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Comparison of the effectiveness of ISSR and SSR markers in determination of date palm (*Phoenix dactylifera* L.) agronomic traits

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Abstract

Date palm (*Phoenix dactylifera*L.) is extensively cultivated in the Middle East and in North Africa. Seven ISSR markers and five SSR loci were selected and used to evaluate genetic diversity in twenty-six Tunisian cultivars. ISSR primers amplified a total of 43 polymorphic DNA fragments. The average was 6.1 fragments per primer. The microsatellites examined in this study were highly polymorphic possessing a great number of alleles with an average of 7.2 alleles per locus. Principal component analyses based on Nei Genetic distances showed groups of cultivars with a common maturity period and a common fruit consistency. SSR markers discriminate the fruit characteristics subpopulations in a more convincing way than ISSR markers. The Mantel test emphasizes a significant correlation between genetic distance and fruit consistency. A significant difference was observed between soft and dry subpopulations using ISSR data and between semisoft and the other fruit consistency subpopulations using SSR data.

Keywords: AMOVA, fruit consistency, Mantel test, maturity period, molecular markers.
Abbreviations: analysis of molecular variance (AMOVA), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), random amplified polymorphic DNA (RAPD), principle component analysis (PCA), random amplified microsatellites polymorphism (RAMPO), amplified fragment length polymorphism (AFLP).

Introduction

The date palm (*Phoenix dactylifera* L.) is one of the most important fruit trees in the arid and semi-arid regions (Munier, 1973). It is extensively grown as a food crop and covers about 3% of the cultivated area of the world (Dowson, 1982). This species has a great value for populations in the Middle East and the north of Africa where it has been intensively cultivated. It provides a wide range of products and services, including many necessities of life. Historical investigations suggest that date palm domestication has been practiced for five millennia (Nixon, 1959). The date palm trunk is bounded by leaf bases, and it can grow to 20m in many localities (Munier, 1973). The leaves cover the top of the plant, and they are pinnate with spines at the base. The fruit is a 'berry' with a single seed in each. The fruit is borne on a bunch, and a productive palm can support up to 13 bunches. The ripening time depends on the cultivar, but generally it takes up to 200 days from the date of pollination to the Tamer stage (Hamza et al., 2009). The date palm has a wide range of geographical and ecological distribution, which is pronounced by its considerable genetic diversity (Elshibli and Korpelainen, 2008). This diversity is the result of the dioecious nature (separate male and female plants) of the specie (Munier, 1981). Many cultivars have been characterized in several areas. This characterization has involved many morphological and molecular tools. Concerning the phenotypic markers, many traits were used to characterise the morphology of leaves, spines and fruit characters. Such morphological features are sensitive to environmental factors (Sedraet al., 1993; 1996), and they can be observed only in mature trees. In Tunisia many reports, have described the importance of morphological traits in identifying the Tunisian date palm cultivars (Ben Salah, 1993; Ben Salah and Hellali, 2004; Rhouma, 2005). In spite of these descriptions, it remains difficult to identify cultivars, especially outside of the fruiting period. In fact, owing to the great adaptive flexibility of this genus, many farmers cannot recognise cultivars outside their oasis (Munier, 1973). However, Hamza et al. (2009) have selected vegetative traits that are steady and unaffected by the environment, and these have been very useful in identifying the fruit maturity period and the consistency. Molecular markers may provide a reliable tool for measuring genetic divergence. Several markers have been used, including random amplification polymorphism DNA (RAPD) (Sedra et al., 1998; Trifi et al., 2000; Al-Khalifa and Askari, 2003), inter simple sequence repeats (ISSR) (Zehdi et al., 2002), random amplified microsatellites polymorphism (RAMPO) (Rhouma, 2008) and amplified fragment length polymorphism (AFLP) (Rhouma et al., 2007). These studies revealed a high polymorphism among date palm cultivars. Simple sequence repeat (SSR) was very useful to identify date palm cultivars, and a high polymorphism has been detected in date palm cultivars (Zehdi et al., 2004; Hamza et al., 2011). All these molecular markers showed a high degree of independence with the geographical origin. The purpose of the present study was to examine the genetic diversity in date palms using two markers: ISSR and SSR. A comparative study was done to search possible relations between the fruit characteristics of *Phoenix dactylifera* L. in Tunisian oases.

Results

A total of seven ISSR primers were screened for their ability to generate consistently amplified band patterns and to assess polymorphism in the tested cultivars. All the primers used have revealed unambiguously scorable polymorphic bands. In

Table 1. List of the ISSR primers and SSR loci and their characteristics.

Primers / locus		Sequence of primer (5'–3') or Repetitive motif	A	Ap	P
ISSR	D9	$(CT)_{10}G$	8	5	62.5%
	D12	$(GA)_6CC$	5	4	80%
	UBC 888	$(GCT)(AGT)(GCT)(CA)_7$	6	5	83.3%
	UBC 890	$(AGC)(ACT)(AGC)(GT)_7$	7	7	100%
	UBC 891	$(ACT)(AGC)(ACT)(TG)_7$	6	6	100%
	PO6	$(AG)_{10}C$	5	3	60%
	PO7	$(AG)_{10}T$	6	4	66.6%
SSR	mPdCIR10	$(GA)_{22}$	8	8	100%
	mPdCIR15	$(GA)_{15}$	6	6	100%
	mPdCIR32	$(GA)_{19}$	7	7	100%
	mPdCIR70	$(GA)_{17}$	8	8	100%
	mPdCIR93	$(GA)_{16}$	7	7	100%

A: Total number of Bands or alleles, Ap Number of polymorphic bands or alleles, P: Percentage of polymorphic Bands or alleles.

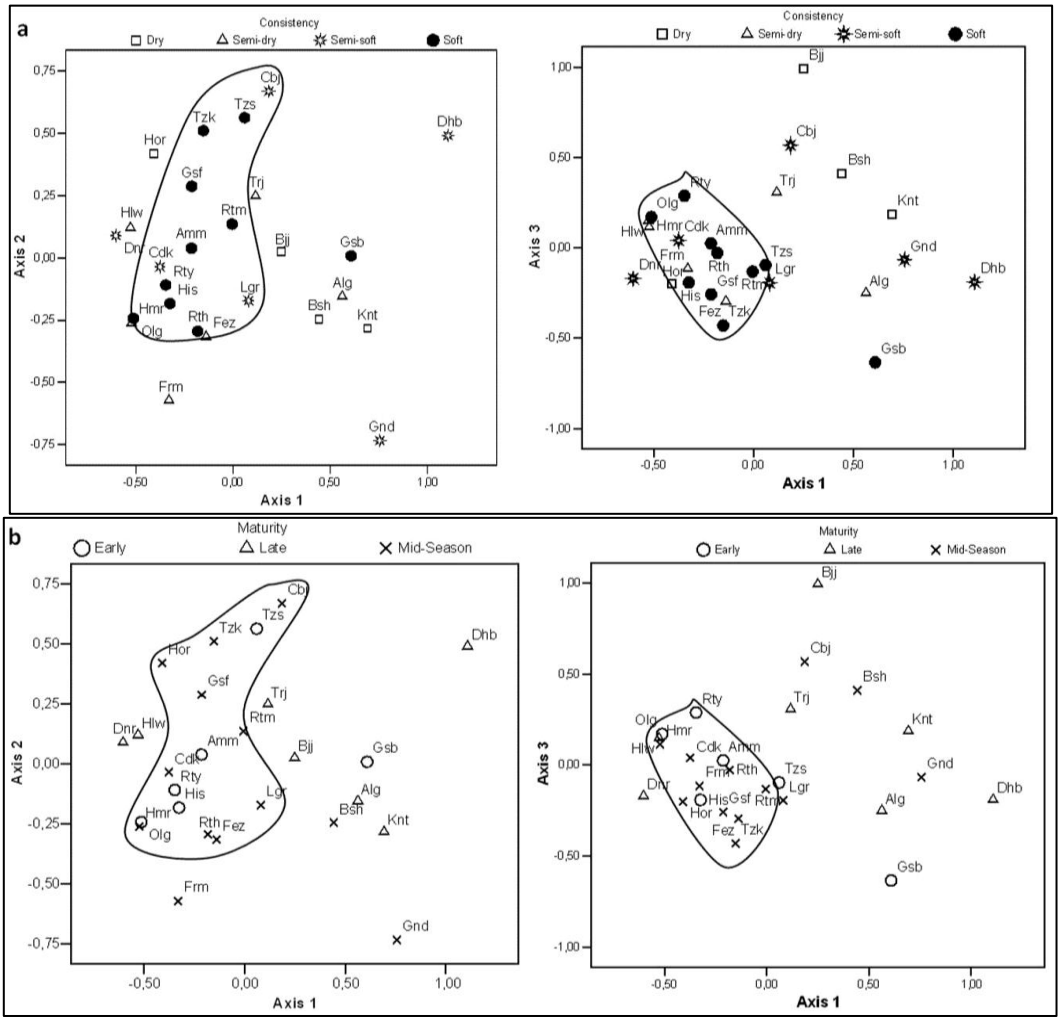

Fig 1. Cultivars distribution on the plan 1-2 and 1-3 of PCA based on ISSR-amplified loci. (a) The scores of fruit-consistency subpopulations (b) The scores of maturity-period subpopulations.

fact, the seven primers amplified a total of 43 polymorphic fragments ranging from 250 to 2,500 bp. The maximum number of fragments was 8 bands that are produced by the primer D9 with 62.5% polymorphism. The minimum number of fragments was 5 bands produced by the primers D12 and PO6 with, respectively, 80 and 60% polymorphism (Table 1). The average was 6.1 ISSR bands per primer. Trimer primers like UBC ones produced the maximum polymorphism; for example, UBC 890 and UBC 891 amplified 100% of polymorphic bands. Concerning SSR profiles, five loci exhibited 36 alleles with an average of 7.2 alleles per locus. The microsatellite markers were found to be highly polymorphic with the number of alleles ranging from six to eight among the 26 cultivar genotypes. All the used loci showed 100% polymorphism (Table 1). Nei genetic distances were used to analyse the variability of the studied cultivars by a principal component analysis. Concerning the ISSR markers, the data showed that the first three axes of the PCA explained 62.51% of the total variability. The distribution of cultivars (Fig. 1) shows a difference between the Dhahbi

cultivar and the others. This cultivar was noted only in the Tamerza oasis (Table 2). No other geographical group was distinguished; the different cultivars are classified regardless of their oasis. However, we can distinguish clusters of cultivars according to their fruit characteristics. In fact, PCA results show a grouping of cultivars with soft or semisoft fruits and with early or mid-season maturities (Fig. 1). On the other hand, the cultivar distribution based on SSR data was, also, independent of the geographical origin. In the scattergram, a subpopulation separation can be observed. For the fruit consistency, the subpopulations are more easily grouped (Fig. 2).Concerning the maturity period, cultivars with early and mid-season maturity are associated (Fig. 2).

Mantel test

The null hypothesis of no correlation between different matrices was tested (Table 3). The ISSR data showed a significant correlation with the fruit consistency matrix (r = -0.120; P = 0.026) but not with the maturity period matrix (r = - 0.054; P = 0.225). The same result was found with the SSR data, in which a significant correlation was found with the consistency matrix (r = 0.110; P = 0.029) but not with the maturity matrix (r = -0.027; P = 0.382). However, no significant correlation was found between the ISSR and SSR data (r = -0.108; P = 0.151).

AMOVA

According to ISSR data, no significant genetic difference (P > 0.05) among fruit characteristics subpopulations was observed, although 3% of total genetic diversity was detected among fruit-consistency subpopulations. Pair-wise comparisons of subpopulations (Table 4) showed that the soft subpopulation and the dry one are significantly different. AMOVA tests based on SSR data showed no genetic differentiation among maturity-period subpopulations; however, significant genetic differentiation was observed among the fruit-consistency subpopulations (P < 0.05) with 7% of total genetic diversity detected among fruit-consistency subpopulations. Pair-wise comparisons of populations (Table 5) show that significant genetic differences exist between the semi-soft subpopulation and the semi-dry and soft groups. Another significant genetic differentiation was detected between semi-soft groups and early-season groups.

Discussion

Molecular markers are efficient tools for cultivar identification and estimation of relatedness through DNA fingerprinting. In the present investigation, ISSR and SSR were employed to assess genetic polymorphisms in Tunisian date palm cultivars. ISSR is a dominant marker that has, in comparison with RAPD techniques, high reproducibility (Williams et al., 1990). In the field of date palms, ISSR markers were found more informative than the RAPD markers (Mitra et al., 2011). Many ISSR primers were tested in the literature and applied on date palm genotypes (Zehdi et al., 2002; Mitra et al., 2011) or on other monocotyledon species such as the genus *Poa* (Arslanet al., 2011) and durum wheat (Pasqualone et al., 2000). A total of seven primers were screened for ISSR-PCR analysis, and they were useful to characterize the samples and produced strongly amplified polymorphic bands. The selected primers generated an appropriate amplification pattern with clear and consistent reproducible bands. In the present study, the number of polymorphic bands (34 bands) and the average number of fragments produced per primer (6.14) are less than those obtained in previous studies of date palms (Zehdi et al., 2002; Mitra et al., 2011). On the other hand, our SSR results indicate the presence of high genetic diversity in Tunisian date palms but less than in Sudanese date palms (Elshibli and Korpelainen, 2008). This may be explained by intensive selection in Tunisian date palm oases (Zehdi et al., 2004). This result agrees with other reports for Moroccan, Algerian and Tunisian date palm cultivars based on analyses using microsatellite markers (Zehdi et al., 2004) and isozyme markers (Bennaceur et al., 1991; Ould Mohamed Salem et al., 2001). The numbers of alleles per locus detected in this study were comparable with those scored by Zehdi et al. (2004): for 46 Tunisian date palm accessions, 100 different alleles were identified at 14 microsatellite loci with an average of 7.14 alleles per locus. In addition, a high degree of independence between the geographical origin and molecular data was indicated. The only distinction is in the case of ISSR data for the Dhahbi cultivar, which is a specific variety of continental mountain oasis (Tamerza), where it is intensively cultivated. The RAMPO and AFLP data applied on Tunisian date palms (Rhouma et al., 2007; Rhouma-Chatti et al., 2011) showed that the studied cultivars clustered independently from their geographic origin. This is in favour of the hypothesis proposed by Wrigley (1995), which suggests a common genetic base of the cultivars in the Tunisian continental oasis. The lack of basic geographical differentiation is explained by the fact that communication often facilitates the exchange of plant material in the studied oases. Molecular studies have proved the efficiency of the molecular markers to assess genetic diversity between date-palm genotypes. However, few studies have shown a correlation between molecular and phenotypic markers. Mirta et al. (2011) have underscored with RAPD and ISSR markers a discrimination of date palm cultivars on the basis of the tree sex. However, Rhouma-Chatti et al. (2011) did not show a significant discrimination of male trees using AFLP and RAMPO markers. The current result unveils a significant correlation between the genetic data and the fruit consistency as shown by the Mantel test, which is partly supported by PCA analysis. The correlation between SSR markers and fruit consistency is more convincing than the ISSR study. These results allowed us to compare genetic structure between groups based on fruit characteristics. The ISSR and the SSR data showed that no genetic differentiation was observed among maturity period subpopulations. However, when the fruit consistency subpopulations were compared, a significant differentiation was detected. Indeed, soft and dry subpopulations were genetically differentiated on the basis of ISSR data (*PhipT* = 0.126, P < 0.05). Another differentiation trend is revealed in this study using SSR data; the F_{ST} values suggest significant genetic differentiation between subpopulations. The semi-soft subpopulation was significantly differentiated from the other fruit-consistency subpopulations and from the early-maturity subpopulation. The discrepancy between molecular markers and maturity period support the statement that the maturity period could be affected by the local environment, whereas the used markers are not, and their variation is based directly on DNA sequence variation (Bruschi et al., 2003).

Material and Methods

Plant materials

Cultivars belong to the continental Tunisian oases were chosen for their fruit importance (Ferchichi and Hamza,

Table 2. Name, origin, and main characteristics of date-palm genotypes studied.

No.	Name	Code	Geographical distribution	Fruit characteristics (at Tamer stage)		
				Colour	Consistency	Maturity period
1	Alig	Alg	Nefzoua&Jerid	Dark brown	Semi-dry	Late
2	Ammary	Amm	Nefzoua&Jerid	Black	Soft	Early
3	Bejjou	Bjj	Nefzoua&Jerid	Brown	Dry	Late
4	BissrHelou	Bsh	Nefzoua&Jerid	Pale brown	Dry	Season
5	Choddakh	Cdk	Nefzoua&Jerid	Dark Amber	Semi-Soft	Season
6	Choddakh Ben Jbir	Cbj	Nefzoua&Jerid	Dark Amber	Semi-soft	Season
7	Dhahbi	Dhb	Temerza	Amber	Semi-soft	Late
8	DegletNour	Dnr	Nefzoua&Jerid	Amber	Semi-soft	Late
9	Fermla	Frm	Nefzoua	Brown	Semi-dry	Season
10	Fezzani	Fez	Nefzoua&Jerid	Amber	Semi-dry	Season
11	Gondi	Gnd	Nefzoua&Jerid	Amber	Semi-soft	Season
12	Gosbi	Gsb	Nefzoua&Jerid	Black	Soft	Early
13	Gharssouf	Gsf	Nefzoua&Jerid	Dark brown	Soft	Season
14	Hissa	His	Nefzoua&Jerid	Honey	Soft	Early
15	Hlwa	Hlw	Nefzoua	Honey	Semi-dry	Late
16	Hamra	Hmr	Nefzoua&Jerid	Amber	Semi-dry	Season
17	Horra	Hor	Nefzoua&Jerid	Amber	Dry	Season
18	Kintichi	Knt	Jerid	Reddish	Dry	Late
19	Loghrabi	Lgr	Jerid	Dark brown	Semi-soft	Season
20	Om Leghlez	Olg	Jerid	Amber	Soft	Early
21	RtobHoudh	Rth	Nefzoua&Jerid	Amber	Soft	Season
22	Rtotbayetelmansoura	Rtm	Nefzoua	Brouwn	Soft	Season
23	Rotbayetyagouta	Rty	Nefzoua	Dark amber	Soft	Early
24	Tronja	Trj	Nefzoua&Jerid	Dark brown	Semi-dry	Late
25	TezerzayetKahla	Tzk	Nefzoua&Jerid	Black	Soft	Season
26	TezerzayetSafra	Tzs	Jerid	Dark brown	Soft	Early

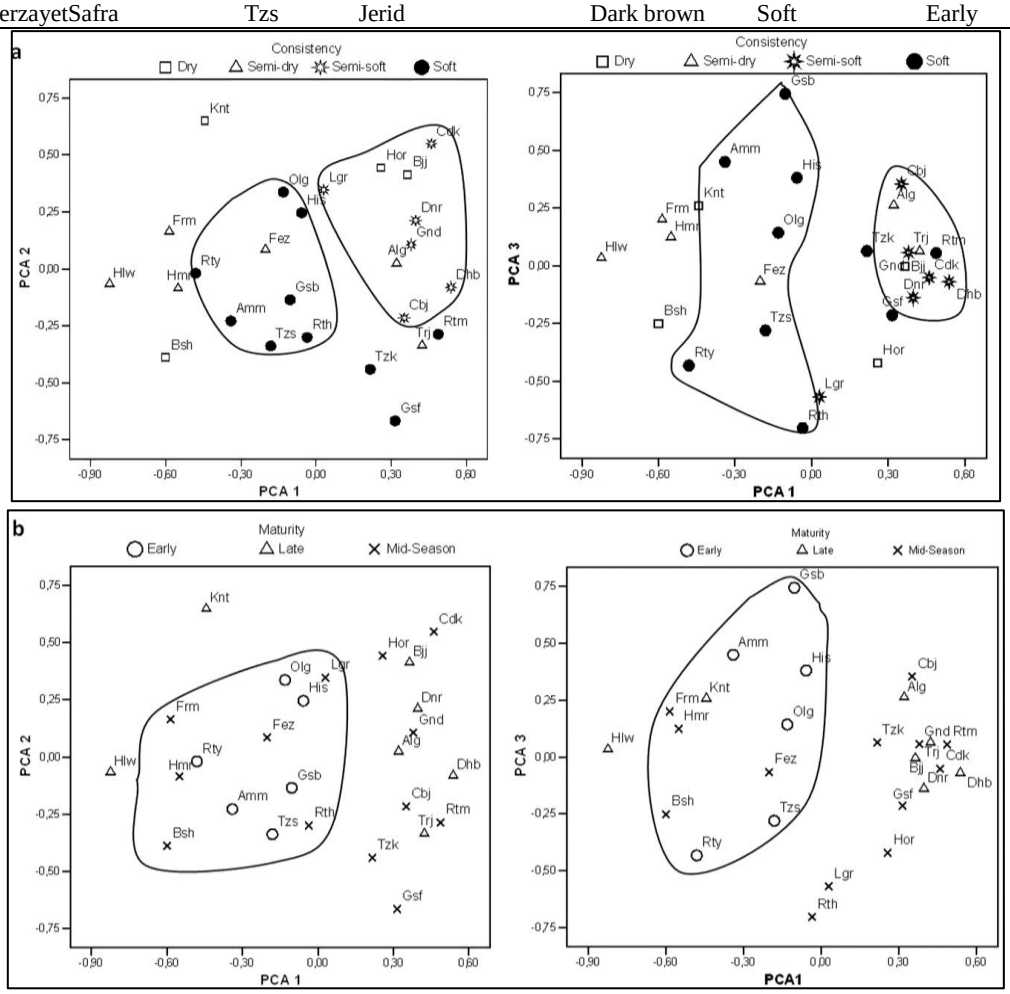

Fig 2. Scattergram showing relative position of date palm cultivars defined by the first three principal components based on the genetic distance of the five microsatellite loci. (a) The scores of fruit-consistency subpopulations (b) The scores of maturity-period subpopulations.

Table 3. Result of Mantel's test of the pair-wise correlations between dissimilarity matrices.

First matrix	Second matrix	Mantel's *r*	Probability
Maturity period	ISSR	-0.054	0.225
Consistency	ISSR	-0.120	0.026*
Maturity	SSR	-0.027	0.382
Fruit consistency	SSR	0.110	0.029*
SSR	ISSR	-0.108	0.151

* Rejection of the null hypothesis of no correlation within a 5% confidence interval.

Table 4. *PhiPT* values between different subpopulations based on seven ISSR primers.

Subpopulations	Early	Late	Mid-season	Dry	Semi-dry	Semi-soft	Soft
Early		0.443	0.460	0.168	0.475	0.417	0.432
Late	0.000		0.443	0.421	0.410	0.493	0.174
Mid-season	0.000	0.000		0.254	0.437	0.419	0.443
Dry	0.057	0.000	0.033		0.081	0.450	0.007*
Semi-dry	0.000	0.000	0.000	0.096		0.496	0.440
Semi-soft	0.000	0.000	0.000	0.000	0.000		0.458
Soft	0.000	0.036	0.000	0.126	0.004	0.001	

*PhiPT*values below diagonal. Negative *PhiPT* values converted to zero. Probability values based on 999 permutations are shown above diagonal.

Table 5. F_{ST} values between different subpopulations based on five microsatellite loci.

Subpopulations	Early	Late	Mid-season	Dry	Semi-dry	Semi-soft	Soft
Early		0.158	0.261	0.222	0.431	0.011*	0.447
Late	0.026		0.436	0.438	0.421	0.420	0.149
Mid-season	0.012	0.000		0.409	0.379	0.272	0.450
Dry	0.026	0.000	0.000		0.244	0.142	0.104
Semi-dry	0.000	0.000	0.004	0.026		0.016*	0.329
Semi-soft	0.093	0.000	0.011	0.049	0.093		0.013*
Soft	0.000	0.020	0.000	0.042	0.007	0.065	

F_{ST} values below diagonal. Probability values based on 999 permutations are shown above diagonal. Negative pair-wise F_{ST} converted to zero.

2008) (Fig. 3). These areas represent more than 85% of the total date palm oases of Tunisia. Analyses were performed on 52 individual trees belonging to the 26 cultivars (Table 2) at the rate of two replications for each cultivar. The replications were done to confirm the intra-cultivar stability underscored by previous work (Zehdi et al., 2002,2004). According to the maturity period, the studied cultivars have three different maturity periods: Early, Mid-season and Late. The fruit consistencies were Soft, Semi-soft, Semi-dry and Dry. Total nuclear DNA was extracted from young leaves according to the Invisorb® Spin Plant Mini Kit (Invitek). The manufacturer's protocol was followed: 20 µl proteinase K was added to the mixture in the lysis step. The final DNA product was eluted in 100 µl of pre-heated Elution Buffer D and incubated for 3 min. This protocol gave a high DNA purity with a concentrationup to 25 ng/µl. DNA polymorphisms were detected by the polymerase chain reaction (PCR).

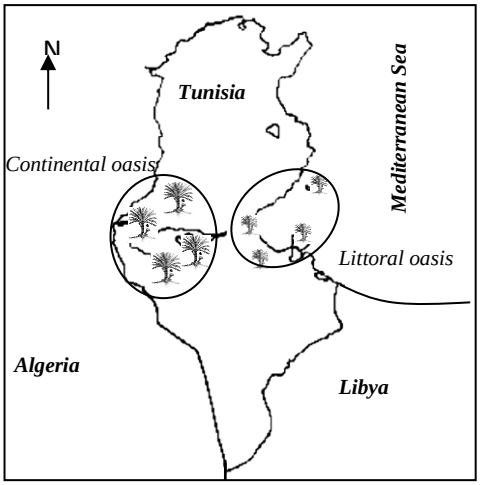

Fig 3. Locations of Tunisian oases

ISSR amplification

Seven ISSR primers were used in this study (Table 1). PCR was carried out in 20-µl final volume using 25 ng of genomic DNA containing, 4 µl of 5×Green GoTaq® (pH 8.5, 7.5 mM MgCl2), 100 µM dNTPs, 150 pmol random primer, and 1.2 units of Taq DNA polymerase. The mixture was made up to 20 µl by the addition of sterilised distilled water. The mixture was amplified in a thermal cycler (GeneAmp® PCR System 9700), which was programmed for one cycle of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, followed by a primer-specific annealing temperature for 55 s and ended by extension at 72°C for 1 min. A final extension cycle was performed at 72°C for 7 min. The PCR machine was adjusted to hold the product at 4°C. The PCR products and 1kb DNA ladder (Promega) were electrophoresed on 2% agarose gels (stained with EtBr). The separated fragments were visualised with an ultraviolet (UV) transilluminator.

SSR amplification

Five microsatellite loci were used in this study were developed for *Phoenix dactylifera* L. by Billotte et al. (2004) (Table 1). The SSR-PCR was performed in a volume of 12.5 µl containing 50 ng of genomic DNA**,** 5X Green GoTaq® reaction buffer (Promega), 0.2 mM of dNTPs, 0.625 U of Taq polymerase (GoTaq, Promega), 2 mM of $MgCl_2$, and 0.2 µM of each primer (Markus, 2000). Amplifications were carried out in a DNA amplification Thermocycler (GeneAmp® PCR System 9700). The conditions for SSR-PCR were an initial denaturation at 94°C for 3 min followed by 10 cycles of denaturation at 94°C for 20 s, annealing at a primer-specific melting temperature for 1 min, extension at 72°C for 40 s, followed by 25 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C with a final extension at 72°C for 8 min. The amplification products were size fractionated by electrophoresis in 1% agarose gels and identified by staining with ethidium bromide. For final analyses, 0.54 µl of amplified DNA and 5 µl of MagaBACE ET400-R DNA size standard were loaded on agarose gels.

Data analysis

For the ISSR results, fragments of the same molecular weight were considered as the same locus. The numbers of bands produced for each primer were scored manually for their presence (1), or absence (0), and a binary matrix was generated and then used for analysis. For the SSR data, genotyping was carried out using an automatic DNA analyser, MegaBACE 1000. The date palm cultivars' scores were coordinated in a bi-dimensional space by principal component analysis (PCA) by a computing matrix based on the Nei genetic distances (Nei, 1976). For pair-wise comparisons between groups, analysis of molecular variance (AMOVA) was tested (Excoffier et al., 1992) using 999 re-sampled individuals. The software was GenALEx 6.1 (Peakall and Smouse, 2006). A Mantel's nonparametric test (Mantel, 1967) was performed to infer possible correlations between matrices of dissimilarity considering fruit characteristics and genetic distances. For the maturity period and fruit consistency data, binary matrices were constructed: the distance was set to "0" between individuals having identical fruit characteristics and to "1" between individuals having different fruit characteristics. These analyses were performed using the software package Mantel (version 2.0) (Liedloff, 1999).

Conclusion

The use of molecular tools, such as ISSR and SSR, is very important to explain genetic diversity and population structures. In the future, the use of these tools should increase in order to fully investigate the relationships between molecular markers and fruit characteristics. This could increase the selection efficiency of date palm cultivars derived by sexual reproduction. In fact, cultivar selection could begin during the seedling stage when the fruit appears only after five years. Furthermore, date palm needs a sustainable management to contribute to the conservation of Tunisian oases where the biodiversity is now endangered.

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