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Molecular identification of Date palm (*Phoenix dactylifera L.*) "Deglet noor" pollinator through analysis of genetic diversity of Algerian male and female ecotypes using SSRs markers

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ABSTRACT

Date palm (Phoenix dactylifera L.) "pollinator or dokkar" influences fruit's quality and quickness of maturity "metaxenia". This study characterizes the genetic diversity and the relationships between different Algerian ecotypes of dokkars. In addition, we report on the use of microsatellites to identify pollinators that are genetically the closest to "Deglet Noor" and "Ghars" cultivars. A total of 25 microsatellites were used on 50 accessions from Algeria, showed high genetic variability. The number of amplified alleles was 135 and ranged from 2 (PDCAT3) to 12 (DP 159), with an average of 5.44. The average of observed heterozygosity ranged between 0.026 (DP169) and 1.00 (PDCAT10, DP171, DP160, DP159, MPdCIR32 and MPdCIR10), with a mean of 0.688 indicating high diversity of date palm accessions. The average of expected heterozygosity was 0.436 and varied between 0.098 (PDCAT3) and 0.6 (PDCAT2). Almost Fis values were negative for markers except for DP 169. Unweighted Pair Group Method with Arithmetic Averages (UPGMA) clustering highlighted close genetic relationships between male and female genotypes for "Deglet Noor" accessions and showed case of homonymy and presence of clones. Principal Coordinate Analysis (PCoA) showed pattern that evidences the genetic diversity existing among pollinators that allowed distinguishing them easily. The combination of two SSR allowed to unambiguously distinguish six dokkars. Overall, UPGMA and PCoA showed that accessions of same type were clustered according to their origin (Oasis). Among the 28 private alleles scored in this study, nine were scored for Deglet Noor". Bayesian analysis showed a conserved genetic structure mainly for "Deglet Noor". Our results suggest that specific SSR marker (MPDCIR35) that characterize both male and female individuals of "Deglet Noor", could be used as potential marker to select, at early stage of seedling, the closest pollinator to the female individual of this important commercial cultivar. In addition, MPdCIR25 represents potential marker to identify male individuals of "Ghars".

1. Introduction

The date palm (*Phoenix dactylifera* L.) is a perennial monocotyledonous diploid (2n = 36) fruit tree (Barrow, 1998), belonging to the family *Arecaceae*. It is a dioecious plant of which there are both male and female' sexes on separate plants. The date palm is an evergreen plant that cultivated in semi-arid and arid regions. It is the pillar element in oasis agro-ecosystems and has a great economic importance in many countries extending from North – West Africa to Asia. Recently, a mitochondrial and chloroplast genome-wide genotyping data showed that date palm cultivation occurred independently in different locations (Mohamoud et al., 2019).

In Algeria, date palm cultivation is considered as a strategic plant and an important source of currency. The date palm covers an area of 168,855 ha, with a production of 1,094,700 T of dates in 2018 (FAOSTAT, 2018) which classified Algeria among the larger producer countries. In fact, there are more than 940 cultivars (Hannachi et al., 1998). However, trends in establishing new date palm orchards is based more on commercial cultivars mainly "Deglet Noor" which represents about 53 % of total date palm production (MADR, 2018). Consequently,

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monoculture would in the future speed up the erosion of the local genetic diversity. Despite this last fact, there is an increase demand of farmers for pollen quality and best pollinators. Actually, by definition, the date palm male is called commonly "Dokkar" or "pollinator". It's availability is very low and it represents only 1–2 % compared to the date palm female plants. There are noticeable differences between dokkars, particularly in terms of the precocity, quantity and quality of pollen produced (Toutain, 1967; Djerbi, 1994).

Often, date palm orchards are planted either with seeded plants or vegetative propagation through offshoots, which have an impact on fruit quality and quantity due to metaxenia and compatibility effects between male and female parents. Khankahdani and Bagheri (2019), indicated that male and female individuals of the same or close date palm cultivars produce dates with higher quality compared with genetically separated ones.

In Algerian oases, overall, farmers use any available pollen source and in mixture for pollination, thinking that there is no incompatibility between male and female palms. In contrast, according to the literature (Nixon, 1934; Chao and Krueger, 2007; Chaouch Khouane, 2012), the contribution of pollen seems to have an effect on the quality and yield of dates. It influences the size, shape, weight and quickness of fruit maturity (metaxenia). Therefore, the study of the genetic diversity of dokkars and their characterization as well as their effects on fruit quality is important to perform either any breeding program or conservation germplasm.

Although, the identification of sex and variety will be after more than 5 years, until the date palm begins to produce the reproductive organs. Pollinators are, in mostly, derived from seeds, followed by accurate selection to choose the best pollinators, which will be successively propagated vegetatively (Chaibi et al., 2002). Unfortunately, overall, there is a dearth of information on morphological characterization of pollinators mainly for the Algerian ones. Currently, in addition to the lack of morphological descriptors for gender identification at early seedling stage, there is no morphological descriptor which is used to identify the best pollinators.

Morphological markers have been widely used for date palm cultivar characterization and their usefulness have been discussed (Elhoumaizi et al., 2002; Ould Mohamed Salem et al., 2008; Ould Mohamed Ahmed et al., 2011; Bedjaoui and Benbouza, 2018). As well, biochemical markers using isozymes have been used in several studies to identity date palm accessions (Baaziz and Saaidi, 1988; Bennaceur et al., 1991; Ould Mohamed Salem et al., 2001). Therefore, several molecular DNA-based markers have been used to study genetic diversity and relationships among date palm cultivars. They have the ability to distinguish among closely related cultivars (Sedra et al., 1998; Ben Abdellah et al., 2000; Trifi et al., 2000; Cao and Chao, 2002; Adawy et al., 2005; Rhouma et al., 2008; Sabir et al., 2014). Yet, few studies have been performed to study dokkar's genetic diversity using morphological characters or molecular markers (Ahmed et al., 2006; Racchi et al., 2014; El Kadri et al., 2019) compared to the number of studies that included a reduced number of pollinators in their investigation on genetic diversity of date palm cultivars (Zehdi et al., 2004; Elshibli and Korpelainen, 2008; Rhouma et al., 2008; Zehdi et al., 2012).

Furthermore, Microsatellite markers have been proven to be very useful and a powerful tool for the evaluation of genetic diversity for plants (Billotte et al., 2004; Al-Ruqaishi et al., 2008; Ahmed and Al-Qaradawi, 2009; Elshibli and Korpelainen, 2011; Elmeer et al., 2011; Arabnezhad et al., 2012; Bodian et al., 2012; Khanam et al., 2012; Racchi et al., 2014; Cherif et al., 2016; Zehdi-Azouzi et al., 2016). They are more recommended on the discrimination between accessions because of their high degree of polymorphism, co-dominant inheritance, high reproducibility, multiallelic nature, relative abundance and extensive genome coverage (Kalia et al., 2011). However, (Akkak et al., 2009) used Algerian cultivars in their study to characterize and evaluate selected Microsatellites loci.

In Algeria, "Deglet Noor" and "Ghars" are important commercial cultivars and farmers are struggling to identify the best pollinator at early seedling stage. Consequently, characterization of date palm pollinators to allow early identification of the best pollinators becomes a substantial request that should be resolved to sustain date palm quality fruit production. Even so, and as far as we know, no molecular markers are known to be used successfully to identify the best pollinators closely related to the "female" cultivar. The first objective in conducting this study was to determine the genetic relationships among some pollinators from Ziban region and their genetic diversity using SSR markers. The second one, was to identify specific SSR markers that characterize male and female individuals of "Deglet Noor" and "Ghars" cultivars to be able to select, at early stage of seedling, the pollinators closely related to the female individual of these two cultivars.

2. Materials and methods

2.1. Plant material

In Algeria, the farmers name the date palm pollinators on the basis of their high morphological resemblance to the female cultivars according to the morphology. Thus, for this investigation, the pollinators were selected on the basis of their morphological resemblance to the female cultivars based on the traditional knowhow of the farmers and each pollinator has been named by the name of the cultivar according to farmer denomination (use of vernacular names). We have sampled, on two sites (Fig. 1), 10 pollinators having similar names of ten female cultivars among the most cultivated ones in Ziban region which is the most important date palm productive area in Algeria (Table 1).

For female cultivars, we have selected "Deglet-Noor", the most important commercial cultivar, and "Ghars" one of the most date palm cultivar used in dates transformation and for pollination. The high number of accessions included in this study was set for "Deglet Noor" male and female cultivar in order to be able to perform the comparative study between the two genders. In total 50 accessions were sampled. Finally, only one accession for both "Horra" and "Unknown" pollinators was sampled (Table 1).

2.2. DNA extraction

Young fresh leaves were collected from 50 accessions, then, they were ground into a fine powder with liquid nitrogen and Silicon dioxyde. Total genomic DNA was extracted from 100 mg of ground leaves using CTAB method (Benbouza et al., 2006a). DNA quality and concentration were estimated using an electrophoresis agarose gel (1 %) and a Nanodrop 2000c. (Thermo scientific, Willington, USA), respectively. The isolated genomic DNA samples were diluted to 50 ng.μl^{-1} in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA).

2.3. SSR analysis

Based on their polymorphism and amplification profiles quality, 25 SSR markers were chosen among those developed for *Phoenix dactylifera* L. in previous studies (Billotte et al., 2004; Akkak et al., 2009; Hamwieh et al., 2010).

PCR amplification for two set of primers, (Billotte et al., 2004; Hamwieh et al., 2010) were performed in a total reaction mixture of 25 μL containing: 50 ng of genomic DNA (5 μL), 1X Green GoTaq® Flexi Buffer (Promega, Madison, USA), 2 mM of MgCl$_2$, 0.2 mM of dNTP (Qiagen, Venlo, the Netherlands), 0.625 unit of GoTaq®G2 Hot Start polymerase (Promega, Madison, USA) and 0.2 μM of each primer. Amplification were performed in VERITI 96 Well Fast thermal cycler (Applied Biosystems, USA). Different amplification conditions were applied to the set of primers pairs. For the first set of primers (Billotte et al., 2004) the following amplification conditions were used: an initial denaturation step at 94 °C for 3 min was followed by; 1) 10 cycles of denaturation at 94 °C for 20 s, annealing at primer specific temperature
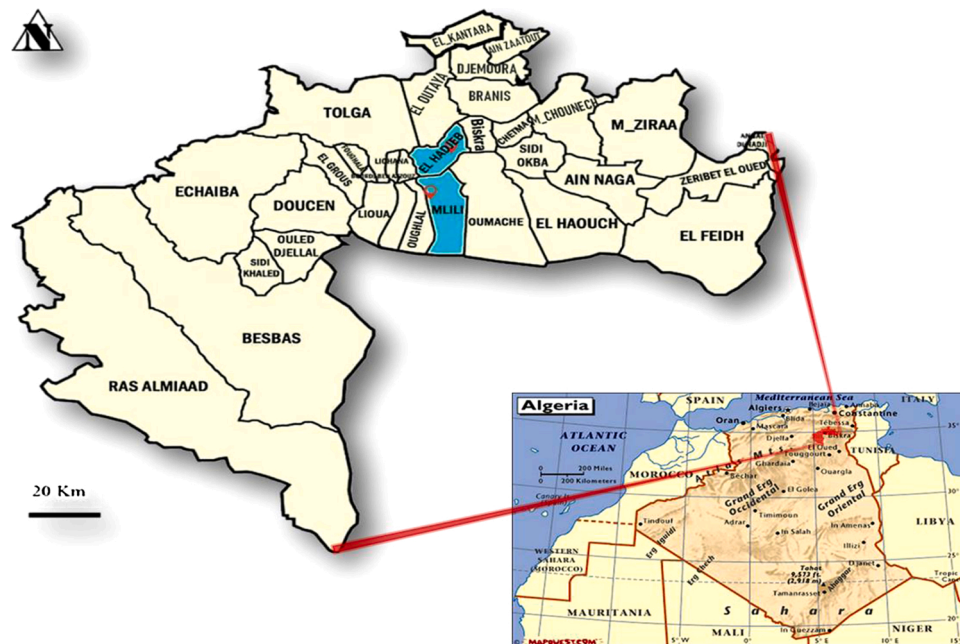
Fig. 1. Map of the sampled areas highlighted by blue color and red dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Name, sex and geographic origin of the 50 accessions collected from Zibans region.

Name	Code	Sex	Number of trees	Spathe's shape[a]	Collection site	Coordinates	Name
Dokkar Deglet-noor	DNM	Male	8	Lanceolate	El Hadjeb (3 trees)	34.7775855	5.6375651
					M'lili (5 trees)	34.651145	5.524592
						34.651942	5.527182
						34.653653	5.533813
						34.650924	5.533631
Dokkar Ghars	GHM	Male	3	Inflated	El Hadjeb	34,7,775,855	5,6,375,651
Dokkar Mech-degla	MDM	Male	3	Inflated	El Hadjeb	34,7,775,855	5,6,375,651
Dokkar Itima	TMM	Male	3	Inflated	El Hadjeb	34,7,775,855	5,6,375,651
Dokkar Safray	SFM	Male	3	Fusiform	M'lili	346,163,579	55,659,109
Dokkar Hamray	HMM	Male	3	Fusiform	M'lili	346,163,579	55,659,109
Dokkar Arechti	ACHM	Male	3	Fusiform	M'lili	346,163,579	55,659,109
Dokkar Halwa	HLWM	Male	3	Fusiform	M'lili	346,163,579	55,659,109
Dokkar Thouri	THRM	Male	3	Fusiform	M'lili	346,163,579	55,659,109
Dokkar Horra	HORM	Male	1	Fusiform	M'lili	346,163,579	55,659,109
Deglet-noor cultivar	DNF	Female	13	Lanceolate	El Hadjeb (6 trees)	34,7,775,855	5,6,375,651
					M'lili (7 trees)	34.650924	5.533631
Ghars cultivar	GHF	Female	3	Inflated	El Hadjeb	34,7,775,855	5,6,375,651
Unknown tree	UNK	Male	1	/	M'lili	346,163,579	55,659,109

[a] Based on descriptors of the International Plant Genetic Resource Institute (IPGRI) (2005).

for 1 min and elongation at 72 °C for 30 s, then 2) 25 cycles of 30 s at 94 °C, 30 s at 53 °C and elongation at 72 °C for 30 s, after that a final elongation step at 72 °C for 8 min.

PCR-SSRs amplification conditions for the second set of primers pairs (Hamwieh et al., 2010) were: a first denaturation step at 95 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at primer specific temperature for 15 s and elongation at 72 °C for 30 s, then a final elongation of 5 min at 72 °C.

For the two first set of primers, a volume of loading buffer (95 % formamide, 20 mM EDTA, 0.5 % (w/v) bromophenol blue, xylene cyanol) was mixed with the same volume of each PCR products. The mixes were denatured at 95 °C for 5 min. Then, 5 μL of each of them was loaded on to 6% polyacrylamide gel and genotyping was carried out using electrophoresis with vertical electrophoresis unit (Apelex "H Vertigel 2″) at 1000 v for 2−3 hours in 1X TBE buffer. The DNA ladder 10 bp (Gene On, USA) was used as a molecular weight marker. The gel was stained with a non-radioactive silver method described in (Benbouza et al.,

2006b).Visualization of DNA banding patterns was performed using BioRadGel DOC TMXR + gel documentation system and Image lab 5.1. Software was used to score and measure the precise size of amplified bands for each genotype.

For the third set of SSR primers (Akkak et al., 2009), PCR amplifications were performed in a total reaction mixture of 20 μL containing: 50 ng of genomic DNA (5 μL), 0.5 unit of AmpliTaq Gold polymerase (Applied Biosystems), 1 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM MgCl2, 200 μM dNTPs and 0.5 μM of each primer. The PCR programme was an initial denaturation of 9 min at 95 °C, 28 cycles of 30 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C and a final elongation step of 45 min at 72 °C. Forward primers were labelled with a fluorochrome (6-FAM, HEX, NED and PET). Amplifications were performed in T100™ Thermal cycler Bio-Rad and PCR products were analyzed by an Abi-Prism 3130 sequencer with Gene Mapper software (version 4.1).

2.4. Data analysis

In order to estimate the genetic diversity and the potential informativeness of the 25 microsatellite SSRs loci tested over 50 accessions in current investigation, several genetic parameters were calculated using the GenAlEx 6.5 software (http://anu.edu.au./BoZo/GenAlEx/) (Peakall and Smouse, 2006), including: the number of alleles (Na), the effective number of allele (Ne), the observed heterozygosity (Ho), the expected heterozyty (He), the Shannon's information index (I), within population inbreeding coefficient (Fis), F statistics (Fst) and private alleles. For each primer pair, the polymorphism information content (PIC), the allele frequency (Fa) and the total number of genotypes (GA) were computed using Power Marker software v3.0 (Liu and Muse, 2005).

Conventionally, the accessions displayed one or two amplified alleles in different sizes, indicating homozygous or heterozygous states respectively. Null allele frequency (R) due to the excess of homozygotes could underestimate the genetic diversity, therefore, the analysis by FreeNA software allows to identify it under the threshold 0.20 (Chapuis and Estoup, 2007) for each locus. The binary matrices based on SSR polymorphic patterns were established for each locus and the SSR amplified fragments were scored as present (1) or absent (0).

In order to elucidate the genetic relationships between studied date palm accessions, a distance-based method using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) (Nei and Li, 1979) and a principal coordinate analysis (PCoA) were performed on the basis of the genetic similarity and distance indices.

Different genetic similarity indices were tested using NTSYS pc 2.11 software (Exeter Software, Setauket, NY) and the Jaccard's index was selected because of its high cophenetic correlation after comparing with the Mantel test the cophenetic matrix obtained from the cluster with the original similarity matrix. Then, the phylogenetic dendrogram was drawn and the bootstrap values were computed over 2000 replications using the Past software 3.18 (Hammer et al., 2001).

Regarding to the principal coordinate analysis (PCoA), this multivariate analysis was performed with GenAlEx 6.5 software (http://anu.edu.au./BoZo/GenAlEx) (Peakall and Smouse, 2006).

STRUCTURE analysis was performed using STRUCTURE software (Pritchard et al., 2000), a model-based Bayesian clustering method applying Markov Chain Monte Carlo (MCMC) estimation in order to inferring population structure and assigning individuals to each inferred population. The program setting was run as following; without and with prior information based on UPGMA clustering, ten independent K value (number of populations) and K ranged from 1 to 10 replicates, with admixture model and allele frequencies correlated among populations. For each run, we employed a burn-in period of 10.000 steps followed by100.000 Monte Carlo Markov chain iterations to estimate individual admixture proportions (Q). To select the most appropriate K as number of populations, an online software STRUCTURE Harvester (Earl and vonHoldt, 2012) was used according to Evanno's method (Evanno et al., 2005), using of the log likelihood L(K) for each K and an ad hoc quantity (ΔK) based on the second order rate of change of the likelihood.

In addition, estimating the partitioning of genetic variation of individuals, among and within populations defined by STRUCTURE, was performed using AMOVA analysis with GenAlEx 6.5 software (http://anu.edu.au./BoZo/GenAlEx) (Peakall and Smouse, 2006).

3. Results

3.1. Genetic variability

The choice of 25 SSR markers used in this study was based on amplification results in similar previous studies. The locus, mPdCIR32, was monomorphic and showed two fragments sized 280 bp and 297 bp, while, all other 24 SSRs loci showed polymorphic amplification with one or two fragments per genotype and amplified a total number of 135 alleles. The number of amplified alleles varied per locus and ranged from 2 (PDCAT3) to 12 (DP159), with an average of 5.44 (Table 3). The lowest allele frequency observed among several loci was 0.010 (Table S1) and the highest one was 0.860 for the allele 260 bp (mPdCIR57 locus) (Table S1). The maximum probability of identity (1) was detected with PDCAT2 and the minimum (0.153) with PDCAT3. PIC values ranged from 0.236 (mPdCIR57) to 0.758 (mPdCIR35), with an average PIC value of 0.55 (Table 2). Yet, high PIC values did not automatically correspond to a high number of distinguished cultivars (NDC) neither to a high number of detected alleles by an SSR marker.

The average of the total rate of heterozygosity (Ht) by locus, for all cultivars, was quite high (0.638), with a 0.83 value scored for mPdCIR10, indicating the high potential of the informativeness of these SSR markers (PIC > 0.5, see Table 2). Out of the 135 alleles amplified, fourteen seems to be fixed (p ≥ 0.5), while sixty-seven (49.26 %) are probably rare alleles (p ≤ 0.05). In addition, most of dokkars showed private alleles except for (Safray, Hamray and Unknown). Out of the 28 amplified alleles, "Deglet Nour" Dokkar had the highest number of specific alleles (9), (Table S1). While, two pollinators "Ghars" and "Horra", amplified only one specific allele sized 101 bp and 235 bp at PDCAT18 and mPdCIR25 loci, respectively (Table S1) and seemed to be fixed ones as their frequencies were equal to 0.5. In addition, the allele 225 bp (PDCAT3 locus) was present in all studied accessions except for "Ghars" both male and female individuals. The PDCAT2 locus had the highest number of genotypes, 15, and the lowest one (1) was scored at mPdCIR32 locus (Table 3).

The observed heterozygosity (*Ho*) was high for almost loci except for 6 loci, where the *Ho* was inferior to 0.5. The *Ho* values ranged between 0.026 (DP169) and 1.00 (PDCAT10, DP171, DP160, DP159, mPdCIR32 and mPdCIR10), with a mean of 0.688 (Table 3) indicating high diversity of date palm accessions. In this study, null allele frequency (R) value (0.20) was considered as the threshold value over which underestimate signification of *He* due to null alleles that can be found (Chapuis and Estoup, 2007). Over all loci, the *He* values were significant as all loci showed a null allele frequency R value under the threshold (Table 3). The average of expected heterozygosity (*He*) was 0.436 and varied between 0.098 (PDCAT3) and 0.6 (PDCAT2), and almost SSR markers showed high values of *Ho* compared to *He*, except for the locus DP169 (Table 3). This tendency is reflected by negative *Fis* values (Table 3) suggesting an excess of heterozygotes.

3.2. Genetic relationships and clustering

Based on the analysis of 25 SSR markers, the UPGMA dendrogram clustered the accessions into two main groups (I and II). Group I included most of the accessions and it was organized in two main subgroups A and B. The subgroup A, contained 8 accessions, and the subgroup B regrouped the highest number of accessions, 33, organized in two sub-clusters B-1 and B-2. The second main group (II) contained only nine accessions distributed on two sub-groups C and D (Fig. 2).

The dendrogram pointed out weak clustering relationships (with weak bootstrap values) mainly for subgroups (A and B). Besides, high variation for similarity values at molecular level was observed among the 50 accessions. It ranged from 0.176 (between 'ACHM1-and 'MDM2') to 1.00 for 32 combination of accessions, between "Deglet Noor" male and female accessions as well as one combination between two male accessions of "Ghars" GHM1 – GHM3 (Data not shown). Overall, accessions of same type were clustered according to their origin (Oasis). Except for i) HMM1, clustered in main Group I, sub- cluster A-1, while the two other accessions in main group II (sub-cluster D-2), ii) THRM1, DNM5 and ACHM1 that were clustered in different sub groups from accessions belonging to the same type of dokkars. In addition, it is interesting to find that accessions of the pollinizer Ghars were clustered in different main group (II) than the accessions of cultivar Ghars (female) (main group I). While, both male and female accessions of 'Deglet Noor' were clustered in the same main group I (sub-cluster B-2).

Table 3
Genetic parameters of the 25 loci examined over Algerian date palm accessions.

Locus	N	Ne	NG	Ho	He	I	Fis	Fst	PIC	R	NDC
mPdCIR10	9	2.562	6	1.000	0.573	0.907	−0.746	0312	0.7499	0	1
mPdCIR25	7	2.096	9	0.635	0.475	0.744	−0.335	0338	0.6853	0.05	2
mPdCIR32	2	2.000	1	1.000	0.500	0.693	−1.000	0000	0.3750	0	0
mPdCIR35	6	2.058	6	0.821	0.466	0.715	−0.762	0372	0.7586	0	0
mPdCIR50	8	2.071	8	0.718	0.473	0.734	−0.518	0293	0.6020	0.024	2
mPdCIR57	3	1.394	4	0.292	0.214	0.324	−0.362	0282	0.2356	0.025	0
mPdCIR93	5	2.000	8	0.708	0.450	0.715	−0.575	0243	0.5458	0	1
DP159	11	2.598	7	1.000	0.584	0.946	−0.712	0256	0.6948	0	2
DP160	4	2.246	2	1.000	0.534	0.791	−0.872	0275	0.6358	0	0
DP168	4	1.626	6	0.375	0.311	0.460	−0.204	0362	0.4713	0.049	0
DP169	4	1.262	5	0.026	0.141	0.200	0.818	0698	0.3586	0.082	1
DP170	5	2.060	4	0.990	0.511	0.734	−0.939	0006	0.4181	0	1
DP171	4	2.063	3	1.000	0.513	0.732	−0.950	0072	0.4444	0	1
PDCAT1	5	1.845	10	0.353	0.334	0.551	−0.057	0556	0.6138	0.036	1
PDCAT2	10	2.726	15	0.974	0.600	1.000	−0.624	0250	0.7347	0	3
PDCAT3	2	1.150	3	0.128	0.098	0.153	−0.304	0712	0.2424	0.001	0
PDCAT5	5	2.284	8	0.974	0.544	0.839	−0.790	0266	0.6766	0	0
PDCAT6	6	2.357	13	0.699	0.527	0.857	−0.325	0324	0.7420	0.014	0
PDCAT8	6	2.433	11	0.776	0.554	0.897	−0.399	0272	0.6738	0.014	1
PDCAT10	3	2.044	2	1.000	0.509	0.718	−0.966	0008	0.3897	0	1
PDCAT12	7	1.911	11	0.500	0.407	0.660	−0.228	0436	0.5810	0.045	1
PDCAT14	6	1.979	13	0.606	0.438	0.706	−0.385	0421	0.6522	0.014	1
PDCAT17	4	1.710	7	0.500	0.349	0.538	−0.434	0444	0.4710	0.011	1
PDCAT18	6	2.075	9	0.760	0.477	0.742	−0.594	0288	0.6145	0	2
PDCAT21	3	1.752	6	0.365	0.330	0.514	−0.107	0441	0.4993	0.049	0

(N) Number of alleles detected, (Ne) Effective number of alleles, (NG) Number of genotypes, (Ho) Observed heterozygosity, (He) Expected heteozygosity, (I) Shannon's information index, (Fis) Fixation index, (PIC)polymorphic information content, (PD)Power of discrimination, (R) Frequency of null allele and (NDC) Number of distinguished cultivars.

The UPGMA dendrogram show four cases of different genotypic profiles with the same denomination that were not clustered in same main groups or sub-groups. These accessions are: THRM1, HMM1, ACHM1 and DNM5. In addition, accessions in subgroups (A, B, C and D) clustered similarly in PCoA analysis (Fig. 6).

3.3. Pollinators and cultivars genetic profiles

Among the 25 SSR loci used in this study, PDCAT2 distinguished the highest number of dokkars /cultivars, while nine SSR loci were not able to distinguish any of them (Table 3). The combination of mPdCIR25 and PDCAT2 markers allowed to distinguish 6 of dokkars /cultivars (Table3). The twenty-five SSRs used in this study identified the 50 accessions. In fact, the 135 alleles revealed 177 genotypes among the studies accessions (Table 3). Out of these genotypes, the number of unique genotypes per SSR locus was 51 and varied from 0 (mPdCIR10, mPdCIR32, DP171, DP160, PDCAT21, and PDCAT3) to 8 (PDCAT2). The accessions DNM5, TMM1 and TRHM1 scored the highest number of unique genotypes. Besides, for all the accessions, mPdCIR32 locus amplified a unique genotype (280−207).

Among, the fifteen genotypes with high frequency (1) found in this study, only three genotypes 241/241 182/191 136/149 were specific to "Deglet Noor" at mPdCIR25, mPdCIR35, and PDCAT6 (SM2) loci, respectively. Among the genetic profiles found in this study, only female accessions of "Deglet Noor" were represented by one multi-locus unique profile (Data not shown), and thereby, can be considered as cultivar genotype.

Among the 28 private alleles scored in this study, the accessions of pollinizers "Deglet Noor" amplified the highest number of private alleles (9) followed by "Itima" (5). Interestingly, for almost both pollinizers and female' accession of "Deglet Noor" two private alleles (182 bp and 191 bp) were scored at mPdCIR35 locus, except for DNM6 which amplified another allele sized 203 bp and the 182 bp (Fig. 3).

Likewise, for "Ghars" three private alleles were scored for all female accessions at loci DP169 (227 bp), DP171 (242 bp), PDCAT2 (183 bp,) and one, at mPdCIR25 locus (235 bp), for all male accessions. In addition, the allele 227 bp at DP169 locus seems to be fixed for "Ghars" cultivar. The lowest rate of polymorphism was scored for "Ghars" with 60 %, while "Arecheti" and "Halwa" showed 100 % of polymorphism (data not shown).

3.4. Genetic structure of date palm accessions

Using STRUCTURE, a Baysian analysis was carried out to infer the population structure among date palm accessions using 25 SSR markers. Analyses were performed with and without incorporating prior information based on UPGMA clustering in order to identify the optimal K value. For both analyses, K = 2 was the optimum of clusters at the highest value of Δk (Evanno method, Evanno et al., 2005) (Fig. 4). Interestingly, all accessions of "Deglet Noor", both female and male, were clustered altogether in cluster 1 with 65 % of accessions having a membership > 0.9, and only 15 % (3 accessions) expressed a membership below 0.6. For remaining accessions, they were clustered in cluster 2 (Fig. 5) where 73.33 % expressed a membership > 0.9. The genetic population structure is in concordance with UPGMA clustering and PCoA, where "Deglet Noor" pollinators and female were grouped all together in the same genetic pool and the remains accessions grouped in the second genetic pool. However, the analysis of population structure without female accessions showed that optimum clusters is Nine (K = 9) (SM. 4).

AMOVA was computed among the two gene pools (Fst = 0.139; P < 0.001) and the greater part of the generic variability explained differences within populations (86 %), while 14 % of the total variance was observed among populations (SM3). PCoA three axis explained 53.55 % (Fig. 6) of total variability three ability and contributed, singly, with CP1 (27.84 %), CP2 (16.83 %) and CP3 (9.28 %) (Fig. 6). The cumulative variability indicates clearly the genetic diversity among the same type of pollinators that enabled to differentiate them easily. Overall, PCoA axis grouped the accessions in two main groups with two sub groups for each following the UPGMA clustering. For all studied accessions, the clustering was according to the sample's origin.

Table 2
Description of the SSR primer pairs used.

Snor	Locus	Repeat motif	Primer sequence (5′-3′)	Annealing temp. (°C)	Range of allele size (bp)
1	mPdCIR10[a]	$(GA)_{22}$	F: ACCCCGGACGTGAGGTG R: CGTCGATCTCCTCCTTTGTCTC	60	105−169
2	mPdCIR25[a]	$(GA)_{22}$	F: GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	51	206−244
3	mPdCIR32[a]	$(GA)_{19}$	F: CAAATCTTTGCCGTGAG R: GGTGTGGAGTAATCATGTAGTAG	54	280−297
4	mPdCIR35[a]	$(GA)_{15}$	F: ACAAACGGCGATGGGATTAC R: CCGCAGCTCACCTCTTCTAT	54	182−203
5	mPdCIR50[a]	$(GAA)_{9}$	F: CTGCGCCAATCTAAACCATT R: GCAAATTGCAACAAATCCTTG	50	159−206
6	mPdCIR57[a]	$(GA)_{20}$	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC	55.4	260−276
7	mPdCIR93[a]	$(GA)_{16}$	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	Touchdown from 60[*]	168−188
8	DP159[b]	$(TC)_{27}$	F:AGCTCCAATTTGCTGCAGAG R:GCTGACCTGGAGTCCAAAAC	55	101−156
9	DP160[b]	$(GAAA)_{5}$	F: AAGAGCGACAATCATGACCA R: GGAAATTGAAGGGCATCTTG	58	196−211
10	DP168[b]	$(CAT)_{8}$	F: GCAGCAAAGCCCTTAGGC R: GGTGTTATGTGCAGCCAATG	55	205−217
11	DP169[b]	$(AAT)_{12}$	F: GCATGGACTTAATGCTGGGTA R: GGTTTTCCTGCCAACAACAT	58	210−227
12	DP170[b]	$(AGGG)_{5}$	F: TCTTTGGGCTTACGACAACC R: GTATGGCCCAAGATGCAGAT	56	205−215
13	DP171[b]	$(TTC)_{10}$	F: GTGGGAGTAGCGAGGTATGG R: GTCCGGCACTTTAGGAAGTT	56	200−242
14	PDCAT1[c]	$(TC)_{21}$	F:CTGAAATCTCTGTTCAAATCC R:AGTTTGGATCTATTTGTGAGTTATTTCTTT	54	79−133
15	PDCAT2[c]	$CTCGCTG(TC)_3(TC)_3T(TC)_3(TTC)_3T$ $(TC)_4TTCTGTCC$	F:GGCCTTCTCTTCCCTAATGGG R:AGTTTCTTGCCCCTGTTCTTTCCCTC	54	145−196
16	PDCAT3[c]	$CT)_3 CG(CT)_3 (CA)_8 - (GT)_3(CA)_4$	F:CAAGGATAGGTGTGATGACC R:ACCGTTTGTCCTTTTAACTTCTTGCTGGAATT	54	221−225
17	PDCAT5[c]	$CG(TC)_{16}T(TC)(AG)_{16}$	F:GGCCCGTCCTTGGATTAGAGC R:TACGTTGTCCCGTCAATTGG	54	67−87
18	PDCAT6[c]	$(CA)_{14}(GA)_{23}$	F:AATCAGGGAAACCACAGCCA R:GTTTAAAGCCTTCTCAAGATAGCCTCAG	54	122−149
19	PDCAT8[c]	$(TC)_{16}$	F:GCTTAAGTGGTTAGTTGCCAA R:GTTTGGCAGAAGTATTGAAAAGTTGA	54	202−238
20	PDCAT10[c]	$(TC)_{16}$	F:CACTGCTCCTGTTGCCCTGTT R:GTAGAAGGGCAGAGGACGG	54	93−111
21	PDCAT12[c]	$(CT)_{19}$	F:CATCGTTGATTCCTAACCCCT R:CGTTTAGATCTTGCATGGCAACGC	54	132−167
22	PDCAT14[c]	$(TC)_{19}(TC)_{16}$	F:TGCTGCAAATCTAGGTCACGAG R:TTTACCCCTCGGCCAAATGTAA	54	121−144
23	PDCAT17[c]	$(GA)_{21}$	F:CAGCGGAGGGTGGGCCTCGTT R:TCTCCATCTCCCTTTTTCTTCTGCTACTC	54	115−137
24	PDCAT18[c]	$(CT)13 G(CT)8CG(CT)3 CG(CT)3$	F:CCTAAACCTGAATGAATCAAAG R:CAACTAACATAAGGACAGTGCT ATGTGATTG	54	101−127
25	PDCAT21[c]	$(GA)_5T(GA)_2TA(GA)_2 GC(GA)_5(GT)_7$	F:GTGTTTGAAGATTGATTTTGT R: GTTATGAGGTTTCGAACTATAGGCATGCACAATAGTATATTG	54	143−150

[a] SSR primer pairs developed by Billotte et al. (2004).
[b] SSR primer pairs developed by Hamwieh et al. (2010).
[c] SSR primer pairs developed by Akkak et al. (2009).
[*] Touchdown program: An initial denaturation of 15 min at 95 °C was followed by 4 steps: step1−3 normal cycles; 94 °C for 1 min, 60 °C for 90 s and 72 °C for 2 min, step2−10 touchdown cycles; 94 °C for 1 min, 60 °C – (0,3 °C/cycle) for 90 s and 72 °C for 2 min, step3−20 touchdown cycles;94 °C for 1 min, 57 °C – (0,2 °C/cycle) for 90 s and 72 °C for 2 min, step4−5 normal cycles; 94 °C for 1 min, 50 °C for 90 s and 72 °C for 2 min. These steps were followed by a final extension at 72 °C for 90 min, then storage until use.

4. Discussion

Date palm cultivation is ancestral and has been the pillar of the arid and semi-arid ecosystems, and is an important economic fruit crop for many countries. The number of date palm orchards cultivated worldwide is increasing with the predominance of commercial cultivars. Many studies have been undertaken to study the genetic diversity of date palm and the importance of the nutritional and medicinal virtues. However, despite the great importance of the dokkars in assuring a good date fruit quality, production (Chaouch Khouane, 2012) and maintaining a genetic diversity of local date palm orchards, there is no germplasm collections established throughout Algeria including at least the best individuals used in pollination. Also, up to now, scarce studies have assessed the morphological and the molecular of variability for the dokkars as well as their genetic identification. Yet, as far as we know, there are no studies highlighting the genetic relationship between female and male date palm individuals of the same type. Hence, this work represents the first study of the assessment of genetic diversity using SSR markers of some Algerian local pollinators maintained in private orchards. The power of SSR markers to identify genetic variability in date palm have been shown by previous studies which is in line with our results. For instance, the twenty-five SSR primers used in this study
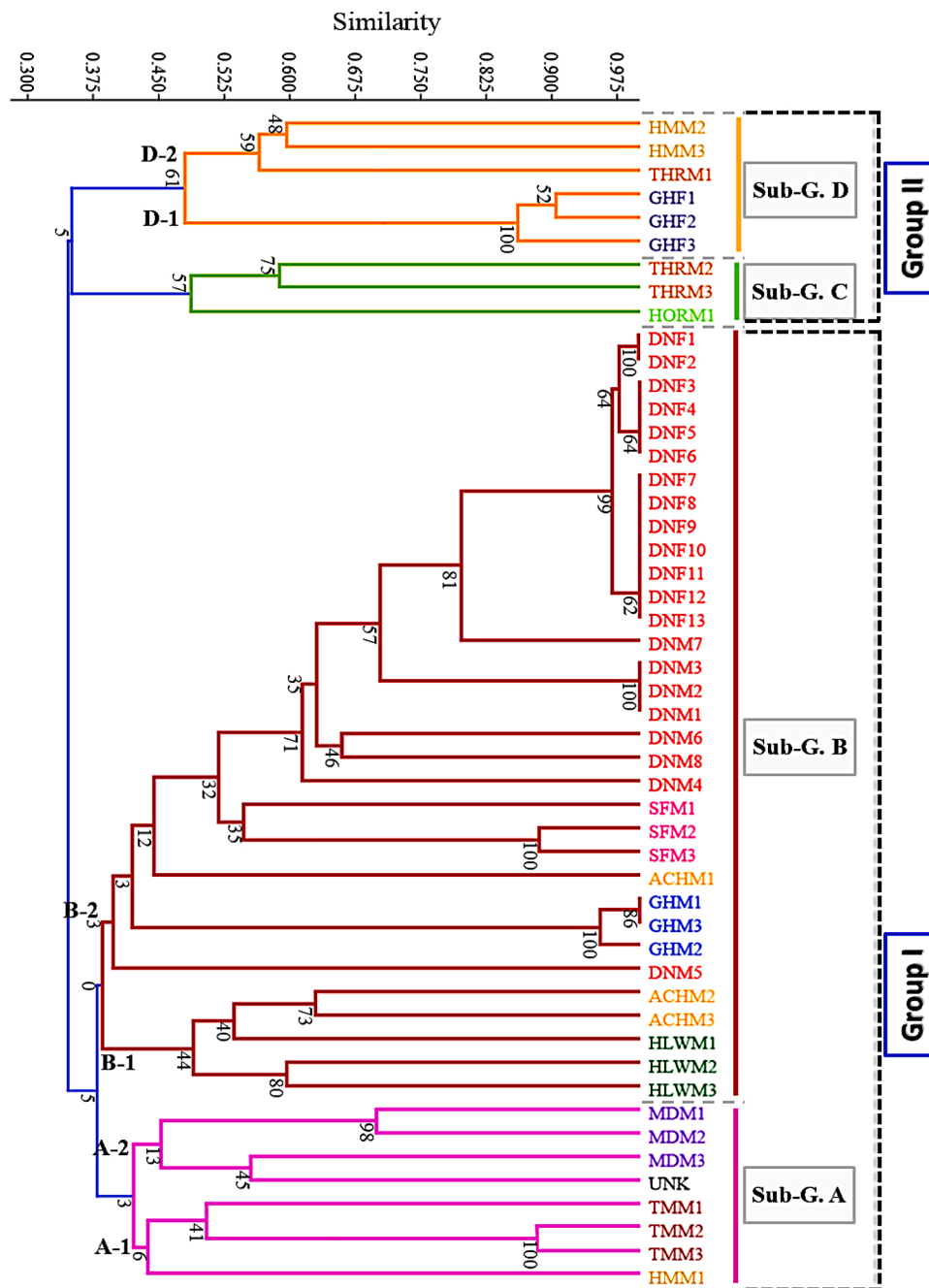
Fig. 2. UPGMA dendrogram constructed for Algerian date palm (dokkars/cultivars) based on Jaccard similarity coefficient.

produced polymorphic bands, with a high average rate of polymorphism (84 %), that highlighted a high genetic variability among the accessions of different pollinators. This high percentage of polymorphism was expected due to the origin of the analyzed material. In fact, the date palm reproduces sexually and asexually. Nevertheless, even the easiness of vegetative propagation in date palm, however, different type of pollinators, named locally "Laachach", are produced by sexual reproduction through seeds that grow from the female individual of the cultivar cultivated in orchards. These "Laachach" means a group of pollinators that are propagated naturally by seeds without any selection and are used, later on, to collect pollen during the reproduction stage. Actually, in private orchards "Laachach" are often observed. One of the reasons of this custom in date palm orchards is probably due to the limited number of offshoots produced in the date life span of date palm and to the fact that a quite high number of them do not survive to adult stage (Zaid and

de Wet 2002). Thus, owing to the importance of these types of pollinators in determining the date palm fruit quality and yield, it would be interesting to use of the organellar genome markers to facilitate understanding maternal contributions in shaping male genotypes. Likewise, the number of private alleles found in this study was relatively high, 28 (Data not shown) compared to the number of the studied accessions, while the number of unique alleles was 20. The highest number of these specific alleles (private and unique) were amplified at PDCAT2 and DP159 loci. The presence either of unique alleles, for an accession, or private alleles, in whole accessions, of a population, might be an indicator of potential genotypes with interesting traits suitable for crop improvement. In this study, DNM5, TMM1 and TRHM1 could be pollinators with interesting characters as they have amplified the highest number of unique genotypes. Yet, this must be checked with further investigation mainly on pollen germination and fruit quality. PDCAT2
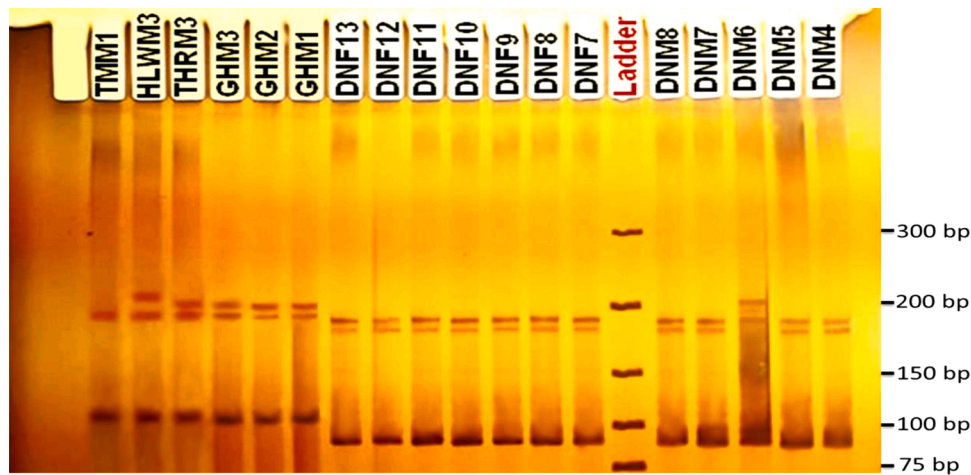
Fig. 3. Amplification profile generated with SSR primer (mPdCIR35) using genomic DNA of male and female date palm plants. Samples abbreviation meaning are reported in Table 1. A unique banding pattern is observed for both 'Deglet noor' male (DNM, except DNM6) and female (DNF).
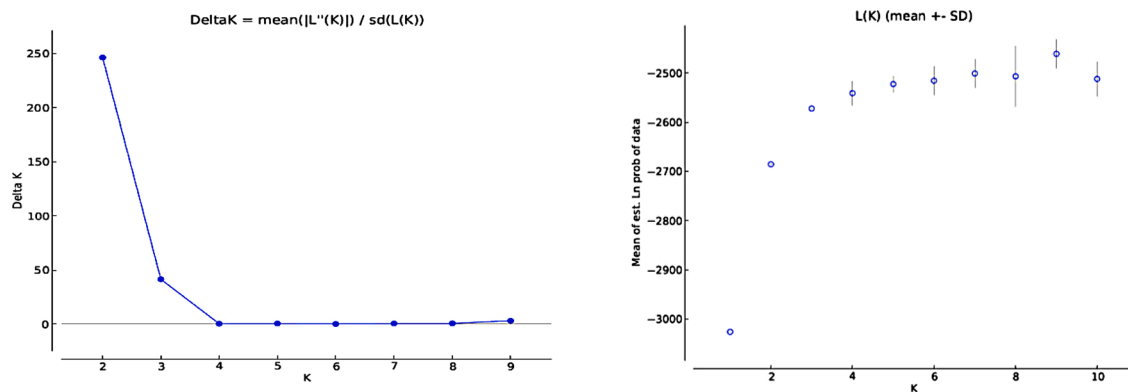

Fig. 4. Select of the most appropriate K to infer population structure according to Evanno's method, using of the log likelihood L(K) for each K and an ad hoc quantity (ΔK) based on the second order rate of change of the likelihood.

1 (DNM1), 2 (DNM2), 3 (DNM3), 4 (DNM4), 5 (DNM5), 6 (DNM6), 7 (DNM7), 8 (DNM8), 9 (GHM1), 10 (GHM2), 11 (GHM3), 12 (MDM1), 13 (MDM2), 14 (MDM3), 15 (TMM1), 16 (TMM2), 17 (TMM3), 18 (SFM1), 19 (SFM2), 20 (SFM3), 21 (HMM1), 22 (HMM2), 23 (HMM3), 24 (ACHM1), 25 (ACHM2), 26 (ACHM3), 27 (UNK), 28 (HLWM1),29 (HLWM2), 30 (HLWM3), 31 (THRM1), 32 (THRM2), 33 (THRM3), 34 (HORM1), 35 (DNF1), 36 (DNF2), 37 (DNF3), 38 (DNF4), 39 (DNF5), 40 (DNF6), 41 (DNF7), 42 (DNF8), 43 (DNF9), 44 (DNF10), 45 (DNF11), 46 (DNF12), 47 (DNF13), 48 (GHF1), 49 (GHF2), 50 (GHF3).
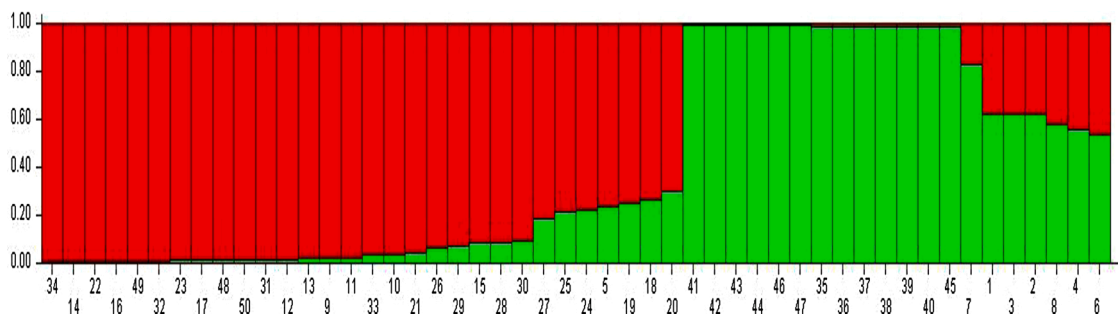
Fig. 5. Inferred population structure of date palm accessions at K = 2 without prior information. Each accession is represented by a vertical bar and the length of each colored segment represents the membership of each gene pool.

and mPdCIR25 markers were the most efficient combination to distinguish between accessions of male populations and hence, represent a good tool for identifying Algerian pollinators.

The mean number of scored alleles (5.44) was low compared to other similar studies (Elshibli and Korpelainen, 2008; Zehdi et al., 2012; Racchi et al., 2014; Yusuf et al., 2015). The heterozygosity for SSR loci ($Ho = 0.688$, $He = 0.436$) was lower than other studies referred above. The main sources of genetic diversity of date palm are often related to its

means of propagation. For instance, growers use a mix of pollen from different male origin obtained either from sexual propagation (seeds) or vegetative propagation, mainly through offshoots. In addition, dokkars are often exchanged among growers. Mean PIC value (0.55) reflects the power of discrimination of the used SSR markers, but it was lower compared to other studies; (Zehdi et al., 2012). However, the comparison made here with other finding on date palm is biased due to the presence of female date palm in these studies.
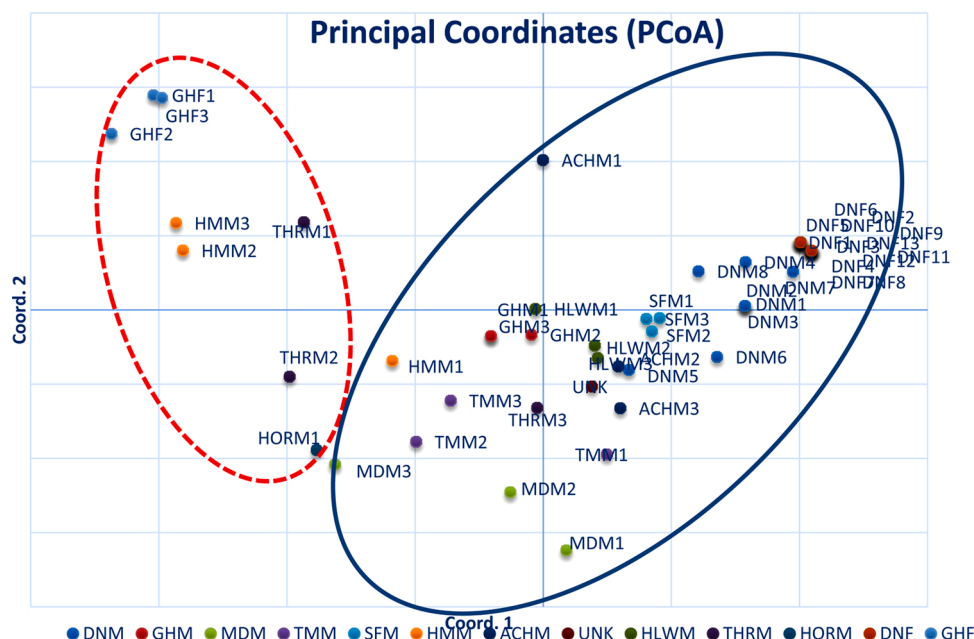
Fig. 6. Plotting of Algerian date palm accessions with 25 SSR markers using principal coordinate analysis (PCoA).

UPGMA clustering highlighted close genetic relationships between male and female genotypes for "Deglet Noor" accessions. They were clustered in the same sub-cluster (B-2). The fact, that both pollinators and female individuals of "Deglet Noor" amplified two private alleles (182 bp and 191 bp) at mPdCIR35 locus, except for DNM6 that amplified only 191 bp allele, could indicate that these two alleles could be used to identify "Deglet Noor" pollinator at early stage of seedling. The accessions DNM1, DNM2 and DNM3 seem to be clones (Fig. 2) as they were propagated by offshoots and had identical genetic profiles over 25 loci. While, the other DNM were issued from seeds and were collected from different oasis. Actually, numbers of offshoots produced in the date life span is limited and a good number of them do not survive until adult stage (Zaid and de Wet 2002).

In addition, all "Deglet Noor" female accessions had the same genetic profiles across the 25 loci and were clustered all together which was expected as all accessions were propagated through offshoots. Yet, it is interesting also to point out that these accessions were grouped according to their sampling site. DNF1-to-DNF6, clustered together, were collected from the same oasis at Elhadjeb. Those from DNF7 to DNF13 were collected from the same oasis at M'lili. All these results suggest that "Deglet Noor" accessions seems to have uniform genetic basis than the other accessions. Moreover, the fact that farmers practice more severe selection for its vegetative propagation based on fruit morphology, shape and quality, because of its economic importance, could also play a key role for this genetic homogeneity. Since "Deglet Noor" was identified by a unique genetic profile, this could be used as mean in certification and control of origin labels of Algerian "Deglet Noor" products. However, increasing the number of accessions should be performed to confirm this result.

While, "Ghars" male and female individuals were not genetically close and were clustered in two different main groups. Despite the fact that they were collected from the same Oasis at Elhadjeb, and were propagated through offshoot, molecular profiles showed differences of alleles over 15 loci among the 25 studied one. However, all female accessions amplified the same private alleles at three loci DP169 (227 bp), with a frequency 1 seems to be fixed, DP171 (242 bp), PDCAT2 (183bp,). Likewise, all male individuals amplified a private allele sized 235 bp, at mPdCIR25 locus, and could be used to select "Ghars" pollinator.

The observed differences for "Ghars individuals" may be due to the

genetic background from initial introduction to the oases. For instance, the maternal plants that could be issued from different seeds followed by selection pression by farmers. Evenly, exchange of propagules which is a mixture of seeds and vegetative propagated material could be another explanation.

The accessions of both sexes were characterized by different private alleles and that could be also used to identify "Ghars" at early seedling stage (Data not shown).

PCoA showed genetic relationships that for the 50 accessions the clustering was according to the sample's origin confirming UPGMA clustering. In addition, UPGMA dendrogram pointed out that most of the pollinators, of the same type, were clustered altogether in the same main subgroups except for TRM1, HMM1, ACHM1 and DNM5. Some studies using SSR markers have reported grouping of date palm genotypes according to geographical pattern but with a narrow genetic base (Papadopoulou et al., 2002; Salhi-Hannachi et al., 2006).

According to the famer, these male accessions were issued from seeds. Indeed, as propagation via seeds gives new genotypes this is probably one of reasons that they had different genetic profiles and were not clustered with their accessions. Additionally, the fingerprinting analyses and largest genetic distance observed between these male accessions having the same denomination but different genetic profiles confirm that they are homonyms. Few homonymies cases have been reported on date palm using phenotypic markers (Ould Mohamed Salem et al., 2008) and isoenzymes markers (Bennaceur et al., 1991). Likewise, for other fruit crops like olive tree and fig tree homonymies cases using SSR markers are often reported (Noormohammadi et al., 2014; Giraldo et al., 2008; Abdessemed et al., 2015; Boudchicha et al., 2018).

STRUCTURE analysis using Bayesian approach generated at K = 2, best value, two genetic pools that are overall similar to UPGMA and PCoA main clusters. However, the analysis using STRUCTURE does not identify populations based on the geographic origin of the accessions. The first genetic pool had a more homogenous genetic makeup and regrouped all "Deglet Noor" female and male individuals, except DNM5. While, the second genetic group contained admixture of genotypes.

Besides, the analysis of population structure, without female cultivars, gave nine (K = 9) genetic clusters according to their highest probability of membership (SM4) that were subdivided accordingly to the studied pollinators population.

AMOVA analysis showed that 86 % of variability is located within

populations, while the variation among population is estimated to be 14 % indicating that there is no significant variability that distinguishes between populations. The average Fst value was 0.317, with positive Fst values, indicate relatively high differentiation of male accessions (Wright, 1965). Yet, these Fst values are biased as two populations were female cultivars.

Both UPGMA and STRUCTURE showed conserved genetic structure mainly for "Deglet Noor" male and female genotypes. Thus, we can suppose, for this study, that pollinators could be named as the female type of the cultivar. This result is in accordance with the local knowhow for pollinators denomination, accumulated for decades, that all pollinators are named according to the female cultivar based on phenotypic similarities. Yet, further investigation should be performed to assume this hypothesis of parental lineage for other type of pollinators.

It is interesting to note that this genetic pooling corresponds also to clustering at phenotypical level when using reproductive morphological descriptors mainly the spathe (Bedjaoui and Benbouza, 2018). For instance, all accessions ("Deglet Noor" male and female individuals) of the first genetic pool had lanceolate form, while the second genetic group clustered accessions that had inflated and fusiform forms (Data not shown). Actually, (Bedjaoui and Benbouza, 2018), reported that "Deglet Noor" cultivar was recognized by the farmers because of its elongated spathe appearance and this characteristic is also used by local farmers to identify the male of this cultivar.

One of the big challenges that date palm seedling producers are facing in Algeria and other countries is to identify the closest genetically pollinators to female genotype belonging to the important commercial date palm cultivars, and more importantly, at an early stage of seedling. The results obtained here could contribute significantly to solve this issue. Indeed, the DNA microsatellites markers used in this study did provide sufficient variation to distinguish between the date palm pollinators, furthermore, the results did supply the first SSR markers that can be used to identify pollinators that are genetically the closest to "Deglet Noor" as well as those that are specific to identify "Ghars" male or female individuals will be effective in cultivar characterization. These markers if combined to sex-linked markers identified in date palm (Al-Mahmoud et al., 2012; Solliman et al., 2017; Intha and Chaiprasart, 2018), they will allow the selection of targeted pollinators at early stage of seedling. This early and targeted pollinators selection, based on these specific molecular markers, is crucial in any breeding programs for date palm. Incorporation of an increased number of accessions, with geographical partitioning, and other SSR markers into genetic studies will provide wider scope in future pollinators studies of Date palm for genotyping and for establishing cultivars identification keys, as well barcodes identification for labels and origin certification.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Asma Chaouch Khouane: Field work, Benchwork, Formal analysis, Visualization, Validation, Contribution in writing the first draft, Writing - review & editing. **Aziz Akkak:** Benchwork, Validation, Writing - review & editing. **Halima Benbouza:** Conceptualization of the study, Supervision, Formal analysis, Validation, Visualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2020.109668.

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