Gene-Targeted markers to assess genetic diversity and population structure within Tunisian *Phoenix dactylifera* L. cultivars

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Abstract

Genetic variability in date palm genotypes collected from different regions of southern Tunisia was analyzed using a Start Codon Targeted (SCoT) marker system. Thirty-one accessions collected from three locations were investigated. One hundred and nine amplicons were produced among which 84 % were polymorphic. The *PIC* value and the *Rp* values testified of the efficiency of used primers. The percentage of polymorphic loci (*Pp*) varied from 44.57 to 83.70 %, Nei's gene diversity (*H*) from 0.175 to 0.273 with a mean of 0.228, and Shannon's information index (*I*) values ranging from 0.257 to 0.409 with an average value of 0.338 were illustrated. The Tunisian date palm populations exhibited high genetic differentiation (*GST* = 0.319) and gene flow (*Nm* = 1.063). The AMOVA analysis presented 70 % of the variation within the population and 30 % of the variation between them. Phylogenetic analysis and the Bayesian clustering approach also revealed high genetic variation among genetic variants with a net divergence of the wild insular population of Kerkennah from other cultivars. The present investigation suggests the effectiveness of the SCoT marker system to estimate the genetic diversity of *Phoenix dactylifera* genotypes.

Keywords: : *Phoenix dactylifera*; SCoT; Genetic diversity; AMOVA; Population structure; Tunisia

Introduction

Palms (Arecaceae or Palmae) are a family of flowering plants that comprise about 2400 species. Most of them distributed in the tropical and subtropical regions of the world (Govaerts and Dransfield 2005). *Phoenix dactylifera*, which belong to this family, is the central Arboricultural crop of oases. It represents, at present, about 800 000 ha and a living income of 10 million oases worldwide (Elhoumaizi et al., 2002). It is one of the most economically important perennial plants in the ecological and socioeconomic structure of sub-Saharan agriculture.

Phoenix dactylifera was hosted in Tunisia by Phoenicians before the Roman occupation and has played a key role in creating oases. Currently, there were about five million palms cultivated in southern Tunisia oases (40,000 ha). Tunisia's input to world date production is very important; it has improved significantly in recent decades (more than 240.000 mt were produced in 2016 (GIF, 2017)). Further, the Tunisian date palm germplasm is distinct by a remarkable richness, represented by the high number of cultivars (over than 250) (Rhouma 1994, 2005). Date production was exceptionally improved with the dominance of *Deglet Nour* variety, which has a particularly appreciated quality leading to high marketing value. Regrettably, this evolution was accompanied by a significant decline in other cultivars with modest or low quality in spite of their tolerances of different diseases similar to brittle leaf disease or bay-oudh. Therefore, this richness served as a greatly effective

natural protection from pathogens and diseases and deserves to be preserved and evaluated in term of genetic diversity.

Recently, several marker methods were developed for the study of genetic diversity. Genome sequence data offers a huge possibility for the improvement of innovative markers in various plant species (Holland et al., 2001). In parallel with the expansion of genomics research, random DNA markers have improved to become markers targeting genes (Andersen and Lubberstedt, 2003; Gupta and Rustgi, 2004).

Start Codon Targeted polymorphism (SCoT), is a marker system based on the short conserved sequence in plant genes adjacent to the ATG translation start codon (Sawant et al., 1999). SCoT marker is easily produced by polymerase chain reaction (PCR). As well, the amplification products of SCoT can simply be figured by standard gel electrophoresis using agarose, tampons, and staining. This practice is more pertinent for research with standard equipment. The efficiency of this technique for the building of genetic maps, fingerprinting and phylogenetic analysis has been established in many plants like mango (Gajera et al., 2014), sugar cane (Que et al., 2014), tomato (Shahlaei et al., 2014), castor (Kallamadi et al., 2015), coconut (Rajesh et al., 2015) and maize (Vivodik et al., 2016).

The goals of this study were to ensure the efficiency of SCoT markers for the study of genetic diversity and relationships among date palm accessions originating from Tunisia. These outcomes could be useful for *Phoenix dactylifera* germplasm collection, conservation, and breeding.

Materials and Methods

Plant material and DNA preparation

Thirty-one accessions of the Tunisian date palm, listed in Table 1, were used in this study. These were collected from key regions of date palm plantations in Tunisia to know the oasis of Degache (Tozeur), Kebili and the island of Kerkennah (Fig 1).

Fig. 1

Tunisia map with locations of Tunisians date palm oases included in the study.

DNA isolation, PCR reactions, and DNA sequencing

Total cellular DNA was purified from young frozen leaves using the InnuPREP Plant DNA Kit (Analytik Jena, Germany) according to the manufacturer's protocol. The DNA concentration was evaluated using a spectrophotometer (Gold S54T, Shanghai) and their integrity was verified by agarose mini-gel electrophoresis (Sambrook et al., 1989). Twelve SCoT primers were randomly selected and only eleven were chosen for their effectiveness and reproducibility (Table 2). Amplification was performed in 25 μ L samples containing 2 μ L 25 ng/ μ L DNA, 2.5 μ L Reaction Mix, 1 μ L 20 μ M primer, 1 μ L 100mM dNTP mix, 0.3 μ L 2.5U/ μ L Taq DNA polymerase, and sterile ddH₂O (Sufficient for 25 μ L). Primers and the PCR protocol were previously described by Jiang et al. (2014). The agarose gel was visualized and photographed under a Compact Digimage System, UVDI series (Major Sciences, USA).

Data Analysis

Amplified bands were noted as presence (1) or absence (0), and only reproducible primers were examined. The resolving power of the primer (Rp) was determined according to $Rp = \sum Ib$ (Prevost and Wilkinson, 1999). Polymorphic information content (PIC) values were considered using the formula provided by Ghislain et al. 1999:

$$PIC = 1 - p^2 - q^2$$

with p is the frequency of present band and q is the frequency of the absent band. The band informativeness (Ib) was calculated as $Ib = 1 - (2 \times |0.5 - p|)$ (Prevost and Wilkinson, 1999).

Numerous genetic parameters (Percent polymorphism (Pp), number of observed (Na), effective alleles (Ne), Shanon's information index (I), total genetic diversity (Ht), population genetic diversity (Hs), Nei's expected heterozygosity (h), genetic identity (I) and genetic distance (D)) were calculated using the POPGENE software version 1.31 (Yeh et al., 1999). Besides, the Analysis of Molecular Variance (AMOVA), carried out in the GenAlEx 6.5 program (Peakall and Smouse, 2012), was employed to calculate the SCoT variation within and among populations. The estimate of gene flow (Nm) among different groups was made according to the formula $Nm = 0.25 \times (1 - GST) / GST$ (Ryman and Leimar, 2009).

Cluster analysis

Pairwise genetic dissimilarities of genotypes using Jaccard coefficient (1908) were calculated using DARwin version 5.0.158 (Perrier and Jacquemoud-Collet, 2006). The dissimilarity coefficients were used to construct Neighbor Joining trees (Saitou and Nei, 1987) with a bootstrapping value of 10,000 replications.

Additionally, Multivariate analysis was performed to examine relationships between accessions by means of Principal Coordinate Analysis (PCoA) carried out on the genetic distance matrix using GenAlEx 6.5 program (Peakall and Smouse, 2012).

Table 1

Date-palm accessions studied and their main fruit characteristics according to Rhouma, 1994, 2005 (male trees are labeled with an asterisk)

Population	Origin	Cultivars	Label	Status	Fruit characteristics		
					consistency	color	Maturity period
Kebili	Tunisia	Rotab	RO	Cultivated	Soft	Light amber	Early September
	Tunisia	Deglet Nour	DNK	Cultivated	Semi-Soft	Light amber	October
	Algeria	Ghars Souf	GS	Cultivated	Soft	Light amber	Early September
	Tunisia	Dhokkar*	DH	Cultivated	-	-	-
Tozeur	Algeria	Tantabecht	TAB	Cultivated	Soft	Black	End of September
	Tunisia	Oukhet Halaoui	OH	Cultivated	Soft	Light amber	End of August
	Tunisia	Tronja	TRO	Cultivated	Semi soft	Amber	October
	Tunisia	Ammari	AM	Cultivated	Soft	Black	End of July
	Tunisia	Boufeggous	BF	Cultivated	Semi-soft	Greenish brown	Middle October
	Tunisia	Kharroubi	KA	Cultivated	Semi-Soft	Dark brown	Early October
	Tunisia	Deglet Nour	DNT	cultivated	Semi-Soft	Light amber	October
	Tunisia	Arichti	AR	Cultivated	Semi-Soft	Brown	Early October
	Tunisia	Guelb Jemel	GJ	Cultivated	Dry	Dark amber	Early October
	Tunisia	Khouet Aligue	KHA	Cultivated	Semi-Soft	Dark brown	Middle October
	Tunisia	Tazerzit kahla	TK	Cultivated	Soft	Black	End of September
	Tunisia	Mahmoudi	MAH	Cultivated	Soft	Light amber	Early September
	Tunisia	Besser Helou	BH	Cultivated	Dry	Light brown	Middle September
	Tunisia	Bidh Hmam	BHM	Cultivated	Soft	Light amber	End of September
	Tunisia	Lagou	LAG	Cultivated	Semi-soft	Light brown	Middle August
	Tunisia	Aligue	ALG	Cultivated	soft	Dark brown	End of October
	Tunisia	Horra	HR	Cultivated	Dry	Yellowish	End of September
	Tunisia	Borhane 3*	BOR	Cultivated	-	-	-
	Tunisia	CRPh1*	CRP	Cultivated	-	-	-
Kerkennah	Tunisia	Sidi Fi Nkhal	SF	Wild	-	-	-
	Tunisia	Ouled Bou Ali	OBA	Wild	-	-	-
	Tunisia	Khouet Ezzedine	KE	Wild	-	-	-
	Tunisia	Sidi Fraj	SF	Wild	-	-	-
	Tunisia	Sidi Youssef	SY	Wild	-	-	-
	Tunisia	Ouled Kacem	OK	Wild	-	-	-
	Tunisia	Mellita Sghaira	MS	Wild	-	-	-
	Tunisia	Mellita	M	Wild	-	-	-

Table 2

SCoT primers used to genotype *Phoenix dactylifera* accessions

primers	Sequence 5'-3'*	TB	PB	PPB (%)	PIC	Rp
SCoT 10	CAACAATGGCTACCAGCC	7	6	85.71	0.486	2.771
SCoT 45	ACAATGGCTACCACTGAC	10	9	90.00	0.479	3.419
SCoT41	CAATGGCTACCACTGACA	9	7	77.77	0.499	3.225
SCoT 5	CAACAATGGCTACCACGA	9	8	88.88	0.494	3.677
SCoT 7	CAACAATGGCTACCACGG	10	9	90.00	0.486	6.193
SCoT9	CAACAATGGCTACCAGCA	8	6	75.00	0.460	2.129
SCoT23	CACCATGGCTACCACCAG	11	9	81.81	0.449	3.806
SCoT26	ACCATGGCTACCACCGTC	13	11	84.61	0.481	4.838
SCoT 30	CCATGGCTACCACCGGCG	14	12	85.71	0.493	6.322
SCoT 35	CATGGCTACCACCGGCCC	13	11	84.61	0.496	6.193
SCoT 42	CAATGGCTACCATTAGCG	5	4	80.00	0.462	1.032
Total		109	92	-	-	43.605
Mean		9.90	8.36	84.00	0.480	-

TB: total number of bands; PB: polymorphic bands; PPB: percentage of polymorphic bands; PIC: Polymorphic information content and Rp: resolving power of primer.

* Collard and Mackill 2009; Luo et al. 2010.

Table 3

Genic Variation Statistics within different geographic region of *Phoenix dactylifera* revealed by SCoT markers

Population	NPB	PPB	I	H	Na	Ne	Ht±SD	Hs±SD	Gst	Nm
Kerkennah	58	63.04	0.349	0.238	1.630	1.429				
Kebili	41	44.57	0.257	0.175	1.445	1.315				
Tozeur	77	83.70	0.409	0.273	1.837	1.476				
Mean	58.6	63.77	0.338	0.228	1.637	1.406	0.337±0.022	0.229±0.019	0.319	1.063

NPB: number of polymorphic band; PPB: percentage of polymorphic bands; I: Shannon information index of diversity; H: Nei's genetic diversity; Na: observed number of alleles; Ne: effective number of alleles; Ht: total genetic diversity; Hs: population genetic diversity; Gst: Coefficient of genetic differentiation; Nm: estimate of gene flow and SD: standard deviation.

Genetic structure analysis

A Bayesian analysis was achieved by means of the program STRUCTURE (version 2.3) (Pritchard et al., 2000). This program helps to estimate the number appropriate of populations (K) in the sample and to assign individuals to one or other of these populations (K). The model was run for with 10 independent replicate runs for each K value (K ranging from 1 to 10), used a burn-in length of 500,000 and a run length of 10^6 iterations. The most probable number of clusters was considered in accordance with the model value (ΔK) described by Evanno et al. (2005).

Results

Polymorphism and genetic diversity of SCoT markers

A sum of 109 clearly identifiable bands was observed. Among them, 92 polymorphic were generated from 11 SCoT primers. The percentage of polymorphic bands for this species was 84 % (Table 2). The total bands (TB) per primer ranged from five (SCoT 42) to 14 (SCoT 30). The average band per primer was 9.90. Polymorphic information content (PIC) varied from 0.449 (SCoT23) to 0.499 (SCoT41) with an average of 0.480 for this species. The resolving power (Rp) of the tested primers ranged from 1.032 (SCoT42) to 6.322 (SCoT30).

The assessment of the genetic variation within different geographic regions of *Phoenix dactylifera* showed an average observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I) and Nei's genetic diversity (H) respectively equal to 1.63, 1.40, 0.33 and 0.22 (Table 3). The population Tozeur exhibited higher values of I (0.40), H (0.27) and genetic polymorphism (PPB: 83.7 %) than the other two populations. The population of Kebili exhibited lower genetic polymorphism (44.57 %), lower Shannon information index of diversity (I = 0.25) and lower Nei's genetic diversity (H = 1.75). Overall, the average SCoT polymorphism in Tunisian date palm population was elevated (63.77 %).

In addition, the total genetic diversity (Ht) and population diversity (Hs) were estimated to be 0.33 and 0.22, respectively. The relative genetic differentiation was high (GST = 0.31), revealing a high gene flow (Nm = 1.06) among the three populations.

Results from AMOVA identified important variability among populations (30 %), along with 70 % within-population genetic variability.

Genetic distances and ecotype relationships

To supplement the cluster analysis information, a multivariate approach was used. Fig. 2 elucidates the distribution of the ecotypes according to the first two components (axis1-axis2). Results showed that about 43.58 % of the total variation was described by the first three PCoA. The three first dimensions of the principal coordinate analysis represented, respectively, 22.80 %, 12.80 % and 7.98 % of the total genetic variability.

Fig. 2

Principal coordinates analysis (PCoA) for the first and second coordinates estimated for SCoT markers (Axes 1 and 2 explain 35.6 % of the total variability)

Moreover, a *Neighbor-Joining* tree was created using data based on the genetic dissimilarity matrix from all the accessions (Fig. 3). The 31 date palm accessions were clustered into three major clusters. The first one included eight accessions collected from the Island of Kerkennah. Based on bootstrap values (87) this one is significantly divergent from other ones regrouping cultivars from Tozeur and Kebili. The relationships observed in the principal coordinate analysis (PCoA) were in harmony with the analysis by the *Neighbor-Joining* approach. Both methods generate similar clustering with the net divergence of the population of Kerkennah from other cultivars.

Fig. 3
NJ dendrogram of Tunisian date palm accessions with a bootstrapping value of 10,000 replications constructed with Pairwise genetic dissimilarities based on SCoT alleles. Green (Tozeur), blue (Kerkennah) and red (Kebili) accessions.

Population Structure

The population structure of the 31 Tunisian date palm accessions was examined assuming the Hardy-Weinberg Equilibrium by means of STRUCTURE V2.3.4 software. Based on maximum likelihood and delta K (ΔK) values, date palm genotypes were assigned to two genetically different clusters (Fig. 4). According to the model $K = 2$, the cluster 1 (green) grouped the accessions from Kerkennah, accessions from Kebili and some accessions from Tozeur ('Oukhet Halaoui', 'Tronja', 'Ammari', 'Boufegous'). Cluster 2 (red) included the remaining cultivars from Tozeur. This separation is not correlated with the characteristics of fruits like consistency, colors or maturity period. At $K = 3$, date palm accessions were clearly differentiated into two geographic groups, the first cluster (red) consisted of only accessions originated from Kerkennah. The second cluster was composed of the accessions from Tozeur and Kebli. This observation is in line with phylogenetic analyses.

Fig. 4
Genetic relationships of 31 *Phoenix dactylifera* accessions inferred from STRUCTURE analysis (a). Each individual is represented by a vertical bar partitioned into colored segments representing the proportion of the individual's genome in the K clusters. The model with $K = 2$ and $K = 3$ showed the highest ΔK value (b). Population 1: Kerkennah; Population 2: Kebili; Population 3: Tozeur.

Discussion

In the early periods, date palm germplasm was studied via standard morphometric and vegetative criteria. In the last two decennaries, analytic fruit parameters, isozyme markers and a large panel of DNA-based markers developed were used. Given that, studies have concentrated on detecting DNA markers appropriate for fingerprinting of date-palm cultivars and/or for varietal identification, as well as for the evaluation of the genetic organization of this crop. In this subject, this study illustrates the development of the SCoT markers to detect polymorphisms in Tunisian *Phoenix dactylifera* L. The genetic investigate using these markers would be more valuable for crop improvement programs. The used SCoT primers, showed that 84 % of the obtained bands were polymorphic, which was high compared to previous studies, on Tunisian date palm, utilizing dominant marker systems like RAPD (Trifi et al., 2000) and ISSR (Zehdi et al., 2002). Therefore, compared with data previously reported with AFLP or else RAMPO markers (Rhouma et al., 2007, 2008), we assume that the designed procedure has permitted to detect the lowest level of polymorphism in this crop; this result might be due to the most conserved regions targeted with SCoT primers. On the other hand, the level of polymorphism detected was higher than find out by Al-Qurainy et al. (2015) within Saudi Arabian date palm using the same marker. We believe that all tested primers are powerful to detect

DNA polymorphisms in the date palm crop. This hypothesis is strongly maintained by regards the high resolving power (R_p) and PIC rates.

Genetic diversity among and within populations of date palm cultivars was, also, analyzed. Among the three geographical regions, the higher genetic diversity was observed in Tozeur ($NPB = 77$; $PPB = 83.70\%$; $I = 0.409$; $H = 0.273$; $Na = 1.837$), followed by Kerkennah and Kebili. Some reasons could cause the differentiation of genetic variation of different geographical regions: climate conditions (Xie et al., 2009), topography (Yan et al., 2006), and sample size (Xie et al., 2010). In many species, genetic variation is frequently related to population size (Xie et al., 2010). Nevertheless, eco-geographical and historical factors may be more significant than the sample size in the assessment of diversity: Tozeur is an oasis in southwest Tunisia. This oasis is situated between Chott el Djerid and the smaller Chott el Gharsa. They are closely associated with specific geology, geomorphology, and water supply with the accumulation of salts in the environment (Hachicha and Ben Aissa, 2014). In addition, in the fourteenth century, Tozeur's date palm agriculture became the most developed than other regions, many varieties grown in this region were the seedling origin or brought by travelers and pilgrims especially from the East (Hamza et al., 2015). The coefficient of genetic differentiation (GST) was found to be high (0.319) and indicate there is a large genetic differentiation base regardless of the level of gene flow ($Nm = 1.063 > 1$).

AMOVA revealed that 70 % of the total genetic diversity occurred within groups, while the leftover 30 % of the total variation occurred among groups. This is probably due to geographical isolation and gene flow. The prospected areas (Tozeur and Kebili and Kerkennah) had a specific geographic location and geomorphologic limits, which make it relatively, isolated areas. As we wrote before, Chott el Djerid (area 7 000 km²) is located between Tozeur and Kebili oasis. In a natural way, this geographic factor could be a crucial barrier, which could avoid gene exchange among situated areas on both sides of the Chott. Moreover, Kerkennah Islands are a group of islands occupy the east border of the country. The gene flow in date palm cultivars occurs through pollens (Balthazard, 2013), which make their move impossible in both cases. In addition, this intragroup diversity may be explicated by the Cross-pollination reproduction due to the dioecy of species considerably, which affects the high dissimilarity within groups and clarifies the level of polymorphism detect.

Cluster analysis and the bayesian analysis of STRUCTURE revealed grouping date palm accessions from the three geographical regions (Tozeur, Kebili, and Kerkennah) in accordance with their breeding status and also with their geographical origins but not with their fruit characteristics. In fact, the studied populations were always subdivided into two major clusters; „Kerkennah“ and „Kebili / Tozeur“. The cluster „Kebili / Tozeur“ is heterogeneous, forming distinct geographical subgroups. This is not in conformity with previous studies involved in Tunisian accessions (Rhouma et al., 2007, 2008, 2014; Zehdi et al., 2012; Zehdi-Azouzi et al., 2016), which propose the hypothesis of a unique population of date palm. Discordance between results could be explained by region coverage by each markers, since SCoT markers unlike other ones are generated from the functional region of the genome (Hajibart et al., 2015).

Accessions from Tozeur were cauterized with the Kebili's ones suggesting a narrow genetic basis among the cultivars studied in spite of their phenotypes distinctiveness. The population from the Island of Kerkennah is characterized by an important number of spontaneous and wild date palms resulting from natural seed-propagation. Our results show an autochthonous behavior and are coherent with anthropogenic pressure and culturing practices and their insular status (Zehdi-Azouzi et al., 2016).

In conclusion, it is decisive to comprehend the genetic polymorphism and variation among and within accessions to decide the efficient plan for conservation and sampling management. In this study, SCoT marker, theoretically similar to the ISSR and RAPD marker, was an efficient technique to estimate the genetic variation and to detect polymorphism among date palm germplasm accessions. Additionally, our results revealed a high genetic variation among tested genotypes, which can be used in date palm breeding programs and for selecting a suitable gene pool for genetic development of this important crop species.

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Table 4

Analysis of molecular variance (AMOVA) of the date palm accessions revealed by SCoT markers

Source	df	Sum of square	Estimated variability	Total variation	P-value*
Among pop	2	113.016	5.251	30	p<0.001
Within pop	28	349.178	12.471	70	p<0.001
Total	30	462.194	17.721	100	p<0.001

df: Degree of freedom; P-value: probability of null hypothesis

*Significance tests after 1000 permutations

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