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Original article

Genetic diversity of endangered date palm (*Phoenix dactylifera* L.) in the oases of Nefzaoua, Tunisia, using SSR markers

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Summary

Introduction – **Date palm (*Phoenix dactylifera* L.) is a dioecious monocotyledon perennial fruit-bearing plant belonging to the palm family Palmae or Arecaceae. The present study consisted of the characterization of the genetic relationships among 21 endangered date palm genotypes from 11 oases of Nefzaoua, Southwestern Tunisia.** *Materials and methods* – **The genetic data was obtained using eight highly polymorphic SSR loci.** *Results and discussion* – **The results revealed that the eight pairs of primers used amplified 48 alleles in the accessions studied, showing a high level of polymorphism with little geographical structure among the oases analyzed. The number of alleles per locus ranged from three (mPdCIR063) to eight (mPdCIR078) with a mean value of 6 alleles per locus. The average of observed heterozygosity (Ho), ranged between 0.38 (mPdCIR078) and 0.80 (mPdCIR010), with a mean of 0.603. The average of expected heterozygosity (He) ranged between 0.54 (mPdCIR035) and 0.78 (mPdCIR025) with a mean of 0.682. UPGMA cluster analysis grouped the date palm accessions analyzed in four groups.** *Conclusion* – **Genetic diversity among the accessions studied is high, as found in previous studies, while the genetic structure is low and does not seem to follow a geographical pattern. The results validate the use of SSR markers in order to analyze the diversity of Tunisian endangered date palm germplasm and optimize conservation measures.**

Keywords
SSRs, characterization, diversity, endangered date palm, Nefzaoua, oases

Significance of this study

What is already known on this subject?

- Date palm (*Phoenix dactylifera* L.) is one of the earliest domesticated fruit-bearing crops and it is currently cultivated in many arid and semi-arid regions of the world, with significant nutritional, health, and economic value. Although its genetic diversity has been already studied in different areas of the world, in this work we use molecular analyses to alert on the risk of losing valuable germplasm in oases of south-western Tunisia.

What are the new findings?

- Genetic diversity among the accessions studied with SSR markers is high as found in previous studies, while the genetic structure is low and does not seem to follow a clear geographical pattern. This could be reflecting the high selection and exchange of date palm accessions between farmers in the region, since date palm is one of the principal economic and food resources of North African oases.

What is the expected impact on horticulture?

- The high degree of genetic variability shows high potential for further selection of endangered cultivars well adapted to local edaphoclimatic conditions before they disappear due to the trend to homogenize the new plantings using elite varieties, such as 'Deglet Nour'.

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious monocotyledon perennial fruit-bearing plant belonging to the palm family Palmae or Arecaceae, subfamily Coryphoideae, tribe Phoeniceae (Dransfield *et al.*, 2008). This species is diploid ($2n = 36$) (Siljak-Yakovlev *et al.*, 1996) and its genome size has been estimated around 650 Mbp (Al Dous *et al.*, 2011; Metoui *et al.*, 2017). The date palm is widely cultivated in arid and semi-arid regions of the Sahara and the Middle East (Barrow, 1998). Recently, it has been proposed that the populations of North African date palms are the product of introgressive hybridization between the cultivated date palm and the Cretan date palm *Phoenix theophrasti* Greuter, a species endemic to Crete and the Eastern Mediterranean (Flowers *et al.*, 2019). Date palm represents an important income to the oases' inhabitants, protects the under-crops from the negative effects of extreme high and cold temperatures, and reduces the damage from sand storms and wind erosion (Rhouma *et al.*, 2014). The date palm is one of the earliest domesticated fruit-bearing crops and it is currently cultivated in many arid and semi-arid regions of the world, due to its significant nutritional and health values (Shabani

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et al., 2016) and one of the most culturally and economically important crops of the Middle East and North Africa (Hazzouri *et al*., 2015).

Tunisia is one of the main date palm producing countries in the world and the first exporter in value (GiFruits, 2018). Moreover, date palm is the most important fruit crop in some regions of the country, such as Djerid and Nefzaoua, both as food and economic resource. Close to 250 date palm cultivars have been described in Tunisia (Rhouma, 1994, 2005). However, the increasing interest in a single variety, 'Deglet Nour', both for the local and international markets (Hamza, 2012; El Kadri, 2019), is accompanied by a considerable decline of the interest in other cultivars, in spite of their adaptation to different climatic factors such as a drought, water stress, or high soil salinity. Therefore, the extant diversity of date palms in Tunisian oases systems is currently endangered due to the severe genetic erosion resulting from the predominance of the elite 'Deglet Nour' cultivar in new plantings (Rhouma, 1994; Zehdi, 2004, 2012). Consequently, there is an urgent need to analyze the extant date palm diversity in Tunisian oases in order to establish appropriate germplasm conservation measures. Different works have addressed the use of either morphological traits (leave, spine and fruit characters) or isozyme markers to identify Tunisian date palm varieties (Reynes *et al*., 1994; Rhouma, 1994; Bouabidi *et al*., 1996; Ould Mohamed Salem *et al*., 2001; Zehdi *et al*., 2012). However, morphological studies of date palm are always difficult to estimate, due to the need of a wide set of phenotypic data (Hammadi *et al*., 2009), and sometimes some parameters can be influenced by climatic and environmental conditions. For this reason, molecular markers can provide more information on the genetic diversity of date palms, several of which can be hardly differentiated with phenotypic data. In this sense, microsatellites or simple sequence repeats (SSR) molecular markers have been proven to be very powerful in genotyping analysis because they are locus-specific, codominant, highly polymorphic and highly reproducible. These markers have also been developed and used to study the genetic diversity of male and female *Phoenix dactylifera* from Tunisia (Zehdi *et al*., 2004; Metoui *et al*., 2017; El Kadri *et al*., 2019). In this work, we report the use of SSR markers to identify female date palm genotypes and assess genetic diversity within 21 accessions in different oases of Nefzaoua, Southwestern Tunisia.

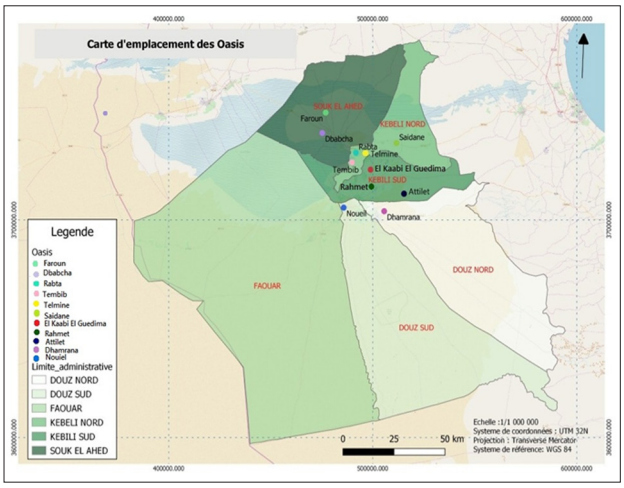

Figure 1. Geographic localization of the 21 date palm accessions studied in this work. Landmap: from OpenStreetMap, Qgis software version Qgis: Qgis 3.6 (Noosa), free multiplatform released under GPL license.

Since date palm is a dioecious species, usually growers maintain their own pollinizer male plants, selected mainly according to their overlap in flowering season with female cultivars, in their farms and often also bring pollen from other areas nearby to hand-pollinate female trees. However, genetic diversity of female plants in Tunisia is highly endangered due to the increasing predominance of the female cultivar 'Deglet Nour' (Hamza *et al*., 2012). As a consequence, some works have been performed to improve knowledge about the diversity of Tunisian female date palms (Hamza *et al*., 2011; Zehdi *et al*., 2004; Metoui *et al*., 2017), which are usually propagated vegetatively through vegetative shoots.

Date palm is a very important crop in these oases and, according to the Regional Commissionership for Agricultural Development (RCAD) of Kebili, 3.2 million palm trees were present in the oases of Nefzaoua in 2012.

Materials and methods

Collection of material

This study was carried out on twenty-one Tunisian date palm endangered accessions, originated in 11 different oases of Nefzaoua (a region belonging to the Kebili Governorate, Southwestern Tunisia) and with different phenotypic traits (Figure 1). Table 1 summarizes the geographical origin of the studied cultivars and Table 2 shows some morphological characteristics of the date palm genotypes studied. These twenty-one cultivars were chosen for their good fruit quality and because they are the most common genotypes in the oases of the studied region Nisia (Ferchichi *et al*., 2008).

DNA extraction

Genomic DNA of each genotype was extracted from dried young leaves. Total nuclear DNA was extracted according to a CTAB based method optimized by Hormaza (2002). After purification, DNA concentrations were determined using a Nanodrop ND-1000 UV-visible spectrophotometer. Resulting DNA solutions were stored at -20 °C.

PCR amplification and genotyping

Eight markers, developed by Billotte *et al*. (2004), were used to study the genetic relationships of the accessions analyzed: mPdCIR010, mPdCIR015, mPdCIR025, mPdCIR032, mPdCIR035, mPdCIR057, mPdCIR063 and mPdCIR078. These loci were selected based on their polymorphic information content among SSR loci previously developed in date palm. Primer sequences are included in Table S1. PCR reaction had a final volume of 15 µL, containing: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 0.01% Tween 20, 2 mM $MgCl_2$, 0.4 mM each primer, 0.1 mM each dNTP, and 0.5 units of BiotaqTM DNA polymerase (Bioline, London, UK). Amplifications were performed in a thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following temperature profile: an initial step of 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature reported in Billote *et al*. (2004) and 1 min at 72 °C, and a final step of 5 min at 72 °C. The amplification products were resolved using a CEQ™ 2000XL capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). After labeling forward primers with a D2, D3 or D4 fluorescent WellRED dye (Sigma-Aldrich, St. Louis, MO, USA) on the 5´-end. Samples were denaturalized at 90 °C for 120 s, injected at 2.0 kV for 30 s, and separated at 6.0 kV for 35 min. Each PCR reaction and capillary

Table 1. Names and localities of origin of the 21 Tunisian endangered date palm accessions analysed in this study.

No.	Accession name	Code	Locality of origin (Delegation)	Oasis name
1	Hissa	His	North Douz	Dhamrana
2	Chabihat Dagla	Cdg	South Kebeli	Attilet
3	Gonda	Gnd	Souk Lahad	Dbabcha
4	Tekremest	Tks	South Douz	Nouiel
5	Gares Souf	Gsf	North Douz	Dhamrana
6	Ammari	Amm	North Kebeli	Saidane
7	Kssebba	Ksb	North Kebeli	Rabta
8	Chaddakh	Cdk	North Kebeli	Tembib
9	Bidh Hmem	Bdh	South Kebeli	Tembib
10	Tezerzeyet	Tzs	Souk Lahad	Faroun
11	Fermla	Frm	North Kebeli	Rabta
12	Korkobi	Krb	South Kebeli	Attilet
13	Malti	Mlt	North Kebeli	Rabta
14	Hamra	Hmr	Souk Lahad	Faroun
15	Gosbi	Gsb	Souk Lahad	Dbabcha
16	Horra	Hor	South Kebeli	Rahmet
17	Fezzani	Fez	South Kebeli	Rahmet
18	Chaddakh Ben Jbir	Cdj	South Kebeli	El Kaabi El Guedima
19	Tronji	Trj	North Kebeli	Telmine
20	Rtob Houth	Rth	South Kebeli	El Kaabi El Guedima
21	Kechdou	Kcd	North Kebeli	Tembib

Table 2. Name, origin, and main characteristics of date-palm genotypes studied (Rhouma, 1994, 2005; Ferchichi and Hamza, 2008).

Accession name	Period of maturity	Color	Consistency
Hissa	Early	Honey	Soft
Chabihat Dagla	Later	Amber	Semi-soft
Gonda	Season	Amber	Semi-soft
Tekremest	Later	Black	Soft
Gares Souf	Season	Dark brown	Soft
Ammari	Early	Black	Soft
Kssebba	Season	Dark brown	Semi-soft
Chaddakh	Season	Dark amber	Soft
Bidh Hmem	Season	Amber	Soft
Tezerzeyet	Season	Black	Soft
Fermla	Season	Brown	Semi-soft
Korkobi	Late	Brown	Dry
Malti	Season	Dark amber	Soft
Hamra	Season	Amber	Semi-soft
Gosbi	Early	Black	Soft
Horra	Season	Amber	Dry
Fezzani	Season	Amber	Semi-soft
Chaddakh Ben Jbir	Season	Dark amber	Soft
Tronji	Late	Dark brown	Semi-soft
Rtob Houth	Season	Amber	Soft
Kechdou	Season	Dark brown	Dry

electrophoresis was repeated at least twice to ensure the reproducibility of the results.

Data analysis

1. Genetic diversity analyses. Genetic diversity was estimated through the calculations of several indexes: number of alleles (A), observed heterozygosity (H_0, calculated as the number of heterozygous genotypes over the total number of genotypes analyzed for each locus), expected heterozygosity ($H_e = 1 - \sum pi^2$), where pi is the frequency of the i^{th} alleles in the cultivars (Nei, 1973); Wright's fixation index ($F = 1 - H_0/H_e$) (Wright, 1965), and departure from HWE (Hardy-Weinberg Equilibrium). Calculations were computed with R using the packages Adegenet (Jombart, 2008) and PopGenReport v. 2.0 (Adamack and Gruber, 2014).

2. Genetic relationships. Genetic relationships within the accessions studied were calculated using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) clustering analysis based on the similarity matrix obtained with the Dice index (Nei and Li, 1979), and principal component analysis (PCA) using the program NTSYS 2.11 (Exeter Software, Stauket, NY). The cophenetic correlation coefficient was estimated by comparing with the Mantel test the cophenetic matrix obtained from the dendrogram with the original similarity matrix.

3. Genetic structure. Genetic structure among the accessions was analyzed using the software Structure v. 2.3.4 (Pritchard *et al*., 2000), which performs a Bayesian analysis using the allelic frequencies and assuming Hardy-Weinberg and linkage equilibrium between loci within populations. This program permits to determine the number of possible populations (K) and the probability of each sample belonging to each population. The program was run 5 times, setting K (number of populations) from 1 to 10 with the admixture model and 10 replications per K. Each run was implemented with a burn-in period of 10,000 steps followed by 100,000 Monte Carlo Markov Chain (MCMC) replicates (Pritchard *et al*., 2010).

Results and discussion

The results obtained in this study show the usefulness of molecular markers to characterize and evaluate the diversity of date palms from different localities in the oases of Nefzaoua.

Genetic diversity indexes

The 8 SSR loci analyzed produced 48 alleles with an average of 6 fragments per locus among the twenty-one cultivars studied. The number of alleles ranged from three to eight. The allelic range size ranged from 119 bp in mPdCIR063 to 302 bp in mPdCIR032. The parameters of variability analyzed are presented in Table 3.

The total number of SSR alleles found in this study (48, varying from 3 to 8 with an average of 6 alleles per locus) is slightly higher than those found studying the genetic diversity of 12 female cultivars of date palms from southern Tunisia with the same SSR loci (42 alleles, varying from 3 to 7 with an average mean of 5.4 alleles per locus) (El Kadri *et al*., 2019). In another study using 22 SSR loci to study genetic relationships among 32 date palms representing common cultivars grown in different geographical regions in Saudi Arabia, 2 to 6 alleles per locus with a mean of 4.14 were encountered (Al-Faifi *et al*., 2016). Other studies have reported a higher number of alleles per locus, such as those established by Zehdi *et al*. (2012, 2004).

High levels of expected (H_e) and observed (H_0) heterozygosity were observed. H_0 values ranged from 0.38 (mPdCIR078) to 0.80 (mPdCIR010) with a mean of 0.60. H_0 showed high values except for mPdCIR078 and mPdCIR035. The average of expected heterozygosity (H_e) ranged between 0.54 (mPdCIR035) to 0.78 (mPdCIR025), with a mean of 0.68, indicating a high degree of genetic diversity among the endangered date palm accessions in the oases of Nefzaoua. H_0 was less than H_e for five loci (mPdCIR025, mPdCIR032, mPdCIR035, mPdCIR063 and mPdCIR078), indicating an excess of homozygosity compared with that expected under random mating and showing positive Fis values: 0.26, 0.19, 0.22, 0.12 and 0.45, respectively (Table 3). In this case, the deviation from Hardy-Weinberg equilibrium was significant for mPdCIR010, mPdCIR025, mPdCIR035, and mPdCIR078 ($p < 0.01$).

The H_e value found in this study (0.68) is similar to the values reported by other studies for Saudian (0.67) (Al-Faifi *et al*., 2016) or Tunisian date palm cultivars: 0.61 (Metoui *et al*., 2017; El Kadri *et al*., 2019) and 0.63 (Hamza *et al*., 2011a). Likewise, the mean H_0 value (0.6) is similar to other studies in Tunisia: 0.67 (Metoui *et al*., 2017), 0.54 (El Kadri, 2019) and 0.7 (Hamza *et al*., 2011a).

These results mean that the Tunisian date palms analyzed are characterized by a high degree of genetic variability (Hamza *et al*., 2011b) which could be explained by an intensive selection of heterozygous trees. According to Zehdi *et al*. (2004), the scored values of diversity are higher at the intra group level than at the inter group level. The same results have been reported in Moroccan, Algerian and Tunisian date palm cultivars using isozyme markers (Bennaceur *et al*., 1991; Fakir, 1992; Ould Mohamed Salem *et al*., 2001). In fact, this genetic diversity values demonstrates a good potential for further enhancing the agronomic characters of date palms (Elshibli and Korpelainen, 2008) that at the end would permit to select some varieties better adapted to the harsh climatic conditions of these oases (Hamza *et al*., 2011a).

Genetic relationships

Four main groups among the genotypes analyzed could be observed in the UPGMA dendrogram obtained (Figure 2). The cophenetic correlation between the cophenetic coefficient and the similarity matrix was $r = 0.8$. The first cluster included eleven cultivars ('Hissa', 'Gonda', 'Fezzani', 'Gosbi', 'Ammari', 'Hamra', 'Kechdo', 'Kssebba', 'Malti', 'Fremla' and 'Bidh Hmem'), while the second cluster comprised three genotypes ('Chabihat Dagla', 'Korkobi' and 'Horra', although the first two seem to be synonyms). The third cluster was

Table 3. Genetic diversity indices for eight microsatellite loci revealed in the studied Tunisian date palm genotypes (A: observed number of alleles per locus; H_0: observed heterozygosity; H_e: expected heterozygosity; Fis: fixation index; and HWE: Hardy-Weinberg Equilibrium).

SSR locus	Size (bp)	A	H_0	H_e	Fis	Departure from HWE
mPdCIR010	121–161	7	0.80	0.69	-0.15	0.003
mPdCIR015	120–136	6	0.76	0.69	-0.10	0.055
mPdCIR025	200–232	6	0.57	0.78	0.26	0.00
mPdCIR032	288–302	6	0.57	0.71	0.19	0.161
mPdCIR035	180–196	5	0.42	0.54	0.22	0.009
mPdCIR057	250–268	7	0.76	0.7	-0.08	0.0105
mPdCIR063	119–151	3	0.57	0.65	0.12	0.749
mPdCIR078	117–149	8	0.8	0.7	0.45	0.00
Mean		6	0.603	0.82	0.11	

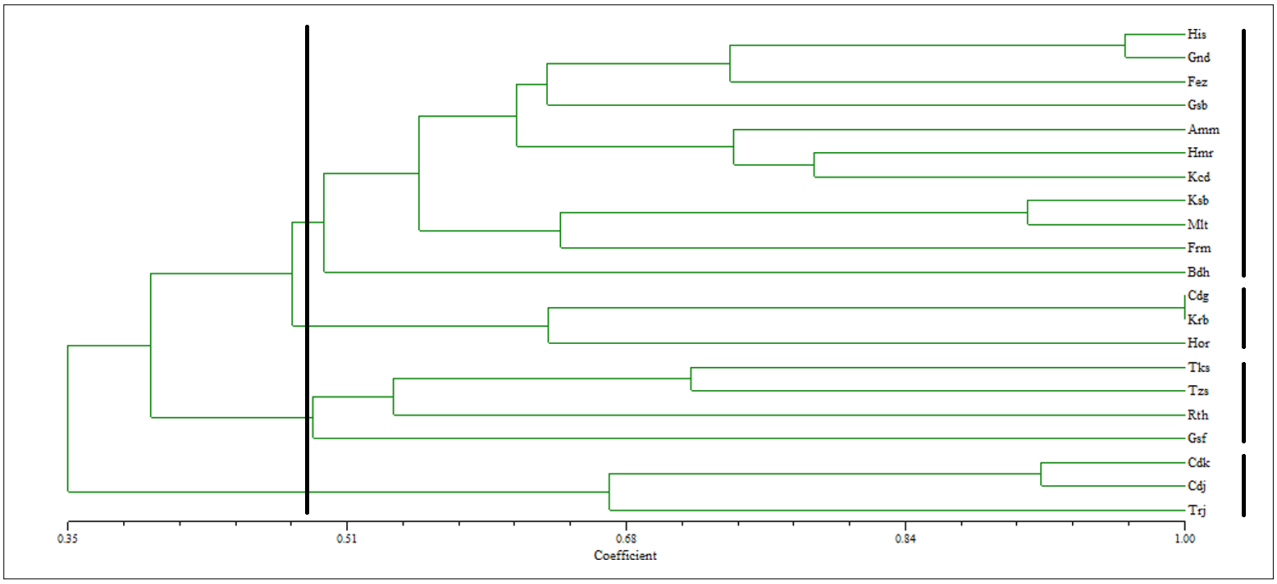

Figure 2. UPGMA dendrogram of the genetic relationships among 21 date palm accessions of oases of Nefzaoua based on the Dice similarity index (Nei and Li, 1979). Black bars indicate the different defined main clusters.

composed of four genotypes, 'Tekremest', 'Tezerzeyet', 'Rtob Houdh' and 'Gares Souf'. Finally, the fourth group includes tree genotypes ('Chaddakh', 'Chaddakh ben Jbir' and 'Tronj'). The highest genetic similarity observed (100%) was found between 'Chabihat Dagla' and 'Korkobi', which are the only samples collected in the Attilet oasis and, consequently, it could be a case of synonymy. The lowest genetic similarity (10%) was detected between 'Chaddakh Ben Jbir' and 'Horra'.

The Principal Component Analysis (PCA) shows that the three first principal components explain 55.22% of the total variability. The contributions of PCA1, PCA2 and PCA3 were 32.20, 12.06 and 10.95 %, respectively. Figure 3 shows the distribution of accessions according to the first two components (PCA1 and PCA2), in which four groups that almost correspond to those found in the UPGMA analysis can be identified. The largest included all the 11 samples included in the first group of the UPGMA dendrogram, obtained from different oases of Nefzaoua. The second cluster just included

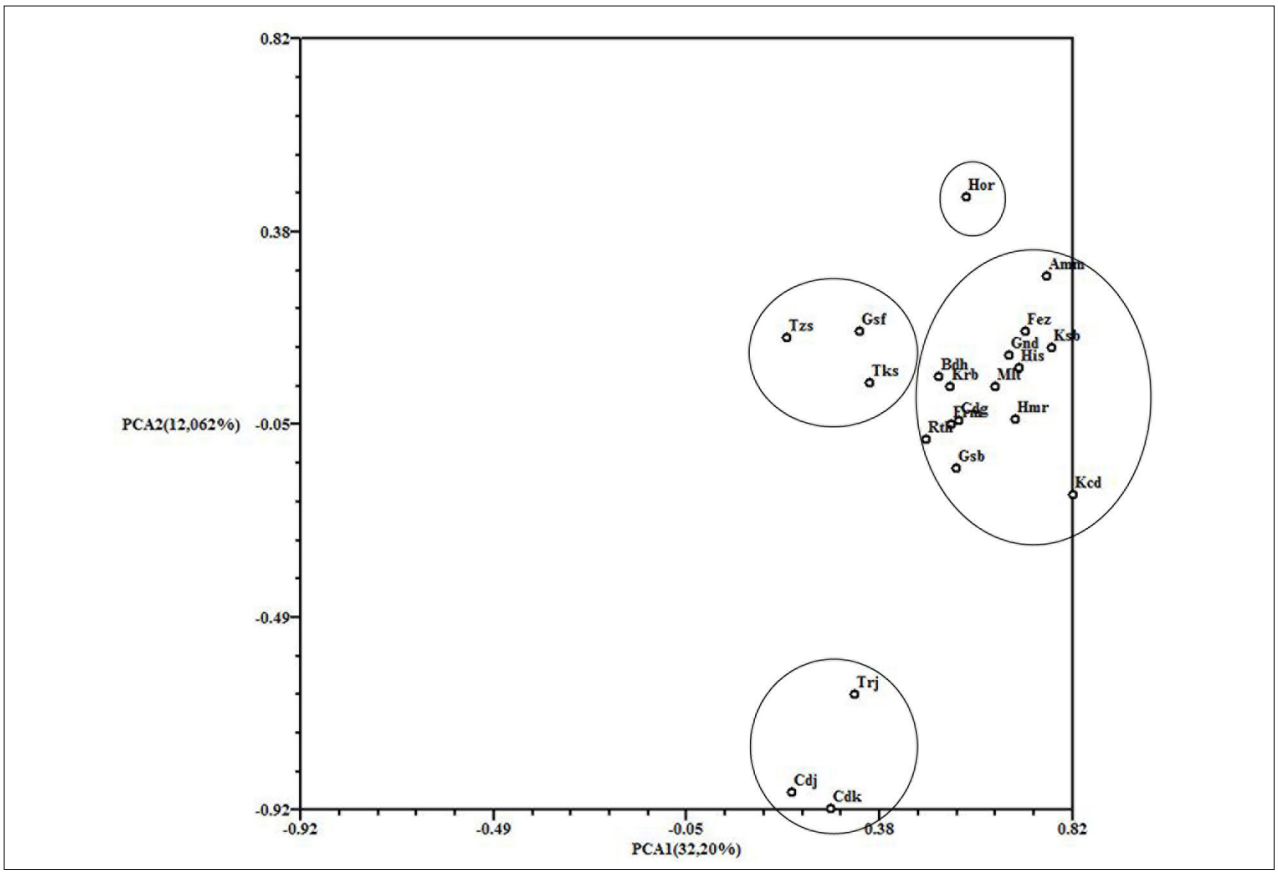

Figure 3. Principal component analysis grouping 21 common date palm accessions of oases of Nefzaoua based on SSR markers.

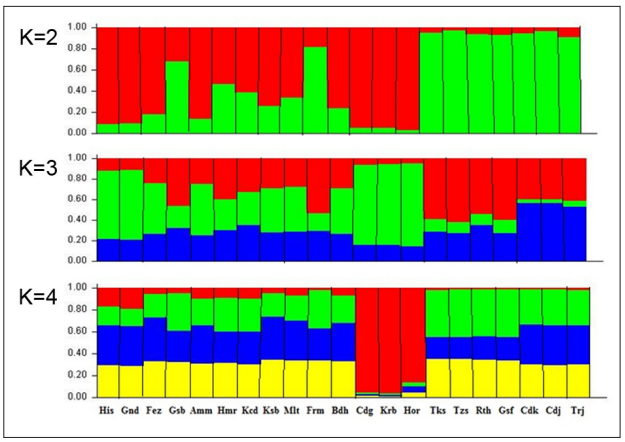

Figure 4. Population structure analysis of 21 date palm genotypes of oases of Nefzaoua using STRUCTURE 2.3.4. Populations derived from STRUCTURE analysis are color-coded. Each accession is represented by a vertical colored bar (K = number of populations).

one sample, 'Horra', which in the UPGMA dendrogram was grouped with the synonyms 'Chabihat Dagla' and 'Korkobi', which now appear in the first cluster. Another cluster was similar to the third UPGMA cluster, except one accession, 'Rtob Houdh', that appears now in the first group. Finally, cluster 4, the most differentiated from the rest, contains only three cultivars ('Chaddakh', 'Chaddakh ben Jbir' and 'Tronji'), which were sampled in geographically very close oases (Tembib, Telmine and El Kaabi El Guedima), and that also form a separated cluster in the UPGMA tree.

Genetic structure

The genetic structure of the accessions analyzed was determined using STRUCTURE software v. 2.3.4. The results for K = 2, 3 and 4, showed a weak genetic structure among the accessions (Figure 4). For K = 2, two populations are distinguished which do not show relation to a geographic pattern. For K = 3 and K = 4, these two populations are no longer found, while only three accessions which also clustered together and separately from the others in the UPGMA dendrogram ('Horra' and the synonyms 'Chabihat Dagla' and 'Korkobi'), seem to differentiate from the rest. Thus, it seems that no clear genetic structure can be found among the accessions analyzed.

The results of the UPGMA dendrogram, Principal Component Analysis and genetic structure indicate that the accessions group independently from their geographic origin, as also proposed by Zehdi *et al*. (2004) based on the existence of one ancestral date-palm population with a unique Mesopotamian domestication origin of this crop (Wrigley, 1995) and a more recent expansion to the African continent (Flowers *et al*., 2019).

Conclusion

In this study, eight simple sequence repeat loci have been used to study the genetic diversity and structure of 21 date palm accessions from eleven different oases of Nefzaoua in the Kebili governorate, situated in Southwestern Tunisia. Similar to previous studies (Trifi *et al*., 2000; Zehdi *et al*., 2004; Hamza *et al*., 2011b), genetic diversity among the accessions studied is high while the genetic structure is low and does not seem to follow a geographical pattern. In fact, several genetic diversity analyses of Tunisian date palms conclude that they could constitute a unique population (Trifi *et al*., 2000; Zehdi *et al*., 2004; Hamza *et al*., 2011). This could be reflecting the high selection and exchange of date palm accessions between farmers in the region, since date palm is one of the principal economic and food resources of the oases. The high degree of genetic variability shows high potential for further selection of endangered cultivars well-adapted to local edaphoclimatic conditions before they disappear due to the trend to homogenize the new plantings using 'Deglet Nour'.

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Supplemental Information – Table S1. Primer name, repeat motif and primer sequences of the primers used in this study (taken from Billotte *et al.*, 2004). Related to Experimental Procedures.

Primer name	Repeat motif	Primer sequence
mPdCIR010	$(GA)_{22}$	F: ACCCCGGACGTGAGGTG R: CGTCGATCTCCTCCTTTGTCTC
mPdCIR015	$(GA)_{15}$	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT
mPdCIR025	$(GA)_{22}$	F: GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC
mPdCIR032	$(GA)_{19}$	F: CAAATCTTTGCCGTGAG R: GGTGTGGAGTAATCATGTAGTAG
mPdCIR035	$(GA)_{15}$	F: ACAAACGGCGATGGGATTAC R: CCGCAGCTCACCTCTTCTAT
mPdCIR057	$(GA)_{20}$	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC
mPdCIR063	$(GA)_{17}$	F: CTTTTATGTGGTCTGAGAGA R: TCTCTGATCTTGGGTTCTGT
mPdCIR078	$(GA)_{13}$	F: TGGATTTCCATTGTGAG R: CCCGAAGAGACGCTATT