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ORIGINAL ARTICLE

Genetic Diversity and Population structure of selected *Phoenix dactylifera L.* Genotypes in Iran

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ABSTRACT
Existing information on genetic diversity of plant species help to gain maximum benefit from breeding programs and underlies the improvement of crops. The success of genetic conservation is dependent on genetic variability that arises from genetic diversity. Although date palm is one of the most food crops in terms of genetic polymorphism studies, but affinities of Iranian date palm and data on the description and variability in these species is not sufficient. In this study, genetic association of 24 date-palm genotypes collected from different part of Bushehr province-Iran was determined for the first time. Analysis of simple sequence repeats markers and inter-simple sequence repeat markers was more or less similar and cluster analysis based on SSR, ISSR and their combined data clearly discriminated the genotypes into different clusters. Result of principal coordinates analysis (PCoA) was in agreement with cluster analysis with a few exceptions. DNA based markers (ISSR and SSR) could be effectively used for genetic diversity evaluation among date-palm genotypes. These results will be useful for the conservation of date-palm genotypes and also for the improvement of the date-palm breeding strategies.
Keywords: *Phoenix dactylifera, PCoA, ISSR, SSR*

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INTRODUCTION

Date palm (*Phoenix dactylifera* L.) (2n = 2x = 36), member of the Arecaceae family, is a perennial, dioecious and one of the great socio-economic importance fruit crop in North African and Middle East countries. The date palm tree is believed to have originated and domesticated since more than 5000 years ago in Mesopotamia and cultivated in arid and semi-arid regions of the world.

Five thousand date-palm cultivars have been reported throughout the world and characterization of date-palm genotypes is essential for the efficient use of these valuable resources to find new sources of genetic variation and better conservation of date-palm populations. In order to exploit this genetic variation at the genotypic level an efficient system such as molecular marker technology is required [14].

In spite of its favorable economic, there has been limited research and extension in date palm compared to other crops in North African and Middle East countries. Molecular marker technologies are a powerful analytical and reliable tool and allow plant geneticists to estimate of genetic resource diversity than phenotypic. The use of functional molecular markers and high-throughput post-PCR analysis by focusing testing resources on genotypes aid the conventional breeding to increase the probability of identifying truly superior genotypes and increase knowledge of and ability to characterize genetic diversity in the germplasm [15, 21].

Recently, high resolution melting analysis (HRM) has been identified as a developed technique and powerful method that can be used for genetic mutations and genotyping (SNP, SSR markers) [10, 11]. The objectives of this study were to assess genetic variation and determine molecular differences within and between 24 different date palms grown in Bushehr Province-Iran by examining both ISSR and SSR

markers, and to determine whether ISSR, SSR, and/or combined data sets can detect geographical differences between these populations.

MATERIALS AND METHODS

Plant materials

The object of the present study was a collection of 24 Iranian date palm genotypes (Table 1). These genotypes represent nine well-known *P. dactylifera* cultivars were collected from different locations in Bushehr Province-Iran for the experiment to facilitate the use of results in future breeding programmes.

Plant genomic DNA isolation

Leaf materials of 24 *P. dactylifera* genotypes were utilized for DNA extraction following the CTAB (hexadecyltrimethylammonium bromide) method (modified from Doyle and Doyle (1990)). DNA was extracted with 2% CTAB buffer. This was followed by protein extraction employing chloroform: isoamyl alcohol (24:1 (v/v)) and finally the isolated DNA was precipitated with isopropanol. The DNA pellet was twice washed with ice-cold 70% (v/v) ethanol, air-dried pellet and ultimately, dissolved in 50µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), pH 8.0 and treated with 1µl RNase. The quality and quantity of DNA was assessed with electrophoresis in 1% (w/v) agarose gel and spectrophotometry, respectively. DNA was diluted to a working concentration of 50 ng/µl as templates for polymerase chain reactions (PCRs).

Inter-simple sequence repeats analysis

Twenty-four ISSR primers were examined for distinguishing the polymorphism patterns, and among those 11 primers produced highly reproducible and polymorphic bands (Table 2) against chosen 24 *P. dactylifera* genotypes. ISSR fragment amplification was conducted in a 15 µl containing 7.5 µl Master Mix (2×) (contains DreamTaq DNA Polymerase, 2 × DreamTaq Green buffer, dNTPs, and 4 mM MgCl2), 0.5 µM of forward and reverse primers and 20 ng genomic DNA. PCR amplification was carried out on a BIO-RAD T100™ Thermal Cycler using Touch-down PCR program.

SSR Amplification and HRM Analysis

For SSR reactions, a total of 28 primers were screened for polymorphism using selected genotypes, and nine primers were selected for the final reactions. The reaction mixture contained 5 µl HRM PCR Master Mix (contains Hot Star Taq Plus DNA Polymerase, Eva Green Dye, optimized concentration of Q-solution, dNTPs, and MgCl2), 0.7 µl of 0.5 µM SSR primers (Table 2), 1 µl of 50 ng genomic DNA and 2.6 µl double distil water. Negative controls were included in each PCR setup to ensure non-contamination of the reagents. The reaction mixtures were carried out on a Rotor-Gene Q using Touch-down PCR protocol starting with five minutes of denaturing step at 94 °C followed by 10 cycles of 94 °C for 60 s, 53 °C for 45 s (decreasing 0.5 °C per cycle), and 72 °C for 59 s. The program was followed by the remaining protocol with 25 cycles of 94 °C for 60 s, 48 °C for 45 s, 72 °C for 60s, and a final extension at 72 °C for 10 minutes. The melting curves were created and the Rotor-Gene Screen-Clust HRM software version 1.10.1.2 was used to classify (autocalls) *P. dactylifera* genotypes. HRM curve for each individual was visually scored.

Data analysis

Amplified fragments were designated based on fragment size and HRM curve; bands were scored as diallelic manually "1" for present, "0"‟ for absent. Information capacity of the primers and polymorphism content of the genotypes were estimated by calculating the total number of bands and of polymorphic bands. Different indices were used for analysis of diversity among the *P. dactylifera* genotypes. These include levels of observed and expected heterozygosity (for SSR markers only), *Nie's* gene diversity parameters, Shannon's information index. Indices were calculated using POPGENE Version 1.32 [22]. The binary data matrix was used to obtain the similarity matrix depending on Jaccard's coefficient by NTSYS-PC software version 2.01 [19]. This similarity matrix was utilized in NTSYS-pc, version 2.2 software for constructing a dendrogram of ISSR and SSR. GenAlEX 6 [17] was used to perform principal coordinate analysis (PCoA) based on a pair-wise genetic distance matrix to show multiple dimensions of the distribution of the genotypes in a scatter-plot. Analysis of molecular variance (AMOVA) was performed to describe genetic structure and variability within and among populations using the software GenAlEX 6 [17].

RESULTS

Genetic diversity in date-palm genotypes

Genetic diversity 24 *P. dactylifera* genotypes were amplified using 11 and nine ISSR and SSR markers, respectively (Table 2; Fig 1 & 2). Genetic diversity parameters (i.e., observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's gene diversity (*He*) [16], Shannon's information index (*I*) were shown in Table 3. All parameters revealed by ISSR primers were found to be higher (2.78, 2.43, 0.92 for

Zahedi and 2.22, 1.96, 0.66, 0.37 for Shokri) than SSR primers (1.57, 1.4, 0.34 for Kabkab and 1.83, 1.43, 0.25, 0.31 for Zahedi) genotypes.

Analysis of molecular variance (AMOVA)

The Analysis of Molecular Variance of the 24 genotypes was shown a highly significant contribution of variance within population by SSR markers (96%) than ISSR markers (80%) (Table 4). No significant genetic variation was observed among population, only 4% for SSR and 20% for ISSR markers.

Genetic similarity, cluster analysis and principal coordinate analysis

Two UPGMA clusters of *P. dactylifera* were constructed based on 37 polymorphic alleles and 252 polymorphic bands amplified by nine SSR and 11 ISSR primers, respectively (Fig. 3.). The SSR dendrogram displaying the molecular relationships among the 24 *P. dactylifera* genotypes tested separates them into six main groups and several subgroups (Fig. 3a).

The cophenetic correlation, as a measure of how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data, was estimated at r = 0.8, corresponding to high correlation of respective clusters to genetic similarity data. The largest cluster (cluster B) contained 13 *P. dactylifera* genotypes. Some same *P. dactylifera* genotypes originating from the different geographic location were found to be clustered closely. Three genotypes, namely Samron (Sa), Sheikh Aali (Sha) and Zahedi (Za) showed the closest relationship.

For ISSR markers, the dendrogram generated from the UPGMA cluster analysis, classified the 24 date-palm genotypes in three main groups with seven sub groups (Fig. 3b). The first major cluster (cluster A) formed by eight date-palm genotypes including genotypes Zahedi (Za), Samron (Sa), Khanizi (Kh), Ali Mehdi (Alm), Shakar (Sh), Sheikh Aali (Sha). Using the SSR marker technology the genotypes were distinguished in contrast to the applied ISSR markers. Therefore the use of SSR markers may reflect a greater degree of genetic resolution than the ISSR markers.

Principal coordinates analysis (PCoA) has been applied to identify multidimensional relationships that describe portions of the genetic variance in a data set. Three-dimensional plots of PCoA based on SSR and ISSR markers were in general consistent with the UPGMA cluster analysis (Fig. 3 and Fig. 4). PCoA based on SSR revealed that the first three principal coordinate components accounted for 23.93, 21.10 and 16.37 % variation, respectively. These first three principal coordinates of the SSR data explained 61.4% of the total variance. For the ISSR markers, the three coordinates explained 37.84, 19.91 and 17.81% of the total variance, respectively. These first three principal coordinates explained 75.56% of the total variance (Table 5).

Table 1. Geographical location of *P. dactylifera* genotypes collected from Bushehr-Iran

Sample code	Genotype	Collection site	Longitude	Latitude
AH1-Be	Berimi	Ahram	51.27258	28.90017
AH2-Ka	Kabkab	Ahram	51.27261	28.90025
AH3-Za	Zahedi	Ahram	51.27308	28.90008
AH4-Kh	Khanizi	Ahram	51.27303	28.90008
AH5-Alm	Ali Mahdi	Ahram	51.27292	28.9
AH7-Sha	Sheikh Ali	Ahram	51.27242	28.90097
AH8-Sa	Samron	Ahram	51.47256	28.89975
AH9-Sh	Shokri	Ahram	51.27228	28.89972
BO1-Ka	Kabkab	Borazjan	51.20925	29.27108
BO2-Sha	Sheikh Ali	Borazjan	51.20775	29.27092
BO4-Za	Zahedi	Borazjan	51.20867	29.27097
BO5-Sh	Shokri	Borazjan	51.20947	29.27083
BO6-Sa	Samron	Borazjan	51.20806	29.27064
TE2-Za	Zahedi	Tange Eram	51.59189	29.09383
TE3-Ka	Kabkab	Tange Eram	51.59197	29.09394
TE4-Sh	Shokri	Tange Eram	51.59217	29.09389
TE5-Sha	Sheikh Ali	Tange Eram	51.59389	29.09656
TE6-Sa	Samron	Tange Eram	51.59272	29.09564
VA1-Ka	Kabkab	Vahdatieh	51.25397	29.47228
VA2-Hab	Haj Bagheri	Vahdatieh	51.25386	29.47222
VA3-Sh	Shokri	Vahdatieh	51.25339	29.47183
VA5-Be	Berimi	Vahdatieh	51.25378	29.47233
VA6-Za	ZAhedi	Vahdatieh	51.25381	29.47258
VA7-Sha	Sheikh Ali	Vahdatieh	51.25425	29.47203

Table 2. SSR and ISSR primers used for amplification of DNA from 24 genotypes of *P. dactylifera*

Marker	Primer code	Repeat motif	Primer sequences (5'–3')	
SSR	mPdCIR010	(GA)22	F:	ACCCCGGACGTGAGGTG
			R:	CGTCGATCTCCTCCTTTGTCTC
	mPdCIR015	(GA)15	F:	AGCTGGCTCCTCCCTTCTTA
			R:	GCTCGGTTGGACTTGTTCT
	mPdCIR025	(GA)22	F:	GCACGAGAAGGCTTATAGT
			R:	CCCCTCATTAGGATTCTAC
	mPdCIR035	(GA)15	F:	ACAAACGGCGATGGGATTAC
			R:	CCGCAGCTCACCTCTTCTAT
	mPdCIR044	(GA)19	F:	ATGCGGACTACACTATTCTAC
			R:	GGTGATTGACTTTCTTTGAG
	mPdCIR048	(GA)32	F:	CGAGACCTACCTTCAACAAA
			R:	CCACCAACCAAATCAAACAC
	mPdCIR078	(GA)13	F:	TGGATTTCCATTGTGAG
			R:	CCCGAAGAGACGCTATT
	mPdCIR085	(GA)29	F:	GAGAGAGGGTGGTGTTATT
			R:	TTCATCCAGAACCACAGTA
	mPdCIR093	(GA)16	F:	CCATTTATCATTCCCTCTCTTG
			R:	CTTGGTAGCTGCGTTTCTTG
ISSR	UBC811			GAG AGA GAG AGA GAG AC
	UBC825			ACA CAC ACA CAC ACA CT
	UBC834			AGA GAG AGA GAG AGA GYT
	UBC841			(GA)8YC
	UBC842			GAG AGA GAG AGA GAG AYG
	UBC880			GGA GAG GAGGAG AGG AGA
	UBC889			DBD(AC)7
	BP11			KKYHYHYCAGCAGCAGCAGCAG
	BP12			KKYRYRYR(AC)10K
	I1			$(GA)_9C$
	I3			$(GA)_9A$

K:G/T, R:A/G, Y:T/C

Table 3. Genetic parameters based on ISSR and SSR analysis of nine genotypes of Date-palm.

Pop	Pop	*Na*	*Ne*	*I*	*P*
SSR	**Kabkab**	2.00±0.24	1.83±0.23	0.58±0.13	77.78
	Zahehi	2.78±0.22	2.43±0.19	0.92±0.08	100.00
	Sheikh Aali	2.11±0.20	1.88±0.18	0.64±0.10	88.89
	Shokri	2.22±0.22	1.96±0.24	0.66±0.12	88.89
	Samroon	2.00±0.17	1.82±0.13	0.61±0.09	88.89
	Berimi	1.78±0.15	1.69±0.14	0.51±0.10	77.78
	Khanizi	0.78±0.15	0.78±0.15	0.00±0.00	00.00
	Ali Mahdi	0.89±0.11	0.89±0.11	0.00±0.00	00.00
	Haj Bagheri	1.11±0.11	1.11±0.11	0.08±0.08	11.11
ISSR	**Kabkab**	1.57 ± 0.49	1.40 ± 0.38	0.34 ± 0.30	57.14
	Zahehi	1.52 ± 0.50	1.37 ± 0.37	0.31 ± 0.30	52.78
	Sheikh Aali	1.47 ± 0.50	1.32 ± 0.36	0.28 ± 0.29	47.62
	Shokri	1.43 ± 0.49	1.29 ± 0.35	0.25 ± 0.29	43.25
	Samroon	1.50 ± 0.50	1.40 ± 0.40	0.31 ± 0.31	50
	Berimi	1.30 ± 0.45	1.30 ± 0.45	0.20 ± 0.31	30.16
	Khanizi	1.00± 0.00	1.00 ± 0.00	0.00 ± 0.00	0
	Ali Mahdi	1.00± 0.00	1.00 ± 0.00	0.00 ± 0.00	0
	Haj Bagheri	1.00± 0.00	1.00 ± 0.00	0.00 ± 0.00	0

Na: different number of alleles; *Ne*: effective number of alleles; *I*: Shannon's information index; *P*: percentage of polymorphic loci

Table 4. Analysis of molecular variance (AMOVA) based on ISSR and SSR markers in date-palm genotypes.

	Source	df	SS	MS	Variation percentage
SSR	Among Pops	3	41.983	13.994	4%
	Within Pops	20	230.058	11.503	96%
	Total	23	272.042	11.924	100%
	Among Pops	3	212.308	7.209	20%
	Within Pops	20	562.358	28.118	80%
	Total	23	774.667	35.327	100%

Table 5. Percentage of variation explained by the first 3 axes for SSR and ISSR

	SSR (PCoA)			ISSR (PCoA)		
Axis	1	2	3	1	2	3
%	23.93	21.10	16.37	37.84	19.91	17.81
Cum %	23.93	45.03	61.40	37.84	57.75	75.56

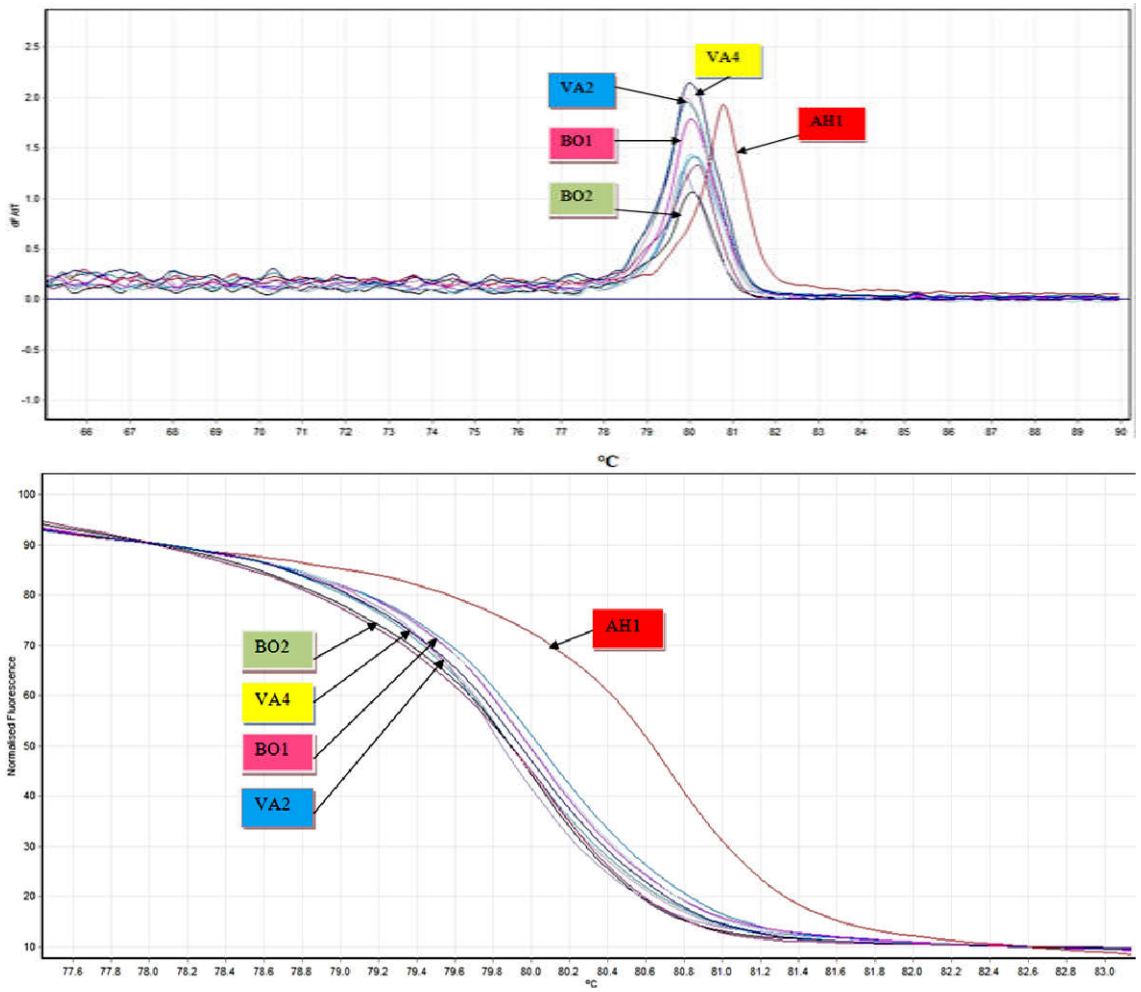

Figure 1. High resolution melting curves of different *Date-palm* genotypes in different regions of Bushehr-Iran.

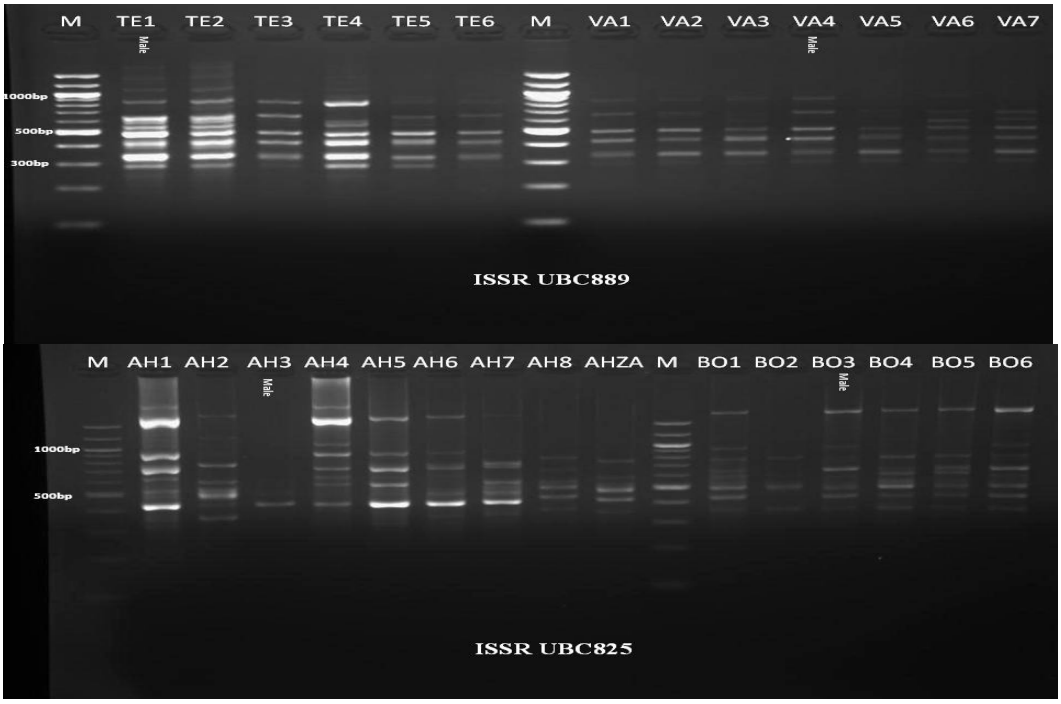

Figure 2 . Amplified products of *P. dactylifera* genotypes, using primers UBC889 and UBC825, showing polymorphism for DNA genetic profile.

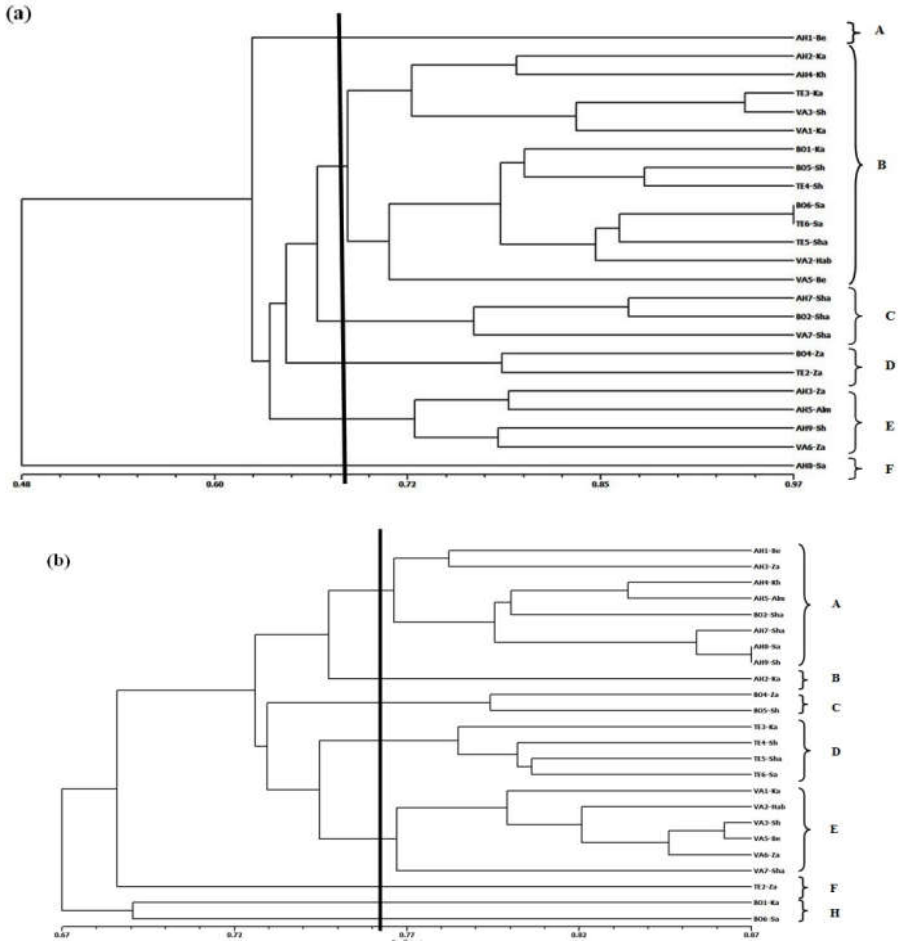

Figure 3. SSR-(a) and ISSR-(b) derived dendrograms of 24 date-palm (*P. dactylifera*) (Kabkab (ka), Zahidi (za), Berimi (Be), Samron (Sa), Khanizi (kh), Ali Mehdi (Alm), Shokri (sh), Sheikh Ali (sha), Haj Bagheri (Hab)) genotypes generated by UPGMA.

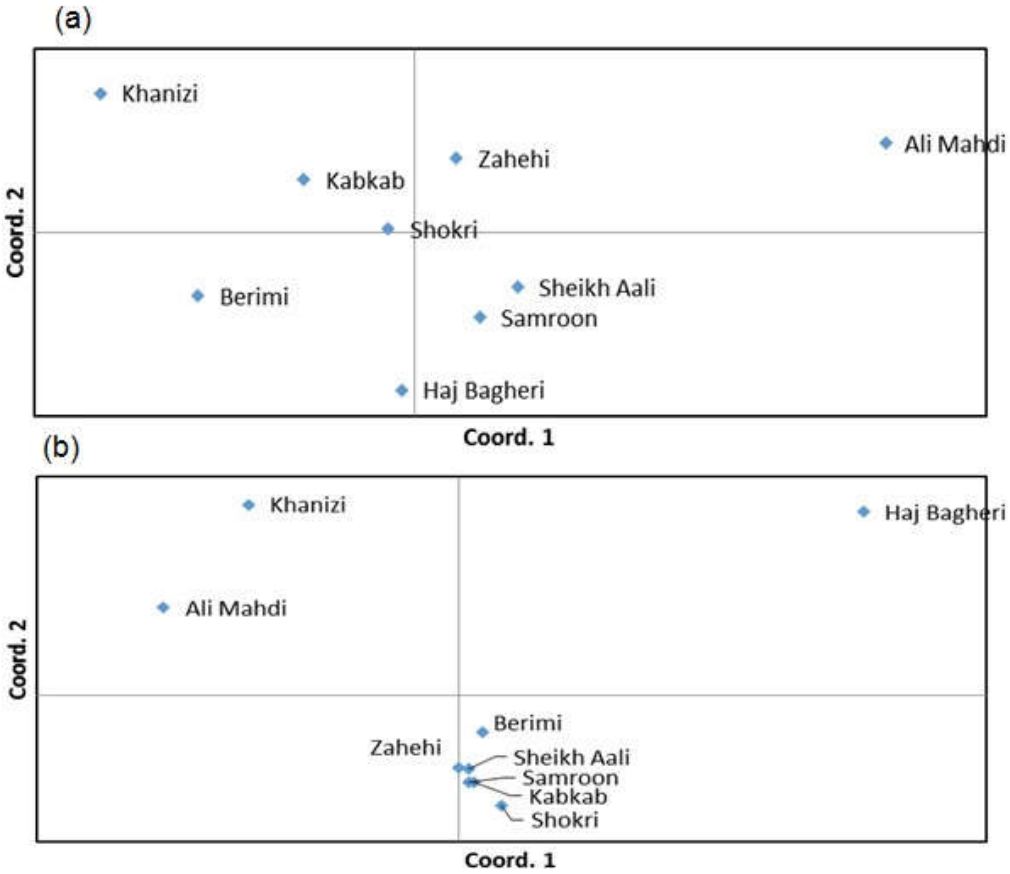

Figure 4. SSR- (a) and ISSR- (b) derived three-dimensional plot based on the first three principal coordinates from a principal coordinate analysis of 24 date-palm (*P. dactylifera*) (Kabkab, Zahidi (Ghasb), Berimi, Samron, Khanizi, Ali Mehdi, Shokri, Sheikh Ali (Kharaki), Haj Bagheri) genotypes.

DISCUSSION

Due to worldwide distribution of *P. dactylifera*, the valuation of the genetic diversity among *P. dactylifera* germplasm from different countries has been performed [20, 12, 18]. Racchi *et al.* [18] analyzed the genetic diversity in 377 female *P. dactylifera* trees belonging to 18 Libyan cultivars from central Libyan oasis of Al Jufrah by SSR markers. With these purposes, nine polymorphic SSR and 11 ISSR markers were successfully used to study the Bushehr-Iran *P. dactylifera*, using 24 samples collected on populations across the Bushehr state-Iran to provide assess of the levels of genetic diversity of date palm within Bushehr Province to help in designing conservation strategies for this species. The total number of loci detected per primer pair and counts and percentages of polymorphic SSR and ISSR data obtained in this study which illustrates the phylogenetic relationships among the samples can be considered enough for evaluating genetic diversity within populations. Differences in the number of polymorphism by ISSR and SSR markers could be explained by differences in the nature of the primers, the germplasm analysed, and in the number of fragments amplified. The average number of alleles per SSR pair detected in this study was almost equivalent to those graded by Arabnezhad *et al.* [3] who recognized 4.82 alleles per locus when examining 17 genotypes grown in different geographical regions using 22 microsatellite loci. Hamza *et al*. [12] identified 7.2 alleles per locus for SSR markers and recognized 6.1 fragments per primer for ISSR markers.

The percentage of polymorphic loci obtained in this study is consistent with the results from previous studies conducted on *P. dactylifera* cultivars using SSR/ISSR markers in Morocco, Oman, Sudan, Qatar, Tunisia, Iran, Saudi Arabia, Egypt and Syria [4, 2, 7, 1, 3, 20, 13]. The results indicated that the percentage of ISSR polymorphic bands (98.81%) was higher than that of SSR (59.26%). The mean number of amplification ISSR bands (22.4) was more than that of SSR (4.1). Moreover, the total number of polymorphic bands (17) detected by three RAPD primers was much higher than that of the three ISSR primers (6), which suggested that the RAPD markers were superior to ISSR markers in the capacity of revealing more informative bands in a single amplification.

The high polymorphism among the *P. dactylifera* cultivars generated by ISSR and SSR markers indicates that the *P. dactylifera* tree is highly diverse due to existence of large number of cultivars distributed across different origin (Enan and Ahamed, 2014). The genetic diversity in the population overall (PPB = 31.22%, H = 0.1, for ISSR markers; PPL = 59.26%, He = 0.29, and Ho = 0.07 for SSR markers) is less than genetic diversity in the Sudan date palms (He = 0.853, Ho = 0.912) [7]. The two dendrograms generated by SSR and ISSR markers show different clustering of 24 *P. dactylifera* genotypes, which can be explained by differences in the number of markers generated by the two different techniques (HRM and gel electrophoresis, respectively) [5].

There was differentiation among the *P. dactylifera* genotypes from various geographic areas. The clustering of *P. dactylifera* genotypes was not clear based on geographical regions of cultivation that has been observed in this study. Nevertheless, with the UPGMA dendrogram, some relationships in positioning of some cultivars were observed. For instance, SSR markers grouped 'Zahedi cultivars' into one cluster, also 'Kabkab' and 'Sheikh Aali' were grouped into another clusters. These results were in agreement with several studies [3, 6, 7] that found no clear correlation between *P. dactylifera* and the geographical zones. The ISSR phenogram based on Jaccard's coefficient also showed a high genetic similarity (more than 67%) among all *P. dactylifera* genotypes with no obvious clustering according to population and/or origin. There is an agreement in the diversity analyses between the PCoAs and the cluster analysis. For the *P. dactylifera*, the results of AMOVA revealed that for among population (4% and 20% for SSR and ISSR, respectively) and within the population (96% and 80% for SSR and ISSR, respectively) indicated that existence of significant levels of genetic differentiation among the cultivars studied.

The knowledge of genetic variation and their distribution can be an important consideration for designing conservation strategies and utilization of *P. dactylifera* germplasm resources. We believe that genetic diversity assessment of *P. dactylifera* collections may provide relevant information for diversity conservation and utilization of local genetic resources. Concentrating conservation efforts only on the small populations can lead to a decrease in the species' ability to survive.

CONCLUSIONS

Date palm is a multi-purpose tree, and extremely important as a subsistence crop. Proper genetic diversity study is critical to enrich the genetic pool of the species and introduce novel germplasm and screening and selection of genotypes. This study revealed a high level of genetic association of 24 date-palm genotypes collected from different part of Bushehr province-Iran; which might be due to the origin and geographical distribution of these genotypes. Genetic diversity analysis with SSR and ISSR markers in the *P. dactylifera* detected a high within population genetic diversity but low genetic differentiation among populations which can be explained by restricted gene flow among populations, dispersal and strongly linked to life-history traits.

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