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Intra-cultivar variability at microsatellite loci in date palm cultivars across the GCC countries

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ABSTRACT

In this study, we characterized 30 date palm trees of Khalas cultivar from the GCC countries, along with seven male trees from Qatar, using 14 microsatellite loci. The results showed that the microsatellites [(GA)n] in the date palm cultivar Khalas varied considerably in allele size (range=120–322 bp, mean=189.78), which revealed a high degree of gene diversity (range=0.66–0.85, mean=0.75) and distinguished all the individual Khalas trees within and among the GCC countries. The extent of polymorphism microsatellite loci was dependent on individual loci itself, which was positively correlated with the number of repeats at the corresponding microsatellite loci. The estimates of the skewness and kurtosis of the allelic distributions showed that none of the distribution of 14 microsatellite loci was considered normal (skewness=0, kurtosis=3), which suggested that the shape of the allelic distribution of these loci varied by chance. In the majority of the cases, the microsatellite allele size of the most frequent allele (mode) was very close to the median (±2 bp or ±(GA)1), which indicated that the most frequent allele was the ancestral allele. Nearly half of the allelic distributions of the 14 microsatellite loci were positively skewed and the other half was negatively skewed, which indicated that the alleles evolved respectively by gaining and losing of (GA)n repeats from the ancestral allele, resulting in intra-cultivar variability in Khalas cultivar over the generations. Based on this analysis, we conclude that the Khalas variety did not evolve independently at multiple origins, but rather it evolved from a single origin and, subsequently, the clones were distributed across the GCC countries. The accumulated mutations of these clones over time resulted in drastic changes among them at the microsatellite loci.

Keywords: Khalas cultivar, intra-cultivar variation, microsatellites, mutation

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1. INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is considered one of the oldest cultivated fruit trees, which is primarily grown in the Middle East and North Africa.[1] It is known for its social, religious and agricultural values. In addition to producing nutritious desert fruit, parts of this tree are used for providing shelter, producing fuel and making handicrafts, rendering it a significant source of revenue for farmers.[2] The date palm also contributes to the sustainable agricultural development of fragile oasis ecosystems.[3] Nearly one-third area of the global date palm lies in the GCC countries, which contributes to approximately one-third of the global production.[4]

Globally, 5000 or more date cultivars are known to exist, among which a rich diversity of over 400 date cultivars is found in Saudi Arabia and the GCC countries.[5] However, the preference of choice of farmers and industries has drastically restricted its genetic resources to producing predominant cultivars with good fruit quality. For example, in Tunisia, genetic erosion in date palms has caused the large-scale cultivation of Deglet Nour variety that has a higher preference over other varieties.[6] As there is no evidence that the majority of cultivars are the breeder's clones, most of the description of date cultivars is based on the farmer's selection.[7] Furthermore, there is an urgent need for their genetic conservation due to their vulnerability to biotic and abiotic stress.[8]

Characterization and identification of date palm is based primarily on the morphology of leaves and spines as well as the physical appearance of fruit such as shape, weight, color, aspect of skin, consistency and texture, which are manifested in mature trees (age 3–5 years). Having limited discriminatory power, these characteristics are known to be heavily affected by environmental conditions. Consequently, this has led to some cultivars with similar morphological characteristics being given the same varietal name.[9]

In addition, there is some confusion due to the naming associated with date cultivars. A date palm can be given multiple names depending on its region. In addition, the nature of dates shows large intra-cultivar variations. This kind of large variations, including differences in fruit characteristics such as size, ripening time or vegetative appearance, was first reported in the early 1920s. Initially, researchers thought the chance that the plant had come from a seedling rather than from an offshoot is the cause of the differences between palms of one variety. Therefore, they differentiated cultivar characteristics as being a hybrid rather than a clone from the parent plant, which were then grown and marketed under the name of the parent cultivar.[10]

To confirm the uniformity and similarity of the cultivars, date palms have always been clonally propagated. Confusion about the nomenclature, preservation and use of the cultivars could be potentially caused by the existence of intra-cultivar variation. There is often extreme difficulty in discriminating among closely related cultivars and clones.[11] However, using DNA markers, we can determine the differences among the closely related cultivars and even among clones.

In contrast, introduction of traditional hand pollination systems along with new genotypes of neighboring countries may be the reason for obtaining recombinant genotypes that cause genetic variation in date palms in various geographical regions. Therefore, it is considered essential to understand, at the regional level, the genetic structure of date palm for the efficient use of these valued resources and the better conservation of date palm populations.[12]

Genetic diversity and phylogenetic relationships proven by DNA markers are the most appropriate techniques for accurately identifying date palm cultivars. In plants, DNA typing is primarily used to identify and protect cultivars/varieties, as well as to estimate genetic diversity, molecular mapping and marker-assisted selection. Conservation of genetic resources of date palms relies on the understanding of the extent and spreading of genetic diversity existing in the present germplasm.[13,14]

In recent years, marker technology for DNA fingerprinting has become increasingly important to discriminate closely related cultivars. Microsatellite or simple sequence repeat (SSR) molecular markers have been proven to be deeply powerful in plant diversity analysis because they are not only locus-specific, but also possess a high degree of polymorphism, have co-dominant inheritance, and are highly reproducible. The SSR marker technique has been used in the study of the relationships of date palm cultivars, as well as their genetic diversity in many countries such as Tunisia,[15] Sudan,[16] Oman,[17] Morocco,[18] Nigeria,[8] Qatar,[19] and Saudi Arabia.[20]

In the Gulf region, a variety called Khalas is popular with a high consumer preference.[1] Being widely cultivated in the GCC countries, Khalas is considered by many as the finest date worldwide, with mostly medium- to large-sized fruits that make a delicacy as both fresh (Rutab) and dry (Tamar) dates

which store well.[21] However, it is not clear whether Khalas cultivars from different countries are genetically similar (clones) or show intra-cultivar variations.

In this study, we collected Khalas cultivars from different countries such as Kuwait, Qatar, Oman and Bahrain, in order to determine using microsatellite markers whether the Khalas cultivar from different countries are the same or different clones. If they are different, what is the extent of variability present among the Khalas cultivars and how they are related? In doing so, we may be able to explain the cause (genetic basis) of intra-varietal variation in Khalas cultivars and their distribution across the GCC countries.

2. MATERIALS AND METHODS

2.1. Plant materials

Matured date palm trees of Khalas variety were randomly selected from different locations in Kuwait, Bahrain, Oman (Khalas Thahra) and Qatar. In addition, seven male palm trees were selected (Table 1). Young leaves were harvested and preserved at -80°C until the DNA was ready to be extracted.

Table 1. Sampling sites of 30 Khalas and 7 male date palm trees.

No.	Country	Cultivar	Location	Code	No.	Country	Cultivar	Location	Code
1	Kuwait	Khalas	Albander	K1	20	Qatar	Khalas	North	Q5
2	Kuwait	Khalas	Alwafrah	K2	21	Qatar	Khalas	North	Q6
3	Kuwait	Khalas	Alahmadi	K3	22	Qatar	Khalas	East	Q7
4	Kuwait	Khalas	Kabed	K4	23	Qatar	Khalas	East	Q8
5	Kuwait	Khalas	Aljahrah	K5	24	Qatar	Khalas	East	Q9
6	Bahrain	Khalas	Almarekh	B1	25	Qatar	Khalas	East	Q10
7	Bahrain	Khalas	Jad Elhaj	B2	26	Qatar	Khalas	East	Q11
8	Bahrain	Khalas	Aljesrah	B3	27	Qatar	Khalas	West	Q12
9	Bahrain	Khalas	Sanabes	B4	28	Qatar	Khalas	South	Q13
10	Bahrain	Khalas	Askar	B5	29	Qatar	Khalas	South	Q14
11	Oman	Khalas	Barka	O1	30	Qatar	Khalas	South	Q15
12	Oman	Khalas	Wadi maaloul	O2	31	Qatar	Male	East	MQ1
13	Oman	Khalas	Alrastaq	O3	32	Qatar	Male	East	MQ2
14	Oman	Khalas	Almasnah	O4	33	Qatar	Male	East	MQ3
15	Oman	Khalas	Almthebi	O5	34	Qatar	Male	West	MQ4
16	Qatar	Khalas	North	Q1	35	Qatar	Male	West	MQ5
17	Qatar	Khalas	North	Q2	36	Qatar	Male	West	MQ6
18	Qatar	Khalas	North	Q3	37	Qatar	Male	West	MQ7
19	Qatar	Khalas	North	Q4					

2.2. DNA extraction and genotyping

To extract DNA, 1 g of the leaf sample was grounded into fine powder using liquid nitrogen, and then the DNeasy Plant Maxi kit (Qiagen Venlo, Netherlands) was used according to the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop spectrophotometer.

As described by Billotte,[22] 14 labeled primer pairs were synthesized by Applied Biosystems (Life Technologies BV, Kwartsweg , Bleiswijk Netherlands), which are listed in Table 2. The PCR was implemented using a 25 µl reaction mixture containing 2 µl (5 ng) DNA, 12.5 µl AmpliTaq Mastermix, 1 µl of 5 µM each forward and reverse primer (labeled) and 8.5 µl nuclease-free water.

DNA amplification was performed in a Veriti 96 Thermal Cycler (Applied Biosystems) under the following conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing temperature depending on the primer (Table 2) for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The analysis of microsatellite loci was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems) by adding 1 µl PCR product to 10 µl Hi-Di formamide and 0.3 µl GS500LIZ size standard followed by denaturation at 95°C for 3 min and cooling on ice. The samples were run on the 3130 Genetic Analyzer. Automatic genotyping and allele scoring were performed using the GeneMapper® Software v4.0 (Applied Biosystems).

Table 2. Date palm-specific microsatellite primers (developed by Billotte et al.[22]).

No.	Primer code	Repeat motif	Primer sequences (5'–3')	Optimal Tm (°C)
1	mPdCIR010	(GA)22	F: ACCCCGGACGTGAGGTG R: GTCGATCTCCTCCTTTGTCTC	55.9
2	mPdCIR015	(GA)15	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT	51.6
3	mPdCIR016	(GA)14	F: AGCGGGAAATGAAAAGGTAT R: ATGAAAACGTGCCAAATGTC	51.7
4	mPdCIR025	(GA)22	F: GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	49.3
5	mPdCIR032	(GA)19	F: CAAATCTTTGCCGTGAG R: GGTGTGGAGTAATCATGTAGTAG	51.5
6	mPdCIR035	(GA)15	F: ACAAACGGCGATGGGATTAC R: CCGCAGCTCACCTCTTCTAT	53.9
7	mPdCIR044	(GA)19	F: ATGCGGACTACACTATTCTAC R: GGTGATTGACTTTCTTTGAG	51.7
8	mPdCIR048	(GA)32	F: CGAGACCTACCTTCAACAAA R: CCACCAACCAAATCAAACAC	51.4
9	mPdCIR057	(GA)20	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC	55.4
10	mPdCIR070	(GA)17	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTGTAGTAT	48.7
11	mPdCIR078	(GA)13	F: TGGATTTCCATTGTGAG R: CCCGAAGAGACGCTATT	49.6
12	mPdCIR085	(GA)29	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA	50.4
13	mPdCIR090	(GA)26	F: GCAGTCAGTCCCTCATA R: GCAGTCAGTCCCTCATA	48.6
14	mPdCIR093	(GA)16	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	51.8

2.3. Data analysis

To determine heterozygosity, major allele frequency, number of alleles, gene diversity and polymorphic information content, data were analyzed using the PowerMarker software v3.0.[23] On the basis of the Hamming similarity index with 100 bootstraps, phylogenetic diagrams were drawn using the Past software version 1.91.[24] The analysis of molecular variance (AMOVA) and the genetic variation within and among the population were analyzed using the GenAlex 6.3 software.[25]

3. RESULTS

The analysis of the 14 microsatellite loci showed high polymorphism, which had a considerable number of alleles across the Khalas cultivars obtained from the GCC countries. When all the 14 microsatellite loci showing polymorphism were examined, each tree had a distinct genotype, which indicated that microsatellites were beneficial for distinguishing intra-cultivar variation in date palms.

3.1. Variability of the microsatellite loci

The number of alleles per locus varied from five alleles in the loci mPdCIR016 and mPdCIR035 to 11 alleles in the locus mPdCIR015 (Table 3). A total of 111 alleles with a mean of 7.93 alleles per locus were recorded. Gene diversity and observed heterozygosity also varied among the loci. The loci mPdCIR093 showed the lowest genetic diversity (0.66), while the locus mPdCIR078 showed the highest genetic diversity (0.85). The heterozygosity index ranged from 0.41 (loci mPdCIR090 and mPdCIR093) to 0.89 (locus mPdCIR010) with an average of 0.62 among the Khalas cultivars from the GCC countries. Polymorphism information content (PIC value), which estimates the marker's discriminatory power to assess the quality of marker genotype data, was also high (average PIC value=0.72; Table 3). All of

these 14 microsatellite loci showed a high number of polymorphism (PIC≥0.63). The inbreeding coefficient (*f*) also varied among the microsatellite loci. Of the 14 microsatellite loci, six and eight showed negative and positive values respectively, indicating an excess and deficit of heterozygotes with respect to the HW equation, with an overall average value of 0.1862.

Table 3. Microsatellite polymorphism at 14 loci in 37 date palm trees of Khalas cultivar from the GCC countries.

Marker or loci	Majorallele frequency	No. of genotypes	Sample size (n)	No. of Observation	No. of alleles	Gene diversity	Heterozy-gosity	PIC	Inbreeding coefficient (*f*)
mPdCIR010	0.26	16	37	37	10	0.845	0.8919	0.8280	-0.0421
mPdCIR015	0.32	13	37	37	11	0.792	0.8649	0.7639	-0.0787
mPdCIR016	0.47	9	37	36	5	0.688	0.6667	0.6434	0.0449
mPdCIR025	0.42	10	37	37	8	0.740	0.5405	0.7069	0.2825
mPdCIR032	0.32	10	37	37	8	0.760	0.5676	0.7217	0.2660
mPdCIR035	0.30	6	37	37	5	0.783	0.8649	0.7481	-0.0914
mPdCIR044	0.46	7	37	34	6	0.705	0.0294	0.6655	0.9595
mPdCIR048	0.37	13	37	31	10	0.729	0.8387	0.6885	-0.1337
mPdCIR057	0.46	8	37	37	7	0.688	0.8108	0.6412	-0.1650
mPdCIR070	0.31	11	37	36	8	0.781	0.4167	0.7487	0.4774
mPdCIR078	0.22	10	37	37	9	0.854	0.4324	0.8372	0.5041
mPdCIR085	0.37	12	37	37	8	0.773	0.9730	0.7430	-0.2468
mPdCIR090	0.43	8	37	37	7	0.729	0.4054	0.6938	0.4551
mPdCIR093	0.54	12	37	37	9	0.658	0.4054	0.6280	0.3956
Mean	0.37	10.36	37	36.21	7.93	0.752	0.6220	0.7184	0.1862

3.2. Size of the microsatellite loci

The size of the microsatellite loci amplified, which was estimated by the average size of the microsatellite fragment amplified across the trees, varied greatly. The locus mPdCIR010 had lowest average size (130.03 bp) of the fragment, while the locus mPdCIR044 had the largest average size (300.62 bp) (Table 4). A two-way analysis of variance (ANOVA) was performed to study the effects of both the microsatellite locus and the Khalas variety from the different countries of origin on the size of the fragment. The analysis indicated that the effect of the locus on generating the variability was statistically different (F=7147.51; df=13; p<0.001). This variation in the size of the amplified fragment among the microsatellite loci was mainly due to the effect of the locus itself, rather than the Khalas trees from different origins. However, the effect of the Khalas trees from the GCC countries on the number of repeats was not significant. This variation in the amplified fragment within the microsatellite loci can be attributed to chance factors.

The extent of variation within a microsatellite locus varied greatly, as indicated by the standard deviation and the coefficient of variation (Table 4). The estimates of the skewness and kurtosis of the allelic distributions showed that none of the distribution of the 14 microsatellites was considered normal (skewness=0 and kurtosis=3), suggesting that the shape of the allelic distribution of these loci also varied by chance. In the majority of the cases, the microsatellite allele size of the most frequent allele (mode) was very close to the median (±2 bp or ±(GA)1-repeat), except for mPdCIR032 and mPdCIR078. Nearly half of the allelic distributions of the 14 microsatellite loci were positively skewed and the other half were negatively skewed. This indicated that alleles evolved respectively by gaining and losing of (GA)n repeats from the most frequent allele or the ancestral allele.

Table 4. Variation at 14 microsatellite loci in date palm trees of Khalas cultivar.

Locus	Range of allele size (bp)	Mean	SD	CV	Skewness	Kurtosis	Median	Mode
mPdCIR010	120–160	130.03	7.59	5.84	2.41	8.04	128	126
mPdCIR015	120–140	130.78	6.14	4.69	0.16	-1.23	130	130
mPdCIR016	130–138	134.42	2.89	2.15	-0.52	-1.27	136	136
mPdCIR025	201–229	216.62	5.78	2.67	-1.03	1.82	215	215
mPdCIR032	286–302	296.49	4.96	1.67	-0.33	-1.21	296	302
mPdCIR035	185–197	189.00	3.55	1.88	1.16	0.69	189	187
mPdCIR044	294–322	300.62	82.73	27.52	3.09	14.61	300	302
mPdCIR048	158–192	183.71	69.11	37.62	-1.18	-0.45	190	192
mPdCIR057	252–276	257.97	3.5	1.35	1.75	9.16	258	258
mPdCIR070	184–204	194.25	32.07	16.51	-0.88	0.5	196	196
mPdCIR078	122–150	138.03	10.76	7.79	-0.36	-1.4	139	150
mPdCIR085	160–182	173.65	7.37	4.24	-0.41	-1.41	178	180
mPdCIR090	144–158	148.59	4.85	3.26	0.94	-0.59	146	146
mPdCIR093	153–175	169.54	6.84	4.03	-1.21	-0.21	173	173

3.3. Molecular variance

The Khalas trees from Qatar as a group were found to be more distantly related to the trees of Kuwait, Bahrain and Oman (Fig. 1), based on genetic relationships. When 30 Khalas trees were compared individually, all the Khalas cultivars from Qatar and the male varieties of Qatar clustered into one group, and the rest of the Khalas varieties from the other GCC countries clustered into the second group (Fig. 2). The AMOVA showed that 53% of variation corresponded to within-population variation and the remaining 47% corresponded to among-population variation.

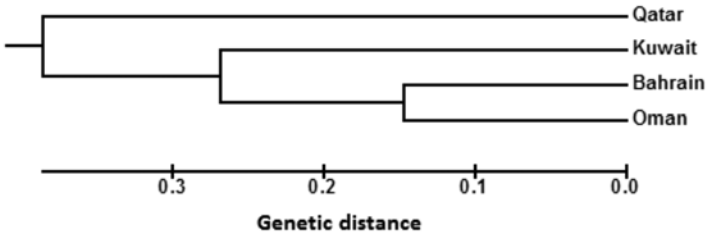

Figure 1. Dendrogram showing relationships among Khalas cultivars from the GCC countries as revealed by the unweighted pair group method with arithmetic mean and based on the shared allele genetic distance.

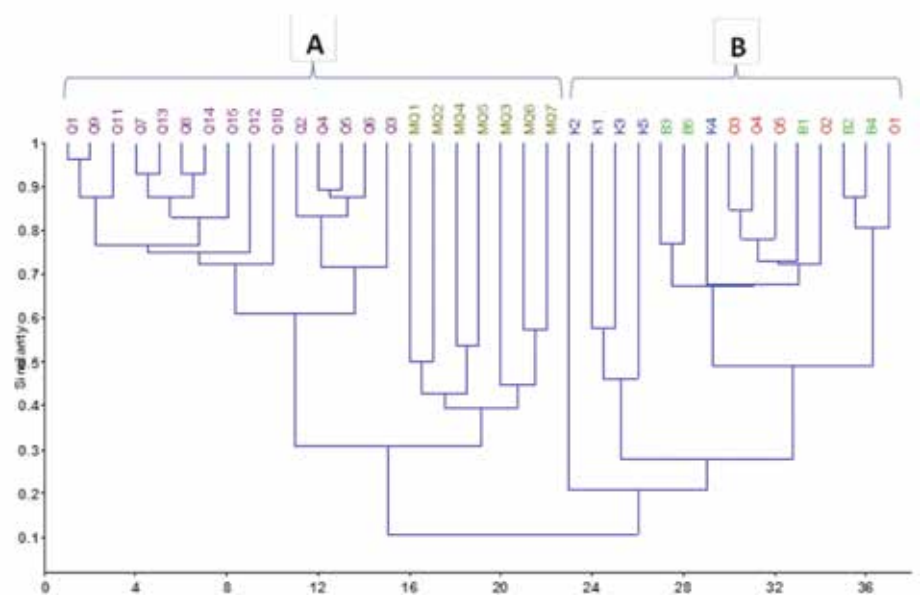

Figure 2. Dendrogram based on the Hamming coefficient analysis showing relationships among the date palm cultivar (A).Khalas and male trees from Qatar (B). Khalas from other GCC countries.

4. DISCUSSION

4.1. Genetic variation

Previous studies have reported that microsatellite loci in date palm are highly variable.[26,27] However, not much systematic studies have been undertaken on date palm to study the variation within a popular cultivar across the GCC countries. In this study, we characterized date palm trees of Khalas cultivar that originated from the GCC countries, and showed that (1) microsatellites [(GA)n] in the date palm variety Khalas varied considerably in allele size (number of repeats), which resulted in high degree of polymorphism and distinguished individual Khalas trees within and among the GCC countries, (2) the level of polymorphism at the microsatellite loci was high, as indicated by a higher number of alleles (7.93) and higher genetic diversity (0.75), (3) the PIC value for the individual microsatellite markers was also very high (PIC value >0.63) with an average of 0.72 across all the 14 markers for all the Khalas trees from the GCC countries, (4) the extent of polymorphism was dependent on the individual microsatellite locus positively correlated with the number of repeats at microsatellite loci, (5) the distribution of variability at individual microsatellite loci in the Khalas trees from the GCC countries was attributed to chance factors, and (6) the skewness of the allelic distribution indicated that some microsatellites varied more preferably by gaining (GA)n repeats and others by losing them.

4.2. Number of alleles

The high degree of polymorphism observed at the microsatellite loci of Khalas cultivar from the GCC countries is comparable to the inter-varietal variability observed in date palms. The average number of alleles obtained in our study (7.93 alleles/locus) was higher than that obtained for date palm varieties of Qatar (4 alleles/locus,[9] 7.7 alleles/locus[26]), Nigeria (7 alleles/locus[8]), Morocco (7.13 alleles/locus[18]), Tunisia (7.14 alleles/locus[15]) and Libya (6.88 alleles/locus[28]). However, a much higher number of alleles were detected in date palm varieties of other countries such as Sudan (21.4 alleles/locus[16]) and Iraq (8.54 alleles/locus[27]).

Cultivars are not of identical genotype unless they are clonally derived from the same original palm tree as date palm is an obligate outcrossing species. The traditional practice of clonal propagation of the Khalas cultivar by offshoots, usually performed by well-skilled farmers, ensures the identity and uniformity of the cultivars. In practice, each variety is derived from a unique descent of seed, which is cloned thereafter by vegetative propagation to ensure the identity and uniformity of the variety. However, intra-cultivar polymorphism could potentially cause errors in cultivar identification.

4.3. Intra-cultivar variability

The intra-cultivar variability observed in the present study may not be due to errors in varietal identification based on morphological traits, because we used true-to-type trees of Khalas. We also rule out the possibility that the intra-varietal variation results from outcrossing and seed-derived plants. If the plants are derived from outcrossing, the majority of loci would have shown a negative inbreeding index (*f*), showing an excess of heterozygotes. However, in our study, we detected positive *f* values for the majority of loci, showing a deficit of heterozygotes.

Due to the high rate of mutations in microsatellite loci, a high degree of microsatellite polymorphism at intra-cultivar level variability is expected. The mutation rate of microsatellite loci in various plants is estimated to be exceptionally high in comparison to unique sequences in the range of 10-2 to 10-4, which are much higher than that observed in microsatellite loci of animals and humans.[29,30] In general, the mutation event at microsatellites results in either the addition or subtraction of a small number of perfect repeats. The predominant mutational mechanism involved in the change of microsatellites in plants is single-strand slippage.[29] Since the chance of replication errors for a longer stretch of microsatellite repeat sequences is higher, a longer microsatellite repeat would tend to show more variation if single-strand slippage is considered an important mechanism.[31] This was tested by determining the relationship between the most frequent (mode) microsatellite allele (ancestral allele of the fragment) sizes and the standard deviation of the microsatellite allele sizes. A positive relationship (r=0.41044; p>0.072) was observed, which supported the view that single-strand slippage is an important mechanism in generating variability at microsatellite loci in date palm.

Another reason for the high degree of polymorphism observed at microsatellites in date palm could be attributed to the perennial life cycle of date palm. Unlike animals, plants do not have a germline as they grow, but rather somatic cells of meristamatic initials of the plant (from which reproductive organs are derived) divide and accumulate mutations,[32] resulting in greater diversity at microsatellite loci in the subsequent asexual progenies. Since this kind of genetic changes at microsatellite loci in somatic cells results in chimeras, the asexual progenies derived from chimeric tissues show a drastic increase in genetic differences at microsatellite loci compared with the mother plant, as shown by the cluster analysis (Fig. 2).

5. CONCLUSION

Based on our analysis of 30 date palm trees of Khalas cultivar from the GCC countries, we showed that the Khalas variety did not evolve independently from multiple origins, but rather it evolved from a single origin and clones were distributed across the GCC countries. The accumulated mutations of these clones over time led to drastic changes among them at the microsatellite loci.

Competing interests

The authors declare that there is no conflict of interest.

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