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Genetic Diversity and Population Structure of Date Palm (*Phoenix dactylifera* L.) Germplasm from Iran using ISSR Assay

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Abstract

Date palm is an important fruit crop cultivated mainly in arid and semiarid regions. The DNA polymorphism among 69 selected Iranian native genotypes belonging to six origin groups and 1 Moroccan genotype was assessed using 10 inter simple sequence repeat (ISSR) primers. Results revealed high genetic variability among studied genotypes. Of the 65 known loci, 46 were polymorphic. Polymorphism information content (PIC) values varied from (6–30 %) with an average 19.4 %,. High number of alleles (Na=1.67), Effective alleles (Ne=1.49), Shanon information index (I=0.40), and polymorphic loci (P= 67.69 %) were detected in "Kerman" population. Based on Nei`s genetic identity, each of the regions of "Morocco", "Fars" and "Bushehr" populations were located in separate groups, but the rest of the populations were located in the same one. Structure analysis of the studied association panel manifested sevev subpopulations and there was no connection between these subpopulations and their geographical distribution. Likewise, most studied date palm genotypes have mixed genotypes related to their common genetic background. Our finding showed that ISSR markers could recognize male stocks of date palm that are important in crossing programs. This research addresses a more comprehensive picture of Iran's date palms genetic variability and structure. This studied panel can apply parental selection for future breeding programs such as mating designs and mapping populations.

Keywords: Association panel; Genetic variability; PIC; Mixed genotype

Introduction

Date palm (*Phoenix dactylifera* L.), belonging to the Arecaceae family, is an economically important fruit crop, dioecious, and monocotyledonous. Date palms is a diploid (2n = 2x = 36), and the predicted genome size is estimated to be approximately between 550 and 650 Mbp long [1]. It is cultivated in arid and semiarid regions of the world. Based on historical records and some very narrow germplasm analyses, it has been argued that human selection, clonal propagation, and movement of germplasm by human migration have been the primary forces that have shaped genetic diversity in date palm [2, 3]. This fruit crop is believed to comprise discrete clones of approximately 5,000 cultivars with a limited genetic exchange [4], although it has not been thoroughly verified by molecular marker analysis. Generally based on ripening time (early, mid or late), date palms were grouped into three cultivars. The early-season cultivar has more market able than others [5]. Its fruits possess high nutritional value and contain about 70% sugar, essential vitamins, and minerals, and different value-added products are produced [6]. Date palm is considered a good source of natural antioxidants and anti-mutagenic [7]. In addition to date palm tree is cultivated for fuel, fiber, and shelter for ground crops. Countries including Egypt, Iran, Saudi Arabia, the United Arab Emirates (UAE), Iraq, and Pakistan as leading producers of date palm have accounted for almost 80 % of the world production, amounting to ~5.8 million metric tons [8]. Assessing the genetic diversity of native tree species such as date palms is critical to assisting the conservation of genetic resources, providing a reservoir of genes for developing novel traits associated with yield enhancement, adaptability to climate change, and pest and disease resistance [9]. DNA fingerprinting is mainly exploited for detecting the genetic diversity of plant species and identifying markers linked with specific traits [3, 10]. Several marker systems have been used to study the genetic diversity of date palm. In brief, randomly amplified polymorphic DNA (RAPD) fingerprints have been used to identify date palm accessions in Tunisia [11], Saudi Arabia [12], and Egypt (Soliman et al. 2003; Adawy et al. 2006). Amplified fragment length polymorphic (AFLP) markers have been applied to study the genetic diversity of date palm cultivars in Egypt and California [13, 14]. Inter-simple sequence repeats ($ISSR_s$) are highly discriminative, simple, fast, cost-effective, firm, and reliable in identifying markers. It requires only a small quantity of the DNA sample and does not need prior sequence information to design the primer [15, 16]. ISSR amplification is a comparatively recent technique that can distinguish closely related genotypes and display its suitability for genetic diversity examination.

Moreover, it is exceedingly reproducible, cost-effective, and requires no earlier sequence data [17]. The use of the ISSR marker for detecting the genetic diversity of date palm was reported earlier by many researchers [18, 19]. Despite using vast traditional varieties propagated clonally by offshoots of female trees, little breeding research has been done on native date palm due to its long growth period and a lack of germplasm information. In the current study, we collected 69 date palm genotypes from 6 regions (provinces) and one genotype from Morocco to (i) evaluate the genetic variability of this association

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panel using ISSR markers (ii) classify of studied regions and identify distinct regions (iii) structure analysis of date palm germplasm using Bayesian approach.

Materials and Methods

Plant material, DNA extraction and quantification

Seventy genotypes were chosen randomly from 7 locations (Bushehr, Fars, Hormozgan, Kerman, Khuzestan, Morocco, Persian Gulf Basin) in Iran (Table 1). The frozen young leaf tissues of date palm collected from each genotype were first cleaned carefully with distilled water to remove the waxy layer. Then, 45 mg of leaf sample was cut into small pieces and ground into a fine powder using liquid nitrogen. Then, DNA was extracted from 45 mg of dried leaf tissue. The samples were ground to a fine powder in a Tissue Lyser II homogenizer (Qiagen) in the presence of steel balls. DNA was extracted using the DNeasy Plant Minikit (Qiagen) following the manufacturer's instructions. DNA qualification was checked by electrophoresis on 1% agarose gel. The gels were stained in ethidium bromide and visualized under UV light by a NanoDrop spectrophotometer.

DNA fingerprinting by ISSR primers

Ten of 12 anchored ISSR primers were given reproducible bands used in current research. The main characteristics of using the ISSR primers are summarized in (Table 2). Polymerase chain reaction (PCR) was carried out in a total reaction mixture of 25 µl of the mixture contained 4 ng.µl genomic DNA, 1U of Taq DNA Polymerase, 1 × PCR buffer, 2 mM of each dNTPs, 2.5 mM MgCl2 and 4 µM of each primer) [20]. DNA amplification was performed in a 96-well thermal cycler (Veriti®, California, USA). Under the following conditions: initial denaturation at 94°C for 5 min, 32 cycles (denaturation 94°C for 45 s, annealing temperature depending on primer for 45 s, extension 72°C for 2 min), final extension 72°C for 7 min. The ISSR-PCR products were separated on 2.5% agarose gel, stained with ethidium bromide and visualized under UV.

Data analysis

The PCR amplification products were scored for the presence (1) or absence (0) of each band marker across all 70 date palm genotypes, and the data were used to construct a binary data matrix. Software

Table 1: Name, origin and pertinent Q-values of studied date palm genotypes.

			Q matrix							
No.	**Name**	**Region (location)**	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**Q6**	**Q7**	**Group**
1	Borhi	Khuzestan	0.663	0.09	0.085	0.039	0.047	0.03	0.046	Mixed
2	Ashrasi	Khuzestan	0.458	0.128	0.122	0.105	0.077	0.062	0.048	Mixed
3	Kush zabad	Persian Gulf Basin	0.03	0.072	0.112	0.23	0.164	0.081	0.311	Mixed
4	Abu Maan	Persian Gulf Basin	0.175	0.05	0.289	0.097	0.239	0.048	0.103	Mixed
5	Abu narenja	Persian Gulf Basin	0.042	0.069	0.294	0.027	0.413	0.021	0.134	Mixed
6	Nabat seif	Persian Gulf Basin	0.134	0.04	0.263	0.04	0.333	0.061	0.128	Mixed
7	Khalas	Persian Gulf Basin	0.017	0.26	0.195	0.051	0.289	0.018	0.169	Mixed
8	Fard	Persian Gulf Basin	0.023	0.071	0.204	0.093	0.365	0.063	0.181	Mixed
9	Ghasb	Kerman (Shahdad)	0.29	0.035	0.156	0.2	0.168	0.048	0.101	Mixed
10	Touri	Hormozgan (Hajjiabad)	0.11	0.031	0.072	0.067	0.041	0.632	0.047	Mixed
11	Sangshekan	Kerman (Bam)	0.331	0.047	0.064	0.25	0.119	0.027	0.163	Mixed
12	Diri	Hormozgan (Minab)	0.905	0.021	0.016	0.014	0.017	0.011	0.016	Red
13	Deglet nour	Persian Gulf Basin	0.912	0.017	0.018	0.011	0.017	0.014	0.011	Red
14	Estameran	Khuzestan	0.068	0.039	0.082	0.41	0.145	0.02	0.237	Mixed
15	Zahedi	Bushehr	0.11	0.122	0.097	0.31	0.131	0.054	0.176	Mixed
16	Piarm	Hormozgan (Hajjiabad)	0.214	0.029	0.183	0.222	0.209	0.023	0.12	Mixed
17	Shahabi	Hormozgan	0.059	0.197	0.076	0.314	0.098	0.032	0.225	Mixed
18	Majool	Morocco	0.052	0.034	0.048	0.035	0.037	0.765	0.029	Mixed
19	Male stock	Kerman (Bam)	0.055	0.425	0.219	0.033	0.151	0.022	0.095	Mixed
20	Male stock	Kerman (Bam)	0.047	0.642	0.112	0.037	0.051	0.058	0.053	Mixed
21	Male stock	Kerman (Bam)	0.016	0.846	0.029	0.022	0.022	0.016	0.049	Green
22	Male stock	Kerman (Bam)	0.023	0.784	0.036	0.023	0.047	0.02	0.066	Green
23	Male stock	Kerman (Bam)	0.038	0.554	0.112	0.064	0.112	0.034	0.086	Mixed
24	Male stock	Kerman (Bam)	0.021	0.889	0.017	0.019	0.018	0.016	0.02	Green
25	Male stock	Kerman (Bam)	0.103	0.292	0.199	0.125	0.103	0.083	0.096	Mixed
26	Male stock	Kerman (Bam)	0.014	0.892	0.021	0.016	0.017	0.019	0.021	Green
27	Male stock	Kerman (Bam)	0.033	0.113	0.023	0.015	0.022	0.778	0.016	Pale blue
28	Male stock	Kerman (Bam)	0.034	0.79	0.03	0.065	0.038	0.013	0.03	Green
29	Zardooni	Kerman (Bam)	0.026	0.042	0.041	0.42	0.078	0.036	0.356	Mixed
30	Kelk sorkh	Kerman (Kahnuj)	0.528	0.041	0.063	0.142	0.081	0.048	0.097	Mixed
31	Mehmooni	Kerman (Kahnuj)	0.089	0.026	0.234	0.146	0.17	0.13	0.205	Mixed
32	Fereh kam	Hormozgan (Hajjiabad)	0.036	0.079	0.295	0.181	0.246	0.056	0.106	Mixed
33	Al mehtari	Kerman (Jiroft)	0.223	0.026	0.105	0.037	0.057	0.523	0.03	Mixed
34	Halileie	Fars (Lar)	0.037	0.024	0.051	0.022	0.024	0.822	0.02	Pale blue
35	Khark	Kerman (Kahnuj)	0.015	0.064	0.131	0.355	0.168	0.026	0.24	Mixed

Table 1: Continued.

No.	Name	Region (location)	Q matrix							Group
			Q1	Q2	Q3	Q4	Q5	Q6	Q7	
36	Amme shanbe	Kerman (Kahnuj)	0.014	0.057	0.269	0.066	0.092	0.412	0.089	Mixed
37	Gerd daneh	Hormozgan (Minab)	0.027	0.161	0.216	0.25	0.18	0.02	0.148	Mixed
38	Negar	Kerman (Jiroft)	0.047	0.71	0.05	0.056	0.039	0.034	0.064	Mixed
39	Zardak	Hormozgan (Roodan)	0.025	0.035	0.211	0.214	0.303	0.044	0.167	Mixed
40	Nozohur	Kerman (Jiroft)	0.036	0.022	0.156	0.059	0.112	0.555	0.06	Mixed
41	Mazafati	Kerman (Bam)	0.036	0.027	0.094	0.332	0.201	0.026	0.284	Mixed
42	Tokhmmorghi	Hormozgan (Hajjiabad)	0.147	0.215	0.114	0.133	0.134	0.105	0.151	Mixed
43	Seloni	Kerman (Kahnuj)	0.05	0.03	0.102	0.404	0.195	0.031	0.186	Mixed
44	Lasht	Kerman (Kahnuj)	0.019	0.021	0.324	0.15	0.205	0.056	0.224	Mixed
45	Nobar	Hormozgan (Fin)	0.018	0.062	0.124	0.242	0.21	0.027	0.316	Mixed
46	Posht nabi	Hormozgan (Fin)	0.022	0.026	0.126	0.32	0.123	0.033	0.35	Mixed
47	Shahani	Kerman (Orzuiyeh)	0.039	0.446	0.082	0.125	0.134	0.046	0.129	Mixed
48	Najmeh	Kerman (Kahnuj)	0.072	0.015	0.212	0.079	0.151	0.33	0.14	Mixed
49	Krut	Kerman (Bam)	0.608	0.02	0.061	0.125	0.041	0.083	0.062	Mixed
50	Halileie	Kerman (Shahdad)	0.192	0.056	0.183	0.145	0.177	0.05	0.196	Mixed
51	Zeinabi	Kerman (Kahnuj)	0.081	0.031	0.13	0.166	0.085	0.284	0.223	Mixed
52	Lilgouni	Kerman (Kahnuj)	0.027	0.046	0.235	0.138	0.337	0.059	0.158	Mixed
53	Peirizi	Fars (Lar)	0.026	0.027	0.148	0.309	0.163	0.03	0.297	Mixed
54	Gand gorbeh	Kerman (Jiroft)	0.472	0.05	0.095	0.146	0.076	0.049	0.111	Mixed
55	Farz	Kerman (Kahnuj)	0.023	0.057	0.188	0.063	0.124	0.458	0.086	Mixed
56	Maktoub	Hormozgan (Roodan)	0.165	0.018	0.123	0.054	0.063	0.538	0.039	Mixed
57	Souzdan	Kerman (Jiroft)	0.123	0.009	0.151	0.247	0.197	0.115	0.158	Mixed
58	Yaghuti	Kerman (Bam)	0.395	0.023	0.082	0.197	0.099	0.055	0.149	Mixed
59	Khosh kang	Kerman (Kahnuj)	0.028	0.024	0.091	0.387	0.251	0.017	0.202	Mixed
60	Nesfei	Hormozgan (Roodan)	0.019	0.013	0.272	0.076	0.147	0.343	0.13	Mixed
61	Halileie	Kerman (Shahdad)	0.058	0.034	0.321	0.082	0.276	0.024	0.204	Mixed
62	Gorbani	Kerman (Kahnuj)	0.032	0.019	0.094	0.342	0.108	0.034	0.371	Mixed
63	Kabkab	Bushehr	0.05	0.022	0.178	0.196	0.296	0.017	0.241	Mixed
64	Morad sang	Kerman (Orzuiyeh)	0.047	0.294	0.096	0.16	0.159	0.031	0.214	Mixed
65	Galami	Hormozgan (Roodan)	0.037	0.023	0.106	0.18	0.096	0.308	0.249	Mixed
66	Sahlaki	Kerman (Kahnuj)	0.024	0.017	0.075	0.33	0.134	0.014	0.407	Mixed
67	Zohrei	Kerman (Kahnuj)	0.017	0.025	0.073	0.267	0.069	0.252	0.297	Mixed
68	Makou	Kerman (Kahnuj)	0.016	0.039	0.089	0.29	0.106	0.144	0.317	Mixed
69	Khsoueie	Kerman (Kahnuj)	0.017	0.041	0.04	0.036	0.026	0.795	0.044	Pale blue
70	Kheli get	Kerman (Kahnuj)	0.063	0.033	0.081	0.309	0.107	0.079	0.328	Mixed

GenAlEx version 6.503 [21] was used to analyze genetic diversity parameters related to each population, including the number of different alleles (Na), the number of effective alleles (Ne), Shannon's Information Index (I), expected heterozygosity (He) and percentage of polymorphic loci (PL%) as well as pairwise population matrix of Nei genetic identity. Population structure was analyzed using a model-based Bayesian approach in the software Structure, version 2.3.4 [22]. Five independent runs were performed, setting the sub-populations (K) from 1 to 10, burn-in time and MCMC (Markov Chain Monte Carlo) replication number to 500,000, and a model for admixture and correlated allele frequencies. Delta K (ΔK) was used to represent the K value based on the second-order rate of change in the likelihood [23]. Inferred ancestry estimates of individuals (Q matrix) were derived for the selected population [22]. Trait-marker association analysis was performed using a mixed linear model (MLM) approach in TASSEL 2.1, accounting for population structure and kinship relatedness (Q+K model). Both kinship coefficients and linkage disequilibrium (LD) were calculated via TASSEL 2.1.

Results

In this research, genetic diversity among 70 date palm genotypes was investigated using 10 ISSR markers and high molecular genetic variability was observed among the studied genotypes. Of the 65 genes studied loci, 45 loci were polymorphic and 19 were monomorphic (Table 2). In this regard, primer P4 with 10 detected loci possessed a maximum number of amplified loci. Resulted that P10 with a PIC value of 30 followed by primers P2 (PIC = 29) and P1(PIC = 26) can effectively be utilized in evaluating date palm genetic variation (Table 2). According to Nei genetic identity (Table 3), the maximum similarity was seen between regions "Hormozgan" and "Kerman"; meanwhile, the minimum value was detected among "Fars" and "Bushehr" regions. According to PCoA analysis of studied populations, regions of "Kerman", "Hormozgan", "Khuzestan" and "Persian Gulf Basin" were clustered in the same group and each of the regions of "Bushehr", "Morocco" and "Fars" located in a separate group (Figure 1). Regarding ISSR marker information of each studied regions, the minimum and maximum values of Na and Ne were possessed in the "Bushehr" and "Kerman" regions, respectively (Table 4). In the current research, the maximum values of He and I were detected for the region of "Kerman". The average percentage of polymorphic loci (PL%) over regions was 34.73% and region "Kerman" had a

Table 2: Information about primer name, sequence, amplified loci, polymorph, monomorphic as well as PIC values of each primer.

Primer Sequence	Number of loci	Polymorphic loci	Monomorphic loci	PIC value(%)
P1	8	7	1	26
P2	5	4	1	29
P3	5	4	1	10
P4	10	8	2	17
P5	5	3	2	16
P6	6	4	2	20
P7	6	6	0	20
P8	7	3	4	20
P9	6	1	5	6
P10	7	6	1	30
Total	65	46	19	19.4

Table 3: Pairwise region matrix of Nei genetic identity.

	Bushehr	Fars	Hormozgan	Kerman	Khuzestan	Morocco	Persian Gulf Basin
Bushehr	1.000						
Fars	0.799	1.000					
Hormozgan	0.89	0.899	1.000				
Kerman	0.888	0.9	0.983	1.000			
Khuzestan	0.855	0.846	0.908	0.918	1.000		
Morocco	0.799	0.867	0.853	0.864	0.815	1.000	
Persian Gulf Basin	0.864	0.869	0.927	0.921	0.89	0.828	1.000

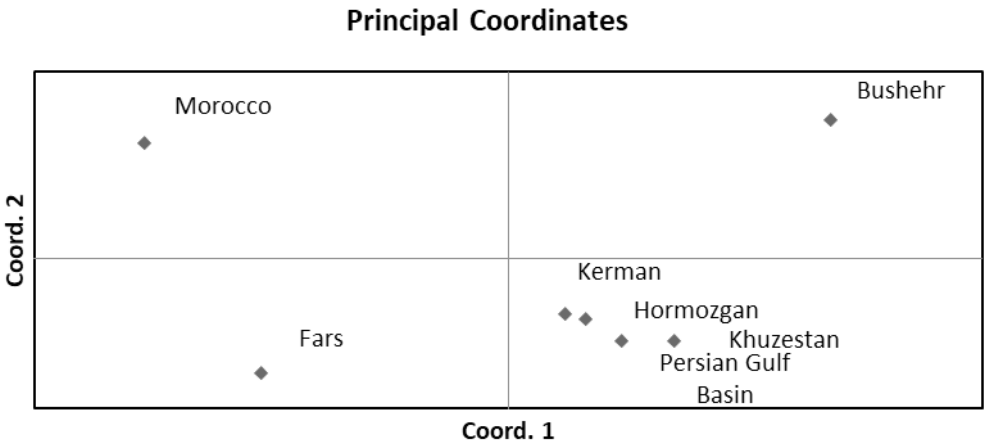

Figure 1: Classification of studied date palm regions using principal coordinate analysis.

Table 4: Mean over loci for each studied regions.

Region	Na	Ne	I	He	%PL
Bushehr	1.154	1.152	0.13	0.089	21.54%
Fars	1.246	1.196	0.167	0.115	27.69%
Hormozgan	1.6	1.475	0.376	0.262	60.00%
Kerman	1.677	1.493	0.406	0.279	67.69%
Khuzestan	1.308	1.235	0.192	0.132	32.31%
Persian Gulf Basin	1.323	1.255	0.201	0.139	33.85%
Grand Mean over Loci and regions	1.299	1.258	0.21	0.145	34.73%

Na = No. of Different Alleles
Ne = No. of Effective Alleles = 1 . (p^2 + q^2)
I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q))
He = Expected Heterozygosity = 2 * p * q
%PL = percentage of polymorphic loci

maximum value of polymorphic loci (67.69%) (Table 4). In order to understand the genetic structure of the association panel, a model-based Bayesian approach in the STRUCTURE software was used to assign each genotype to the corresponding subgroup. The group of 70 date palm genotypes was partitioned into seven subgroups (Figure 2A). These subgroups included Red (Diri and Deglet nour genotypes), Green (5 male rootstock genotypes), Pale blue (Halileie and Khasoueie genotypes) and Mixed (the rest of the studied genotypes) based on each genotype Q value (Figure 2B and Table 1).

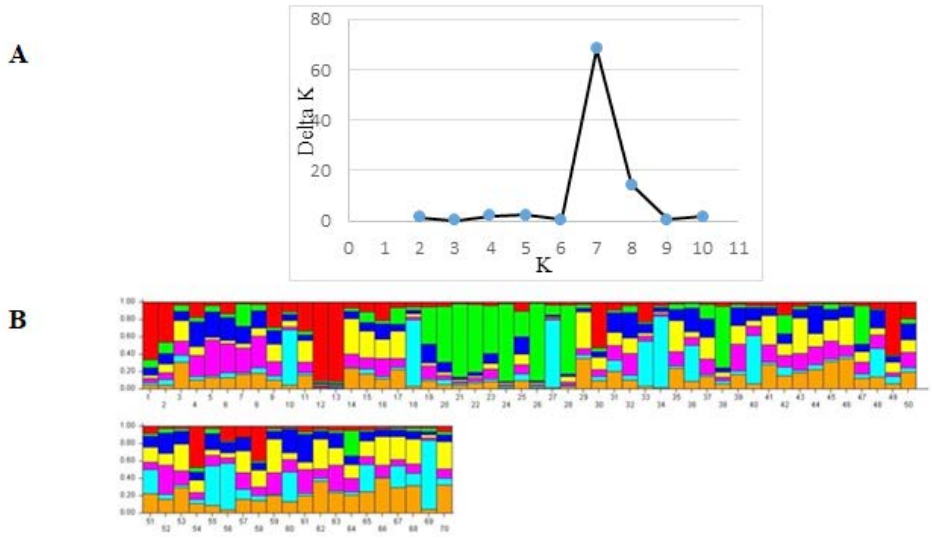

Figure 2: (A) Bilateral charts to access the optimal number of sub-populations in the studied date palm panel (K = 7). (B) Genetic relatedness of individuals from 70 date palm genotypes using STRUCTURE software. Numbers on the y-axis show the membership coefficient to sub-populations. The numbers on the x-axis show that the individual code belongs to date palm genotypes.

Discussion

The date palm can be assumed as an important fruit crop due to its great potential to generate income in extreme environmental conditions and unproductive lands. As well as, there are vast and worthy cultivars and genotypes of it have been planted in southern regions of Iran for many years [24]. Accordingly, characterization of date palm germplasm from Iran is a prerequisite for its improvement through breeding programs. This study implemented ISSR markers to fingerprint date palm genotypes from six regions (provinces) of Iran, accompanied by a genotype from Morocco. The current study implies that ISSR markers are effective tools to discriminate various date palm genotypes. Several studies reported the efficiency of ISSR markers in fingerprinting and distinguishing date palm genotypes (Srivashtav et al. 2013; Marsafari and Mehrabi 2013; Purayil et al. 2018; Abdelaziz et al. 2020). In this research, the percent of polymorphism revealed by ISSR markers was 70.76% (46 loci out of 65 detected loci), meanwhile previously reported as 28.6% by Hussein et al. [25], 64.1% by Adawy et al [14], and 95% by Marsafari and Mehrabi (2013).

The most important parameter proposed to calculate for a molecular marker data set is the polymorphism information content (PIC) value that measures the ability of a marker to detect polymorphisms and has enormous importance in selecting markers for genetic studies [26]. Herein, P10 among studied primers possessed the highest PIC value and will be effectively recommended to evaluate date palm germplasm. The average PIC (19.4) observed in the present work is comparable with previous reports by Purayil et al. (2018) and Abdelaziz et al. (2020), with values of 0.23 and 0.45, respectively. Despite low PIC values, these ISSR markers can be utilized to estimate relationships between varieties based on their geographic origin [27]. The pairwise Nei genetic identity between studied regions was calculated and then PCoA analysis revealed four major clusters for studied regions. In this regard, the region "Morocco" has been constructed as a separate class which is logical. There was no coincidence between geographical distribution and Nei genetic identity in other studied regions. For instance, the regions "Bushehr" and "Fars" have also been located separately; they were geographically near each other. Oppositely, regions that were far from together ("Kerman", "Hormozgan", "Khuzestan" and "Persian Gulf Basin") were located in the same group (Figure 1). Similar to our findings, in a study of desert date (*Balanites aegyptiaca* Del.) genetic variation, five geographically distant populations was located in the same group (Abdelaziz et al. 2020). Overall, moderate genetic differentiation was detected among studied regions in this research. This finding is similar to what was reported for oil palm by using an SSR [28]. Among studied regions, the "Kerman" and then "Hormozgan" showed maximum Ne, I, and PL values, which implied their importance for future date palm breeding programs. However, it seems that "Kerman" should be considered more than others because of its male rootstocks and the male genotypes that could influence the quantity and quality of progenies [29]. According to the model-based clustering for the genetic structure of date palm individuals, seven genetically distinctive subpopulations were presented that were not formed in line with their geographical location. The most probable clustering of genotypes was observed at this K level and showed admixture structure among genotypes. Our result displayed studied populations with a common genetic background and shared common alleles among them. The most differentiated genotypes were observed in "Kerman" region, which showed fewer admixtures than the rest regions. Generally, the structure results showed shared ancestry between date palm genotypes and cultivars that were early introduced [30].

Conclusion

Genetic diversity is desirable for long-term crop improvement such as date palm and reduction of vulnerability in plants to important crop diseases. In the present study, the ISSR marker was handled to evaluate the genetic diversity of date palm germplasm and confirm the potential use of ISSR markers in date palm fingerprinting. The studied region "Kerman" manifested remarkable genetic variability compared with other regions. That is expectable because "Kerman" is the first producer of date palm in Iran and evolved most cultivated varieties and genotypes. The genetic structure of inspected germplasm depicted 7 subpopulations with mostly mixed genetic backgrounds implying a common genetic background and shared alleles between them. In this study, the clustering pattern of genotypes was independent of their geographical distances. In summary, the analysis of ISSR markers of the date palm genotypes documents the significant variation present in

the *in situ* found in the regions and confirms the need to conserve this valuable resource.

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