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REGULAR ARTICLE

Genetic diversity analysis of date palm (*Phoenix dactylifera* L.) in the Kutch region of India using RAPD and ISSR markers

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Abstract

First line in abstract should be Date palm is an important fruit crop of the arid and semiarid region. In the present investigation two types of DNA based marker RAPD and ISSR were used to characterize 8 date palm genotypes grown in the Kutch region. Amplification of genomic DNA of 8 genotypes using 13 RAPD analyses yielded 88 fragments, of which 35 were polymorphic, with an average of 2.69 polymorphic fragments per primer. Two ISSR primers produced 13 bands of which 3 were polymorphic. RAPD markers were more efficient than ISSR assay with regard to polymorphic detection; RAPD markers detected 39.77% as compared to 23.07% of ISSR markers. Cluster analysis by UPGMA showed that the dendrogram obtained by RAPD and RAPD + ISSR were similar. Cluster A consisted of Early maturing, Ghanshyam and Late maturing female genotypes with 0.81 to 0.88 Jaccard's similarity range. Cluster B consisted of Seasonal female, Male-1, Male-2, Male-3 and Male-4 genotypes with 0.82 to 0.91 similarity range. Genotypes Male-1 and Male-2 were most closely related with the highest value in similarity for Jaccard's coefficient (0.91). Principal coordinate analysis differentiated one group of genotype Male-1 and Male-4 while other genotypes were randomly distributed.

Key words: Date palm, Diversity, Molecular markers, RAPD, ISSR

Introduction

The date palm (*Phoenix dactylifera* L.), 2n=36, is a dioecious long-lived monocotyledonous plant, which belongs to the family Arecaceae. It is one of the excellent candidate crops in arid and semiarid regions of the world with high tolerance to environmental stresses. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops. The annual world production of dates has reached 6-8 million mt (metric tons), representing a market exchange value of over 1 billion USD. The fruit is nutritionally rich; several products are made that generate employment and thus benefit the socioeconomic status of local people (El Hadrami and Al-Khayri, 2012). Moreover, this crop has great potential as a source of renewable energy due to the high carbohydrate content, 44-88%, of date palm fruit (Jain, 2012).

Date palm is one of the important horticultural crops of the Kutch region which enjoys the monopoly of commercial cultivation of this crop in India. The total area under cultivation has increased to 16,000 ha during 2010-2011, from 8,973 ha during 2000-2001, with production of 1.2 lakh tons. (Shandilya, 2012). The Kutch is the largest producer of date palm in India having the most suitable climate for date palm plantations. But available germplasm in the Kutch region lacks information about its genetic base. Due to the crosspollination mode of reproduction and seeds thrown by army camps and partly from the seeds and offshoots planted by the settlers, there is tremendous variation in the genotypes grown in Kutch. Hence, there is an urgent need to document these genotypes and identify characteristics of the best genotypes growing under Kutch conditions in order to expand/replace cultivars for higher and better-quality yield.

Morphological characterization of cultivars requires a large set of phenotypic data that are normally difficult to collect, and statistically variable due to environmental effects (Sedraet al., 1998). Biochemical markers (isozymes and

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proteins) have proven to be effective in cultivar identification as well (Bennaceuret al., 1991). However, they provide limited information and are usually an indirect approach for detecting genomic variation.

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 (Khanam et al., 2012). Molecular markers based on RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) are powerful techniques, which can be used to identify and determine plant genomes or to estimate the phylogenetic relationship among genotypes of date palm (Cullis, 2011; Elshibli and Korpelainen, 2011). The RAPD technique has been used for cultivar genotyping (Ben-Abdallah et al., 2000; Trifi et al., 2000) and for analyses of phylogenetic relationships and genetic diversity (Al-Khalifah and Askari, 2003; Al-Moshileh et al., 2004; El-Tarras et al., 2007; Khalifah et al., 2012). Estimating the genetic distance assists in studying genetic diversity, a trait that is important for parent selection associated with genetic mapping and for marker-assisted selection in breeding programs (Lapitan et al., 2007; Trethowan and Kazi, 2008). The ISSR targets multiple genomic loci, and usually yields dominant markers (Zehdi et al., 2004; Khanam et al., 2012). Therefore, the present investigation was conducted to define the genetic diversity among female and male genotypes grown in the Kutch region using RAPD and ISSR markers.

Materials and methods

Date palm genotypes

Fresh juvenile leaf samples of date palm germplasm were collected from the Kutch district of Gujarat. This included 8 date palm genotypes comprising 4 females, cvs.: Early maturing, Ghanshyam, Late maturing and Seasonal (also known as Timely maturing) and 4 males plants of different characters designated as Male-1, Male-2, Male-3 and Male-4. Morphological characters of these genotypes are given in Table 1 and their growth locations are shown in Figure 1.

Table 1. Morphological characters of 8 date palm genotypes under study.

Sr. No.	Female trees	Fruit color	Fruit size	Resistance to rain water	Fruit Taste	Plant age (yr)
1	Early maturing	Red	Large	Resistance	Sweet	16
2	Ghanshyam	Red	Medium	Resistance	Much sweet	8
3	Late maturing	Yellow	Large	Low resistance	Medium sweet	17
4	Seasonal	Brown red	Large	Susceptible	Medium sweet	12
Sr. No.	Male trees	Location	Quality	Source	Plant age (yrs)	
5	Male-1	Bhuj (Kutch)	Medium	Offshoot	4 years	
6	Male-2	Bhuj (Kutch)	Medium	Main plant	15 years	
7	Male-3	Mandvi (Kutch)	Medium	Offshoot	15 years	
8	Male-4	Bhuj (Kutch)	Good	Offshoot	4 years	

Figure 1. Location of Kutch region in India. All genotypes were collected from Bhuj except male-3 which was collected from Mandvi.

DNA extraction

Total genomic DNA of 8 date palm genotypes was extracted from 500 mg of young leaf samples using the CTAB (Cetyltrimethylammonium bromide) method of Doyle and Doyle (1990) with a few modifications. The leaves were first ground to a fine powder in liquid nitrogen using autoclaved and pre-chilled mortar and pestle. It was then transferred into a prewarmed extraction buffer (1.5M NaCl, 100 mM Tris-Hcl pH-8, 40 mM EDTA, 1% PVP, 3% C-Tab and 1% β-mercaptoethanol) and incubated at 65°C for 1 hr. Equal volume of chloroform : isoamyl alcohol (24:1 v/v) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. After centrifugation, the pellet was washed in 70% ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified using UV-spectrophotometer and diluted to 50-60 ng/µl and used in PCR.

PCR conditions and electrophoresis

Polymerase chain reaction (PCR) for both RAPD and ISSR analysis was performed in 20µl volume containing 1x PCR buffer (Banglore Genei), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.1µM of primer, 50 ng of genomic DNA and 1 U Taq DNA polymerase(Banglore Genei). The reaction mixture was placed on a DNA thermal cycler (Biometra). RAPD program was performed as 1 cycle of 94°C for 5 min and 40 cycles of 94°C for 1 min, 38°C for 1 min and 72°C for 2 min, then, a final extension step 72°C for 8 min. The ISSR program was performed as 1 cycle of 94°C for 5 min and 35 cycles of 94°C for 1 min, 45°C for 45 sec, 72° C for 1.5 min and a final extension step of 72°C for 8 min. PCR products were electrophoreses on 1.5% (w/v) agarose gels, in 0.5X TBE Buffer at 80 V for 1 hr and then stained with ethidium bromide (0.5 µg/ml). Gels with amplified fragments were visualized and photographed under UV light.

Scoring and data analysis

The DNA bands were scored for computer analysis on the basis of presence or absence. If a product was present in a genotype it was designated "1", if absent it was designated "0" after excluding irreproducible bands. Pair-wise comparisons of genotypes, based on presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based on similarity matching. Using a distance matrix, a principal coordinate analysis to construct a three-dimensional array of eigenvectors was performed using a DCENTER module of NTSYS 2.2 program.

Results and discussion

The use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetic studies. In this work, the utility of RAPD and ISSR markers for variation and identification of date palm germplasm was studied.

RAPD banding patterns and dendrogram analysis of germplasm

Eight date palm genotypes obtained from the Kutch region were amplified using 13 RAPD oligonucleotide (Table 2). Amplification products of 8 genotypes with these 13 RAPD primers yielded a total of 88 scorable bands, out of which 35 (39.77%) were found to be polymorphic. The size of the amplification product ranged from 200 to 4000bp. The highest number of bands (12) were obtained with primer OPE-5, while the lowest number of bands (3) were obtained with primer OPE-9. The percentage of polymorphism ranged from 16.66 to 80%. Primer OPE-10, revealed the highest polymorphism (80%) while primer OPJ-1 and OPL-3 exhibited the lowest polymorphism (16.66%). Total number of bands amplified ranged from 16 (OPE-9) to 63 (OPM-7). Similarly, Sedra et al. (1998) observed 66.07% polymorphism among 43 date palm accessions using 19 RAPD primers. The polymorphism was much higher than our study which might be due to a higher number of date palm accessions.

The primer OPE-17 showed 5 monomorphic bands among which 4 bands were in the range of 1.1 to 1.5kb and 1 band of 600bp (Figure 2). A band of~1kb was present only in late maturing and seasonal female genotype, while absent in other genotypes. A band of ~300bp was present in Seasonal, Male-2 and Male-4 while absent in the other 5 genotypes. The primer OPE-3 has amplified a total number of 8 scorable bands in the ranges from 300 to 1000bp. Genotypes from lane 1-4 (Female) and lane 5-8 (male) exhibited first 6 monomorphic bands. One band of ~475 bp was present in Late maturing, Ghanshyam and Early maturing female genotypes which are rain water resistance while absent in the other 5 genotypes. Bands of ~300bp was present in 4 female genotypes (lane 1-4) and absent in 4 male genotypes (lane 5-8) which may be for female specific. Similarly, 3 female specific markers were

identified in date palm by Younis et al. (2008). Male specific alleles were identified by Cherif et al. (2012) in 52 male genotypes of date palm using microsatellite markers. They found three genetically linked loci which were heterozygous only in males.

Table 2. Details of amplification obtained with different RAPD primers.

Sr. No.	Name of primer	Sequence (5'—3')	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1	OPE-3	CCAGATGCAC	8	2	6	25	55
2	OPE-5	TCAGGGAGGT	12	6	6	50	61
3	OPE-9	CTTCACCCGA	3	2	1	66.66	16
4	OPE-10	CACCAGGTGA	5	4	1	80	24
5	OPE-17	CTACTGCCGT	8	3	5	37.5	53
6	OPE-18	GGACTGCAGA	7	3	4	42.85	45
7	OPJ-1	CCCGGCATAA	6	1	5	16.66	45
8	OPJ-5	CTCCATGGGG	8	2	6	25	59
9	OPJ-18	TGGTCGCAGA	5	1	4	20	35
10	OPK-8	GAACACTGGG	5	2	3	40	35
11	OPL-3	CCAGCAGCTT	6	1	5	16.66	42
12	OPM-7	CCGTGACTCA	10	5	5	50	63
13	D07	TTGGCACGGG	5	3	2	60	26
Total			88	35	53	39.77	559

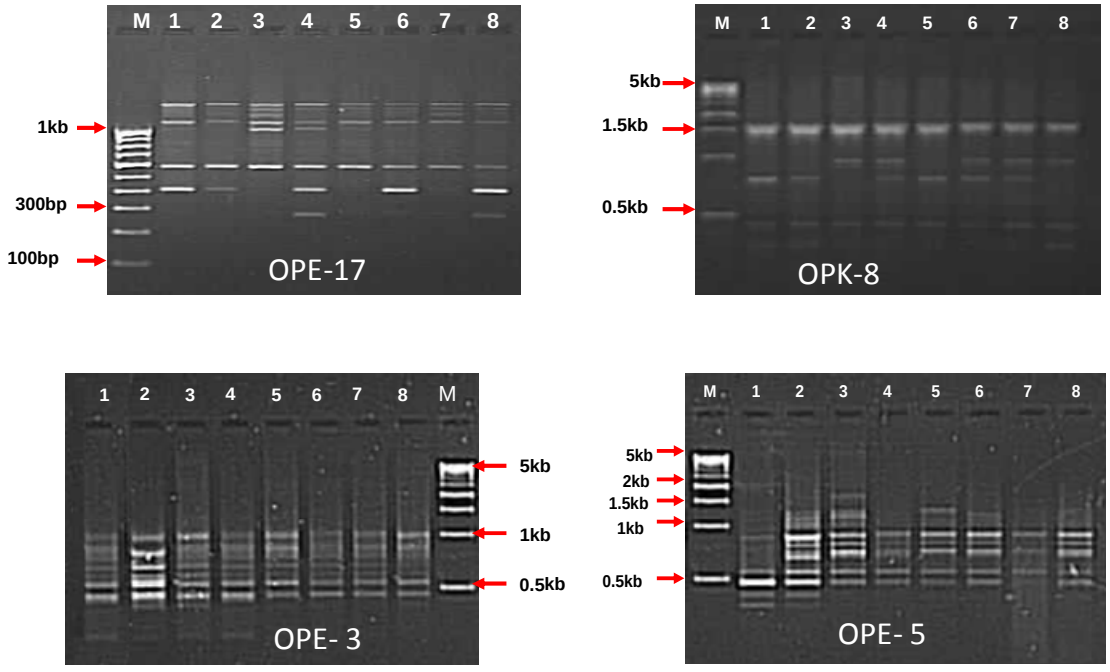

Figure 2. RAPD amplification pattern of 8 date palm genotypes obtained with 4 random primers. M is the DNA marker, lanes 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and lanes 5-8 are the males (M1, M2, M3 and M4).

The primer OPK-8 generated 3 monomorphic bands of size ~350, 400 and 1500bp while 1 band of 1kb was absent in Early maturing, Ghanshyam and Male-1, and present in the other genotypes. A band size of ~800bp was present in Late maturing and Male-4, while absent in the other 6 genotypes. In the case of primer OPE-5 it amplified a total number of 12 scorable bands. All the 8 genotypes reveal 6 monomorphic bands of which 4 bands ranged from ~750bp to 1000bp and the other 2 ranged from ~480 to 510bp. A band of ~700bp and 1.5kb was unique to Ghanshyam (very sweet fruit) and Late maturing (yellow color fruit) respectively. A band size of ~300 and 400bp was unique to Early maturing and Ghanshyam but absent in the other 6 genotypes and may be for red color or resistance to rain water.

A dendrogram (Figure 3) based on UPGMA analysis grouped the 8 genotypes into 2 main clusters, with Jaccard's similarity coefficient ranging from 0.78 to 0.93. Similarly, Al-Moshileh et al. (2004) observed similarity coefficient ranges from 0.70 to 0.84 in 6 cultivars of date palm using RAPD profiles. This higher similarity reflects less diversity of germplasm due to collections coming from the same geographic region. The dendrogram obtained 2 main clusters (A and B) with 3 and 5 genotypes respectively. The cluster A has 2 subclusters (A1 and A2). Subcluster A1 has Early maturing and Ghanshyam with red fruits and high resistance to rain water and subclusters A2 contains Late maturing with yellow fruit and medium resistance to rain water. Cluster B bifurcates into 2 subclusters. Subcluster B1 with 1 female genotype Seasonal (not resistance to rain water) while subcluster B2 contained 4 male genotypes (Male-1, Male-2, Male-3 and Male-4) in which Male-1 and Male-2 were the most similar genotypes and Male-4 was a good quality male genotype with most variable characteristics in subcluster B2. These results suggest that RAPD may differentiate genotype based on morphological characters and also gave some unique markers in some genotypes.

ISSR banding patterns and dendrogram analysis of germplasm

Eighteen ISSR oligonucleotides were used for amplification of all the 8 genotypes out of which only 2 primers gave reproducible amplification products (Table 3), while 16 primers did not give any polymorphism. Amplification products of 8 genotypes with these 2 ISSR primers yielded a total of 13 scorable bands, out of which 3 were found to be polymorphic (Figure 4). The size of the amplification product ranged from 300 to 4000 bp. Primer HB-9 showed the highest percentage value of polymorphism of 40%, compared to HB-10 with 12.5%. Similarly, Hussein et al. (2005) reported polymorphism at a low level (28.6%) using 10 ISSR primers among 14 date palm accessions. The present result of ISSR showed very low polymorphism (23%). This does not mean that ISSR is inefficient to detect polymorphism. There is a need to screen a larger number of primers to evaluate the present set of genotypes.

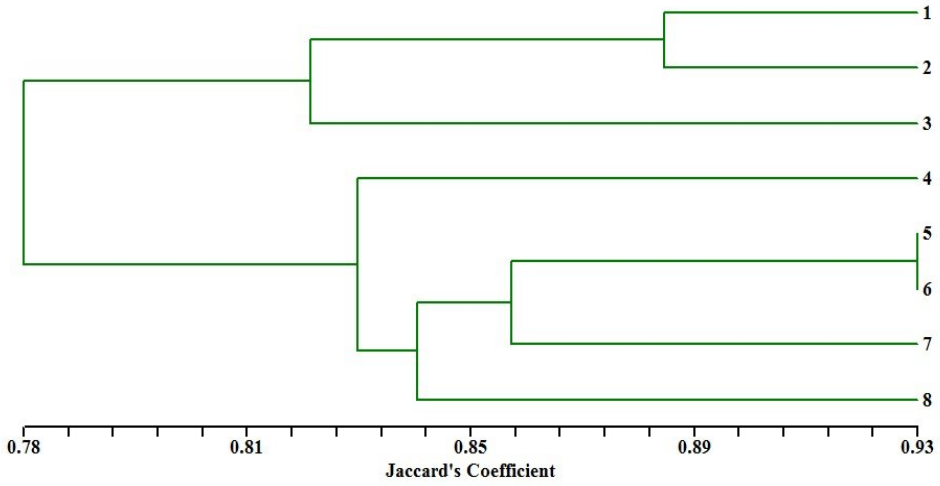

Figure 3. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on RAPD data.

Table 3. Details of amplification obtained with different ISSR primers.

Sr. No.	Name of primer	Sequence (5'-3')	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percent (P %)	Total No. of bands amplified
1	HB-9	$(GT)_6GG$	5	2	3	40	29
2	HB-10	$(GA)_6CC$	8	1	7	12.5	61
Total			13	3	10	23.07	90

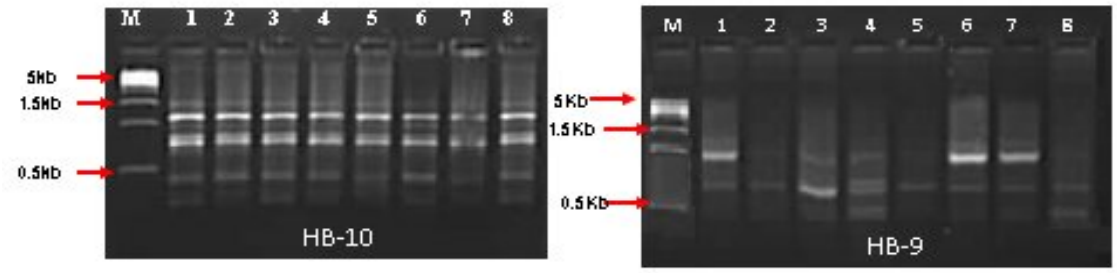

Figure 4. ISSR amplification pattern of 8 date palm genotypes obtained with two random primers. M is the DNA marker, lanes 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and lanes 5-8 are the males (M1, M2, M3 and M4).

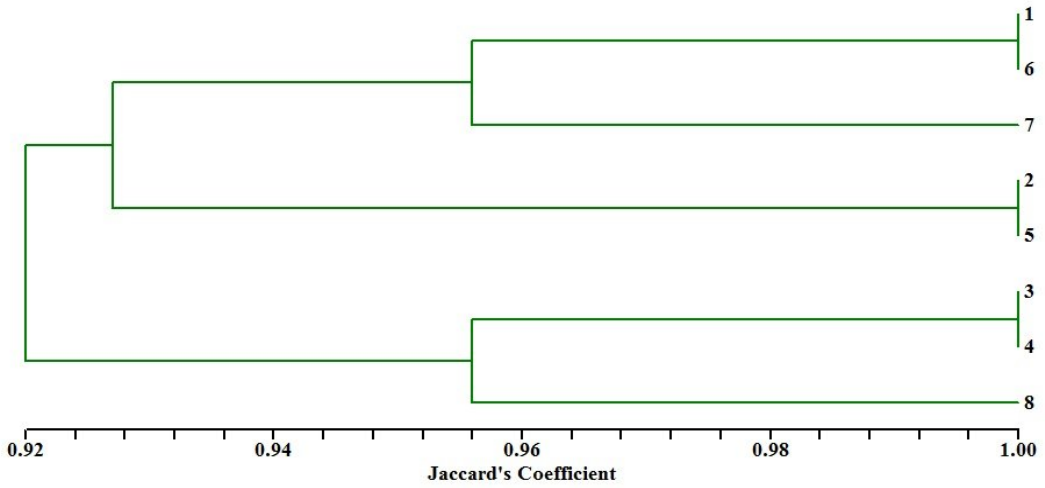

Figure 5. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on ISSR data.

The primer HB-9 showed a total of 5 scorable bands, out of which 2 were polymorphic. A band size of ~400, 650 and 900bp showed 3 monomorphic bands, while a band size of ~670bp was present in only Late maturing and Seasonal which have large fruit size and Medium sweet, while absent in other 6 genotypes. A total of 7 bands were amplified by primer HB-10, out of which 6 were monomorphic ranging in size from ~400 bp to 1.5 kb and 1 polymorphic band which was absent in Ghanshyam, Male-3 and Male-4, while present in other genotypes.

A dendrogram (Figure 5) based on UPGMA analysis grouped the 8 genotypes into 2 main clusters, with Jaccard's similarity coefficient ranging from 0.92 to 1.00. The dendrogram showed 2 main clusters (A and B) with 5 and 3 genotypes respectively. Cluster A has 2 subclusters (A1 and A2). Subcluster A1 has Early maturing, Male-2 and Male-3 genotypes in which Early maturing and Male-2 with nearly similar ages (15 and 16 years), while Male-3 genotype was from a different location. Cluster B was further divided into 2 subclusters. Subcluster B1 consisted of Late

maturing and Seasonal (female) which were most similar, while subcluster B2 including Male-4 (a good male genotype) were different from Late maturing and Seasonal genotypes.

Combined analysis using RAPD and ISSR data

The combined analysis of RAPD and ISSR with similarity coefficient ranging from 0.77 to 0.91, showed polymorphism of 37.62% using 15 selected primers among 8 date palm genotypes. These results are consistent with Hussein et al. (2005) working with 14 date palm accessions, collected from different locations in Egypt, using a combination of RAPD and ISSR markers. They found low levels of intervarietal polymorphism and higher genetic similarities ranging from 91.4% to 99.6%. These results suggest a narrow genetic background in collected germplasm. Contrary to our results, Haider et al. (2012) observed higher polymorphism in 23 date palm cultivars from Syria representing 18 female and 5 male cultivars. The average polymorphism detected by the RAPD assay (58.5%) was higher than that observed for ISSR (50.6%). These results might be due to more genotypes and primers used in the study.

The amplification data obtained from RAPD and ISSR primers were used for a similarity matrix. This combined dendrogram (Figure 6) was similar to the RAPD dendrogram. In RAPD and ISSR combined marker data, the UPGMA dendrogram obtained had almost the same sample distribution pattern. On the other hand, the dendrogram from ISSR, with a high similarity matrix value, suggests that RAPD was more efficient than ISSR in date palm.

Principal co-ordinate analysis

PCA was performed to visualize the dispersion of genotypes in relation to the first 3 principle axes of variation (Figure 7). The genotype Male-1 (5) and Male-4 (8) genotypes were grouped together as good males while other genotypes were randomly distributed. Genotype no. 4 (female Seasonal), rain water sensitive, was in the central position. Furthermore, females no. 1, 2 and 3 (Early maturing, Ghanshyam and Late maturing) were on the same axis, while male samples no. 6 and 7 (Male-2 and Male-3) were on the other axis. Hamza et al. (2012) found grouping of date palm cultivars according to their fruit characteristics. PCA results showed the grouping of soft or semi-soft fruits and early or mid-season maturities. From PCA analysis, we conclude that Male-1 and Male-4, which are of same age (4 years) and the same location (Location-1) and taken from offshoots were grouped together. These results may further be confirmed with a larger number of genotypes and primers.

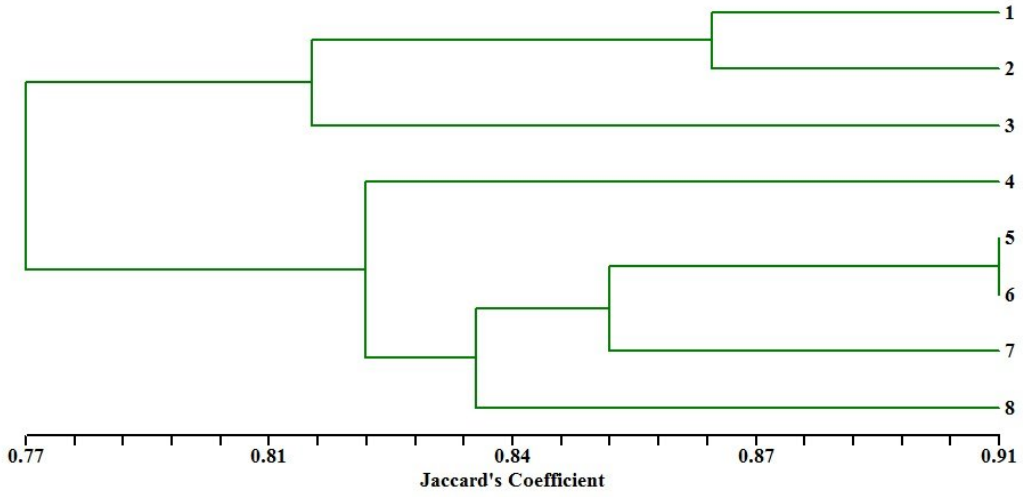

Figure 6. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on pooled RAPD and ISSR data.

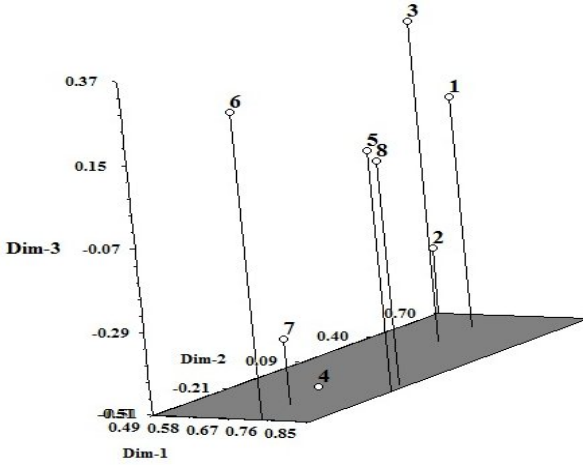

Figure 7. Principle coordinate analysis of 8 date palm genotypes, 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4).

Conclusion

The date palm is known as *kulpvriksh* in the Kutch region, due to its great potential to generate income in extreme environmental conditions and unproductive lands. Characterization of germplasm is a prerequisite for crop improvement and systematic study. The current study implies that RAPD and ISSR markers are effective tools to discriminate various date palm genotypes. Development of other date palm molecular markers like microsatellite (SSR) would facilitate distinguishing various genotypes based on particular traits. Molecular markers are indispensible for modern breeding to achieve date palm genetic improvement considering the lengthy and dioecious nature of date palm.

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