophrastii* accessions we examined. Within the species *P. dactylifera*, the iPBS system separated the cultivated date palm accessions into clusters according to locality and sexuality. The level of information generated suggests that accessions collected from the same geographical region or breeding program tended to group together, indicating that despite the widespread distribution of date palm in the tropical world and more than 120 years of cultivation in Australia, germplasm exchange among regions has been limited. Furthermore, results indicated that only a small number of date palm cultivars have been used in breeding programs.

The iPBS method is a good molecular marker system because it requires no previous knowledge of the genome (Odong et al., 2011), it is polymorphic and evenly distributed across the entire genome, it distinguishes between genetic differences, it is cheap, quick, easy to use, and requires minimal amounts of DNA. The main drawback of the iPBS method is the reproducibility of the PCR (Wünsch, 2009). However, the results from our study confirm that the iPBS method exhibits all the advantages listed above.

In this study, 111 iPBS bands were obtained from five primers. The average numbers of iPBS bands were far more than those reported by Gailite and Rungis (2012), Baranek et al. (2012) and Guo et al. (2014). The 12-mer primer 2380 produced the lowest number of 19 bands whereas another 12-mer primer 2374 generated the highest number of 25 bands. Hence the length of the individual primer (in this case 12-mer) was not relevant to the number of bands amplified in this study. This finding contrasts with the results of Baranek et al. (2012) and Guo et al. (2014).

When studying date palm germplasm grown in the Northern region of Sudan using microsatellite markers, Elsafi (2012) reported a Shannon's Information Index of 1.78, expected heterozygosity of 0.8 and unbiased expected heterozygosity of 0.82. Our iPBS gave much lower average

Table 2. Five iPBS primers used in the detection of polymorphism among 66 date palm (*Phoenix dactylifera*) accessions.

iPBS primer	Sequence (5'-3')	Ta°C	Number of bands*	Mean PIC value[1]
2074	GCTCTGATACCA	31°C	23	0.2445
2271	GGCTCGGATGCCA	55°C	20	0.3198
2276	ACCTCTGATACCA	34°C	24	0.3289
2374	CCCAGCAAACCA	45°C	25	0.3013
2380	CAACCTGATCCA	36°C	19	0.2135

* Total accountable bands consistently appearing in two or three repeated experiments [1]PIC value is calculated as PIC = 1-[f^2+ $(1-f)^2$], where f is the frequency of the marker in the data set

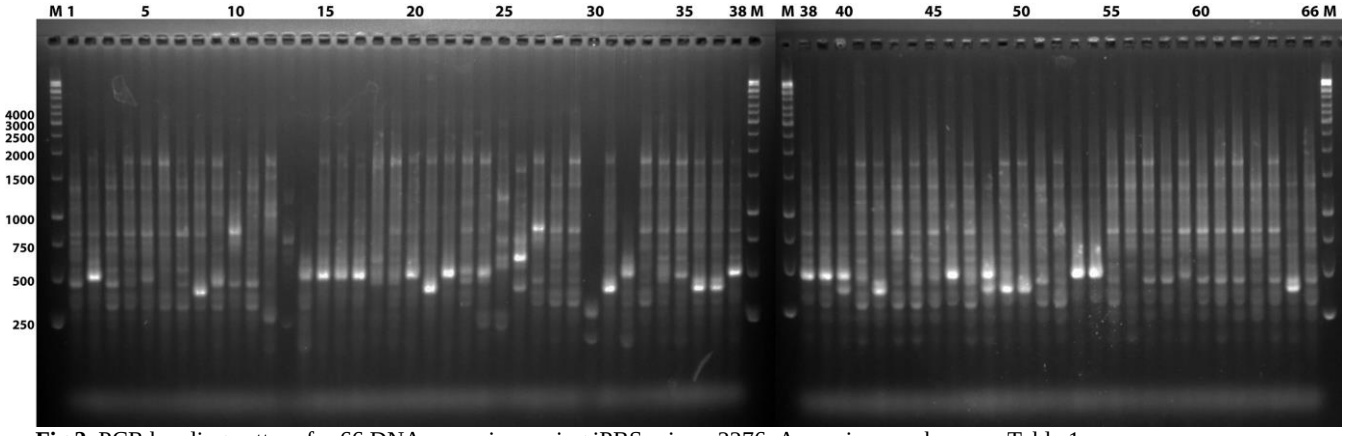

Fig 2. PCR banding pattern for 66 DNA accessions using iPBS primer 2276. Accession numbers see Table 1.

Table 3. Summary statistics for 66 date palm (*Phoenix dactylifera*) accessions assessed with 5 iPBS primers (Kalendar et al. 2010)

Groups	N	Na	Ne	I	He	uHe	PPL
AF	21.0	1.378	1.383	0.336	0.224	0.229	67.57%
AM	28.0	1.505	1.388	0.350	0.230	0.235	74.77%
IR	12.0	1.234	1.290	0.261	0.172	0.180	54.05%
SP	5.0	1.568	1.403	0.374	0.245	0.272	74.77%
Mean	16.5	1.421	1.366	0.330	0.218	0.229	67.79%

N, number of sample size; Na, number of different alleles; Ne, number of effective alleles; I, Shannon's information index; He, expected heterozygosity; uHe, unbiased expected heterozygosity; PPL, percentage of polymorphic loci.
AF: Australian female collections; AM: Australian male collections; IR: Iraqi female cultivars; SP: Other species.

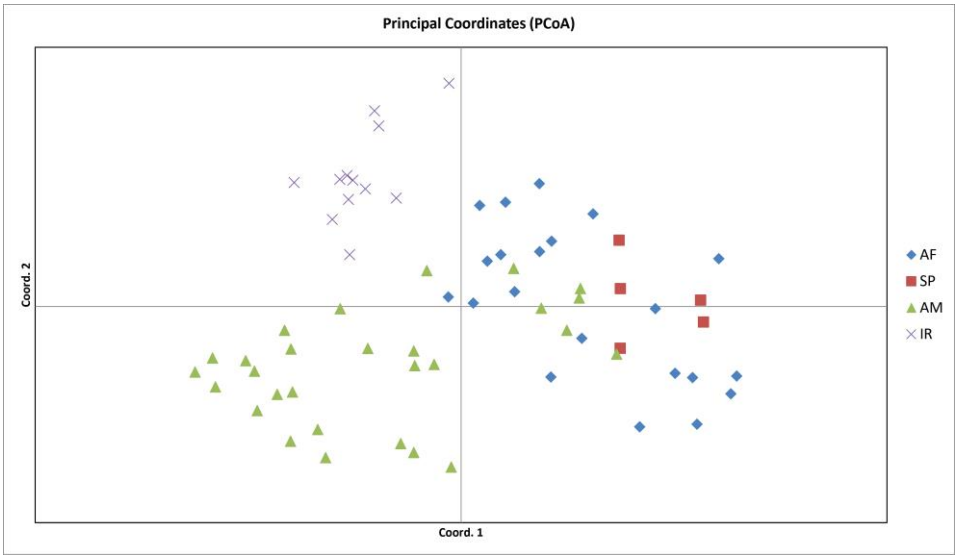

Fig 3. Principle coordinate analysis of 66 date palm accessions with five iPBS primers.
AF: Australian female collections; AM: Australian male collections; IR: Iraqi female cultivars; SP: Other species.

Table 4. Pairwise population matrix of Nei genetic identity.

	AF	SP	AM	IR
AF	1.000			
SP	0.924	1.000		
AM	0.952	0.884	1.000	
IR	0.887	0.821	0.918	1.000

AF: Australian female collections; AM: Australian male collections; IR: Iraqi female cultivars; SP: Other species

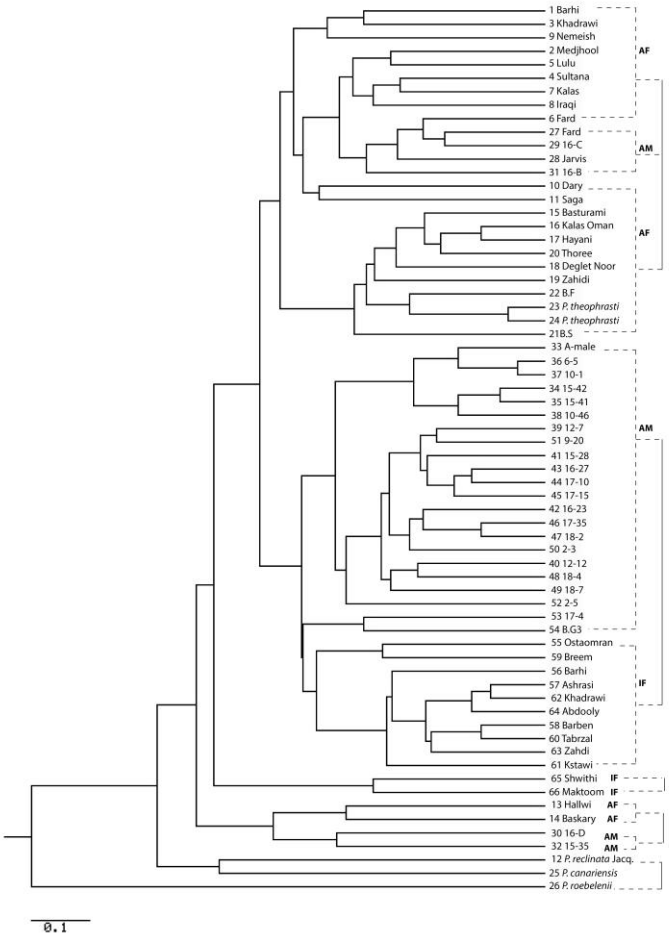

Fig 4. Dendrogram of 66 date palm accessions generated with data from five iPBS primers.
AF: Australian female collections; AM: Australian male collections; IR: Iraqi female cultivars; SP: Other species

values for Shannon's Information Index (0.33), expected heterozygosity (0.218) and unbiased expected heterozygosity (0.229). These lower values suggest that cross-incompatibility may hinder the creation of agriculturally useful hybrids from parental clones as suggested by (Elshibli and Korpelainen (2009). Consequently, controlled hybridization between cross-compatible parents may be required for the development of new date palm cultivars. Since we evaluated only a small amount of iPBS data, there is a possibility that the low observed heterozygosity and Shannon's Information Index could be due to sampling bias of the iPBS markers used. Tests with additional iPBS markers on more diverse date palm germplasm could clarify this situation.

Whilst the cultivars Barhi and Khadrawi originated in Iraq, the Barhi (1) and Khadrawi (3) cultivars collected in Australia clustered in a different group to those collected in Iraq. This observation suggests that a range of genetic variation within each of the Iraqi cultivars exists. Our finding is similar to that of Elhoumaizi et al. (2006) who showed that the Moroccan cultivar Medjool, collected from different regions in that country, had a significant range of genetic variation. These observations suggest that targeted intra-varietal selection within major date cultivars such as Medjool, Barhi and Deglet Noor could be an important crop improvement strategy.

In Iraq, date palm breeding is highly dependent on seed propagation with subsequent selection based on specific characteristics such as fruit quality and plant vigor as determined by local farmer preferences (Khierallah et al., 2011). Results indicated that cultivars collected from the Diyala area of Iraq tended to have a close genetic basis. Consequently, it would be advantageous for Iraqi farmers to access cultivars not only from other countries, but also from other Iraqi districts to overcome the limitations of inbreeding arising from current practices.

The situation is different in Australia. Since recent Australian quarantine laws prohibit the introduction of overseas date palm accessions, the genetic variability of Australian dates cannot be enhanced through introduction. However, our study reveals that there is significant genetic diversity in the established Australian date germplasm. This

infers that intra-crossing between genetically distant Australian cultivars could enhance hybrid vigour that could be fixed using asexual propagation. Specifically, among the Australian date palm accessions, two male (16-D, 15-35) and two female (Hallawi, Baskary) accessions were genetically distant from the rest of the collections. To maximize the metaxenia effect of crossing, one of these cultivars should be used as a parent to widen the parental genetic distance thus increasing the probability of identifying hybrids with increased quality and productivity. Species classification within the *Phoenix* genus is complicated by compatible interspecific hybridization occurring in nature (Krueger, 1995). Barrow (1998) questioned the separate species status of *P. theophrasti* and *P. dactylifera* based on the diversity of some intergenic spacer regions of the 5S DNA units which formed a low resolution cluster of *P. dactylifera*, *P. theophrastii* and *P. sylvestris*. Additionally, using SSR and ITS (internal transcribed spacer) marker systems, Rivera et al. (2008) differentiated date cultivars with multi interspecific characteristics derived from *P. theophrastii, P. dactylifera* and *P. sylvestris*. The iPBS results from this study confirmed both the Barrow (1998) and Rivera et al. (2008) finding that *P. dactylifera* and *P. theophrastii* are not distinctly separate species. Therefore, more morphological and molecular experimentation on a larger number of accessions of *P. dactylifera* and *P. theophrastii* is required to confirm the actual genetic relationships of these two species. Overall, this study affirms that the iPBS marker system can be a very powerful tool to explore genetic diversity at the species level. Future research using the iPBS system could target other species within the *Phoenix* genus.

Materials and Methods

Plant materials

The sixty six accessions used for the iPBS analysis are listed in Table 1. Four were collected from the Royal Botanic Garden, Sydney. Fifty cultivars including 23 females and 27 males were collected from the Desert Fruit Company (a date palm orchard in Alice Springs, Northern Territory, Australia). This orchard preserves the major date palm varieties (and their derivatives) introduced into Australia over the last 120 years from the Middle East, North Africa and the USA. Another 12 female cultivars were collected from the Diyala district of Iraq (Fig. 1).

DNA extraction and quantification

Young fresh leaves were collected from the listed accessions and were used for the DNA isolation. DNA was extracted from 200 mg of fresh leaves using the plant DNA isolation Mini Kit (Bioline, Australia) in accordance with the manufacturer's protocols. The quality and quantity of the DNA was checked using 2.0% agarose gel electrophoresis in comparison with known λ DNA concentrations. Portions of the isolated DNA were diluted in molecular grade water to 10 ng/µl concentration and used as templates for subsequent PCR.

iPBS PCR amplification

The iPBS primers listed by (Kalendar et al., 2010) used in this study were from Sigma Aldrich (Castle Hill, NSW, Australia). The DNA amplification was conducted using a slightly modified protocol from Kalendar et al. (2010). The PCR were performed in 20 µl reaction mixtures containing 10 ng genomic DNA, 1 time GoTaq buffer (Promega), 0.5 µM of primer (single primer), 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (GoTaq, Promega) and 2.0 mM $MgCl_2$. The PCR program had an initial hot start at 95°C for 3 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 31-58°C for 30 s and with an extension at 72°C for 2 min. There was a final extension at 72°C for 5 min and the program was terminated by holding at 10°C. The reaction was performed in a Bio-Rad T100™ Thermal Cycler using 0.2 ml tubes or 96-well plates. A 10 µl sample of each PCR product was electrophoresed at 70 V for 3.5 hours in a 1.5% (w/v) thin agarose gel with 1× TAE buffer (0.04 M Tris-acetate, 0.001M EDTA). A Thermo Scientific Gene-Ruler 1 kb ladder from Fermentas (Australia) was used to estimate fragment lengths. Gels were post-stained with GelRed (Biotium) for 15-20 min and photographed using the BIO-RAD Gel Doc™ XR+ with Imaging Lab™ Software.

Data scoring and analysis

For each primer, the PCR was performed three times to confirm band pattern consistency. DNA bands were sized and scored by the Image Lab™ Software and carefully checked manually. Only clear bands were scored and faint bands were ignored. Bands with the same size were assumed to represent a single locus. For each locus, data were recorded using '1' for presence of a band and '0' for absence to build a binary matrix.

Summary statistics for each group of accessions related to allelic richness, heterozygosity, genetic diversity, number of alleles and Shannon's Information Index were computed using GenAIEx 6.5 (Peakall and Smouse, 2012). Shannon's Information Index was calculated following the method of Lewontin (1972). GenAIEx 6.5 was also used to perform principal coordinate analysis (PCoA). Dendrograms were constructed using a Dice genetic similarity coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) (Nei and Li, 1979). The matrix data were imported into Tree Drawing software from PHYLIP (Felsenstein, 2005) for the dendrogram construction.

Conclusion

The iPBS marker system established in this study is very important to reveal genetic diversity and population structures for date palm. Within the species *P. dactylifera*, the iPBS system grouped the cultivated date palm accessions into clusters according to locality and sexuality. Results from this study provided valuable information for future genetic improvement for our collected Australian date palm germplasm and the distinguishing power at inter-specific level within the genus. In the future, the use of the iPBS markers should extend in order to demonstrate the relationships between molecular markers and some other important specific morphological traits. This could increase the selection efficiency of date palm cultivars derived by sexual reproduction.

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