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Genetic Diversity Analysis of Date Palm (*Phoenix dactylifera L.)* Cultivars from Morocco Using SSR Markers

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Abstract

In Morocco, date palm is the most important arboricultural crop and little is known about its germplasm. Thus, this work aimed at analyzing genetic diversity among 200 date palms sampled from three oases (Figuig, Tata and Zagora) of Morocco using microsatellite markers. Among these palms, 191 were females, belonging to 26 cultivars, and 9 were males. Eighteen primers were used for the analysis of their genetic diversity. Only 15 primers amplified successfully all the samples. The total number of alleles was 116 and the percentage of polymorphic loci was high and ranged between 60 and 100% with an average of 93.33%. The genetic similarity values ranged from 0.146 to 0.745. The molecular variance analysis showed 64% of variability among cultivars. The obtained dendrogram showed three groups and generally, a good structuring of cultivars. However, we noticed one case of homonymy among cultivars. In fact "Tadmant" cultivar of Figuig was different from "Tadmant" of Tata and Zagora. Males were clustered in two main subgroups.

Keywords: Cultivars; *Phoenix dactylifera*; Genetic diversity; Microsatellite markers; Morocco

Introduction

Date palm (*Phoenix dactylifera L.*) is a dioecious perennial monocotyledon plant with long generation times (a period of 4 to 5 years is necessary to reach the first flowering) that belongs to Arecaceae family [1]. It is a diploid (2n=2x=36), and the predicted genome size is estimated to be approximately between 550 and 650 Mbp [2]. Date palm has traditionally been vegetatively propagated from offshoots produced by elite individual trees. In Morocco, more than 220 clonally propagated varieties are known [3] and date palm is the most important arboricultural crop. There are 4.7 million palm trees covering a surface area of approximately 44,000 ha. More than 220 clonally propagated varieties are known [3]. All commercial varieties are female and there is no method yet of producing male palms of these varieties. However, the effects of pollen on date quality through metaxenia are well documented, and male genotypes with desirable qualities are maintained in the plantations and commonly used to hand pollinate female trees.

The most serious fungal disease threatening date palm plantations in North Africa, especially in Morocco is "Bayoud" [4]. This vascular wilt caused by *Fusarium oxysporum f. sp. albedinis* has recently affected about 67% of Moroccan palm trees and has continued to spread to the East, demolishing a large portion of the palm groves in its path. This disease has destroyed more than 12 million palms in Morocco [5].

Despite all these problems, little is known about Moroccan date palm genetic diversity and resistance of different cultivars to Bayoud.

Date palm varieties can be differentiated using morphological markers viz., shape, size, weight, color, aspects of fruit skin, consistency, texture, etc. [6,7] and biochemical markers like isozymes and proteins [8-10] but these traits are greatly influenced by environmental factors as well as the developmental stages of the plant. The random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), random amplified microsatellite polymorphism (RAMPO) and microsatellites (SSR) have earlier been employed for germplasm characterization of different date palm cultivars from Saudi Arabia and other countries like Qatar, Egypt, Tunisia, Sudan, Mauritania and Morocco with similar climatic conditions [11-24]. In most of these studies, considerable genetic diversity has been detected in date palm germplasm.

Microsatellites or SSR (simple sequence repeats) or STR (short tandem repeats) are repeating sequences of 2 to 6 nucleotides in non-coding regions generally [25]. They are distributed in all regions of the genome [26-29] and are present in all eukaryotes [30,31] and in some prokaryotes [32] and are evenly spread across the genome [26-29]. Microsatellites are preferential markers used in the study of genetic diversity because they are abundant [33,34], typically co-dominant and of a high variability [35]. The objective of this work is therefore to analyze the genetic diversity of common cultivars of three date palm oases from Morocco using SSR markers.

Materials and Methods

Study area

Samples were collected in three oases (Figuig, Tata and Zagora) (Figure 1). Figuig is situated in North East Morocco (latitude: 32°07'1" N, longitude: 1°13'37" W, altitude: 903 m); Tata is in North West Morocco (latitude: 29°44'34" N, longitude: 7°58'21" W, altitude: 670 m) and Zagora is in South East Morocco (latitude: 30°19′56″ Nord, longitude: 5°50′18″ Ouest, altitude: 698 m).

Figure 1: Representation of Figuig, Tata and Zagora oases on Morocco map.

Plant material

A set of 200 date palm samples were used in this study (Table 1). They consisted of 26 female cultivars and 9 males from 3 Moroccan oases. In Figuig oasis, 128 samples (121 belonged to 11 cultivars and 7 males) were collected; in Tata, 20 samples (18 belonged to 9 cultivars and 2 males) and in Zagora, 52 samples (belonging to 10 cultivars). Cultivars were the most common genotypes in these oases while males were less frequent as they only serve as pollinators. Young leaves of adult trees were dried and conserved in silicagel.

Oases	cultivars	Number of samples
	Afroukh n'tijent	10
	Admam	4
	Aziza bouzid	15
	Aziza manzou	15
	Assiane	17
Figuig	Boufeggous	16
	Boufeggous gharas	11
	Mejhoul	4
	Taâbdount	11
	Tadmamt	3
	Tgharas	15
	Males Total	7 128
	Admam	2
	Amanane	1
	Boufeggous	4
	Bouhassa	1
	Bouyatoub	1
Tata	Bouskri	2
	Jihel	4
	Taghanimt	1
	Tiguemi n'tanout	1
	Sayer laalat	1
	Males Total	2 20
	Ahardane	2
	Aguellid	10
	Boufeggous	10
Zagora	Bousthammie noire	2
	Bouzgagh	2
	Iklane	2
	Jihel	10
	Meh albayd	2
	Oum nehl	2
	Tadmant Total	10 52

Table 1: List of collected date palm cultivars in different Oases.

DNA isolation

Genomic DNA was extracted using a modified preparation procedure according to Bousquet et al. [36]. After purification, DNA yields were determined by a NanoDrop 800 spectrophotometer (PEQLAB Biotechnologie GmbH) and diluted to a working concentration of 25 ng/l.

Microsatellites amplification and electrophoresis

A PCR was carried out using 18 date-palm SSR markers developed by Billotte et al. [37] and Akkak et al. [38] (Table 2). PCR reactions were performed in a total reaction mixture of 10 µl containing: 25 ng template DNA; 1 µl of PCR buffer 10 X; 2.0 mM $MgCl_2$; 0.2 mM dNTP; 0.06 µM of the universal Cy5-labeled M13- primer (5´-tgtaaaacgacggccagt-3´); 0.08 µM of M13-tailed forward primer at the 5´end [39], (F); 0.1 µM of reverse primer (R); 0.5 U of Taq DNA polymerase (Tina Taq). Amplifications were performed using a MJ Research PTC 200 thermal cycler (Bio-Rad Laboratories Inc.) with the following cycle profile: initial denaturation for 1 min at 95°C followed by 35 cycles with 30 sec at 94°C, 1 min at primer-specific annealing temperature (Table 2), 2 min at 72°C and a final extension step at 72°C

for 8 min. All PCR products were separated on a 6% polyacrylamide denaturing gel using an automatic laser fluorescence-sequencing machine (ALFexpress DNA Sequencer, GE Healthcare) by loading 2 µl of PCR product diluted 4 X in loading buffer.

Primer names	Primer sequences	Repeated motif	Size (bp)	Annealing temperature (°C)
MPdCIR010	F: ACCCCGGACGTGAGGTG R: CGTCGATCTCCTCCTTTGTCTC	(GA)22	180	55,9
MPdCIR015	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT	(GA)15	253	51,6
MPdCIR016	F: AGCGGGAAATGAAAAGGTAT R:ATGAAAACGTGCCAAATGTC	(GA)14	209	51,7
MPdCIR025	F:GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	(GA)22	269	49,3
MPdCIR032	F: CAAATCTTTGCCGTGAG R: GGTGTGGAGTAATCATGTAGTAG	(GA)19	376	51,5
MPdCIR035	F: ACAAACGGCGATGGGATTAC R: CCGCAGCTCACCTCTTCTAT	(GA)15	341	53,9
MPdCIR044	F: ATGCGGACTACACTATTCTAC R: GGTGATTGACTTTCTTTGAG	(GA)19	340	51,7
MPdCIR048	F: CGAGACCTACCTTCAACAAA R: CCACCAACCAAATCAAACAC	(GA)32	439	51,4
MPdCIR050	F: CTGCCATTTCTTCTGAC R: CACCATGCACAAAAATG	(GA)21	568	48,5
MPdCIR057	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC	(GA)20	360	55,4
MPdCIR063	F: CTTTTATGTGGTCTGAGAGA R: TCTCTGATCTTGGGTTCTGT	(GA)17	301	49,8
MPdCIR070	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTTGTAGTAT	(GA)17	265	48,7
MPdCIR078	F: TGGATTTCCATTGTGAG R: CCCGAAGAGACGCTATT	(GA)13	260	49,6
MPdCIR085	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA	(GA)29	375	50,4
MPdCIR090	F: GCAGTCAGTCCCTCATA R: TGCTTGTAGCCCTTCAG	(GA)26	269	48,6
MPdCIR093	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	(GA)16	230	51,8
PdCAT01	F: AACCACGGGGATCTATTTGT R: AACTTCTACTTTGCTTGCCATCA	(TC)21	161	51

PdCAT20	F: CCATTTCAGACACATCAAGTAAC R: GGATATAAGTAGCTAACCCGAACC	(GA)29	219	53

Table 2: Characteristics of 18 used microsatellite primers.

Analysis of SSR data

The targeted fragments and allele scoring were performed by the ALFwin Fragment Analyser Software. For each marker, the average number of alleles per locus, the expected heterozygosity (He) and the observed heterozygosity (Ho) were calculated by GenAlex 6.3 software. The fixation index or F-statistic (Fis, Fst) were computed according to Wright [40]. Values of FST (fixation index) ranged from 0 (completely undifferentiated) to 1 (completely differentiated). The genetic similarity and the analysis of molecular variance (AMOVA) were also calculated using GenAlex 6.3 software [41]. DARwin 5.0 software was used to make dendrograms which showed the distribution of different individuals.

Results

Microsatellite amplification

Among the 18 microsatellite makers used, 15 showed a net amplification of DNA fragments. MPdCIR044, MPdCIR048 and MPdCIR063 primers amplified only a few or no samples. In addition, these amplifications showed no polymorphism. Therefore, these three loci were not considered in the following statistical analyses.

Figure 2 represents an example of polyacrylamide gel showing the pattern of DNA fragments amplified with MPdCIR25 marker. The different sizes of the DNA fragments amplified, showed the polymorphism of the markers. This polymorphism is used for the determination of differences between samples and to calculate the genetic parameters.

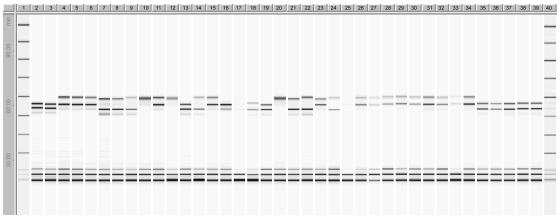

Figure 2: Pattern of DNA fragments with marker MPdCIR25.

- lanes 1 and 40: Ladder
- lanes 2 and 3: Taâbdount
- lanes 4 to 6: Jihel
- lanes 7 to 21: Tgharas
- lanes 22 to 34: Tadmant
- lanes 35 to 39: Mejhoul

Allele number and percentage of polymorphic loci

A total of 116 alleles were detected for 15 selected SSR loci. The number of alleles per locus varied from 4 (MPdCIR35) to 11 (MPdCIR50 and MPdCIR70) with a mean of 7.7 alleles per locus (Table 3).

Primers	**Pd10**	**Pd15**	**Pd16**	**Pd25**	**Pd32**	**Pd35**	**Pd50**	**Pd57**	**Pd70**	**Pd78**	**Pd85**	**Pd90**	**Pd93**	**AT01**	**AT20**
Allele number	8	7	5	6	11	4	11	6	10	11	10	6	5	8	8

Table 3: Allele number per primer of Moroccan cultivars calculated with GenAlex 6.3 software Pd10: MPdCIR010; Pd15: MPdCIR015; Pd16: MPdCIR016; Pd25: MPdCIR025; Pd32: MPdCIR032; Pd35: MPdCIR035; Pd50: MPdCIR050; Pd57: MPdCIR057; Pd70: MPdCIR070; Pd78: MPdCIR078; Pd85: MPdCIR085; Pd90: MPdCIR090; Pd93: MPdCIR093; AT 01: PdCAT01; AT20: PdCAT20.

The percentage of polymorphic loci per cultivar varied between 60% and 100% with an average of 93.33% (Table 4).

Cultivars	**Percentage (%)**
Assiane	80,00
Boufeggous (Figuig)	100,00
Tgharas	86,67
Aziza bouzid	100,00
Aziza manzou	100,00
Boufeggous gharas	100,00
Taâbdount	93,33
Boufeggous (Zagora)	100,00
Jihel (Zagora)	93,33
Tadmant (Zagora)	93,33
Afroukh	100,00
Aguelid	60,00
Mâles (Figuig)	100,00
Boufeggous (Tata)	100,00
Jihel (Tata)	93,33
Admam (Figuig)	100,00
Mejhoul	93,33
Tadmant (Figuig)	100,00
Mâles (Tata)	93,33

Admam (Tata)	80,00
Mean	93,33

Table 4: Percentage of polymorphic loci of Moroccan cultivars calculated with GenAlex 6.3 software.

Heterozygosity and fixation index

The total rate of heterozygosity (Ht) by primer for all cultivars was very high and varied between 0.696 (MPdCIR93) and 0.945 (PdCAT20) (Table 5). The average of expected heterozygosity (MHe) ranged between 0.464 (MPdCIR16) and 0.677 (PdCAT20) and the average of observed heterozygosity (MHo) ranged between 0.816 (MPdCIR78) and 0.950 (PdCAT20) (Table 5). For all the markers, the observed heterozygosity value was higher than the expected one. The Fis values were negative for all markers and varied between -0.895 (MPdCIR32) and -0.404 (PdCAT20) per marker with an average of -0.757 (Table 5). The Fst values for their part varied between 0.267 (MPdCIR93) and 0.472 (MPdCIR78) with an average of 0.363 (Table 5).

	Pd10	Pd 15	Pd 16	Pd 25	Pd 32	Pd 35	Pd 50	Pd 57	Pd 70	Pd 78	Pd 85	Pd 90	Pd 93	AT01	AT20	Mean
Ht	0.800	0.736	0.736	0.798	0.718	0.811	0.847	0.819	0.828	0.906	0.853	0.831	0.696	0.850	0.945	
MHe	0.516	0.512	0.464	0.518	0.517	0.524	0.541	0.491	0.478	0.479	0.506	0.512	0.510	0.484	0.677	
MHo	0.913	0.898	0.822	0.935	0.980	0.950	1.000	0.856	0.846	0.816	0.907	0.844	0.902	0.900	0.950	
Fis	-0.769	-0.755	-0.772	-0.805	-0.895	-0.813	-0.849	-0.744	-0.772	-0.706	-0.790	-0.649	-0.767	-0.861	-0.404	-0.757
Fst	0.355	0.305	0.370	0.350	0.280	0.354	0.361	0.401	0.423	0.472	0.406	0.384	0.267	0.431	0.284	0.363

Table 5: Heterozygoty and fixation index of Moroccan cultivars calculated with GenAlex 6.3 software Ht: total rate of heterozygosity; MHe: average of expected heterozygosity; MHo: average of observed heterozygosity; Fis: fixation index of individuals relative to subpopulations; Fst: fixation index of subpopulations compared to the total population.

Genetic similarity

Table 6 showed the degree of similarity between cultivars. The genetic similarity values ranged from 0.146 to 0.745. The highest similarity value was observed between “Boufeggous” et “Boufeggous gharas” while “Aguelid” and “Mejhoul” had the smallest similarity value. “Mejhoul” and “Boufeggous” had also a high genetic similarity (0.702).

Bfgs	Assia	Tghar	Azi b	Azi m	Jihel	Tadm	Bf gh	Taâb	Afr nt	Aguel	Mâles	Adm	Mejh	
1.000														**Bfgs**
0.226	1.000													**Assia**
0.636	0.549	1.000												**Tghar**
0.324	0.428	0.461	1.000											**Azi b**
0.283	0.372	0.507	0.489	1.000										**Azi m**
0.380	0.335	0.380	0.578	0.393	1.000									**Jihel**
0.544	0.440	0.700	0.309	0.357	0.416	1.000								**Tadm**
0.745	0.395	0.649	0.417	0.386	0.306	0.495	1.000							**Bf gh**
0.410	0.317	0.397	0.262	0.280	0.264	0.403	0.476	1.000						**Taâb**
0.482	0.573	0.580	0.465	0.531	0.429	0.553	0.524	0.353	1.000					**Afr nt**
0.184	0.238	0.392	0.310	0.187	0.211	0.246	0.172	0.275	0.244	1.000				**Aguel**
0.486	0.590	0.680	0.417	0.437	0.427	0.572	0.566	0.359	0.585	0.331	1.000			**Mâles**
0.522	0.590	0.629	0.616	0.498	0.481	0.527	0.624	0.472	0.686	0.485	0.656	1.000		**Adm**
0.702	0.309	0.555	0.233	0.205	0.360	0.619	0.531	0.293	0.476	0.146	0.457	0.503	1.000	**Mejh**

Table 6: Genetic similarity between Moroccan cultivars calculated with GenAlex 6.3 software Bfgs: Boufeggous; Assia: Assiane; Tghar: Tgharas; Azi b: Aziza bouzid; Azi m: Aziza manzou; Tadm: Tadmant; Bf gh: Boufeggous gharas; Taâb: Taâbdount; Afr nt: Afroukh n’tijent; Aguel: Aguelid; Adm: Admam; Mejh: Mejhoul.

Analysis of molecular variance between Moroccan cultivars

Molecular variance analysis showed 64% of variability among Moroccan cultivars (Figure 3).

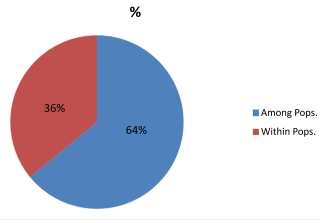

Figure 3: Analysis of molecular variance between Moroccan cultivars.

Dendrogram of similarity of moroccan cultivars

The similarity dendrogram (Figure 4) showed 3 main groups (a, b and c). The first group (c) was divided into 2 subgroups (one subgroup constituted by 2 individuals of "Aziza manzou", 1 individual of "Boufeggous" from Figuig, 6 individuals of "Tgharas", and 2 males from Figuig and one subgroup constituted by the rest of "Tgharas" individuals). The second group (b) was divided into 2 subgroups also. The first subgroup was constituted by individuals of "Boufeggous" from Zagora and from Tata, 1 individual of "Boufeggous" from Figuig, 2 individuals of "Mejhoul", 1 "Admam", 1 "Tadmant" from Figuig, 2 "Boufeggous gharas" and 2 "Ahardane". The second subgroup was constituted by 1 "Admam" and 1 "Tadmant" from Figuig. The third group (a) was also divided into 2 subgroups. The first one was constituted by the 5 individuals of "Tadmant" from Zagora, the 5 males from Figuig remaining and the 2 males from Tata. The second one was constituted by the individuals of "Aguelid", 2 "Taâbdount", 2 "Admam", "Jihel" individuals and by the rest of samples.

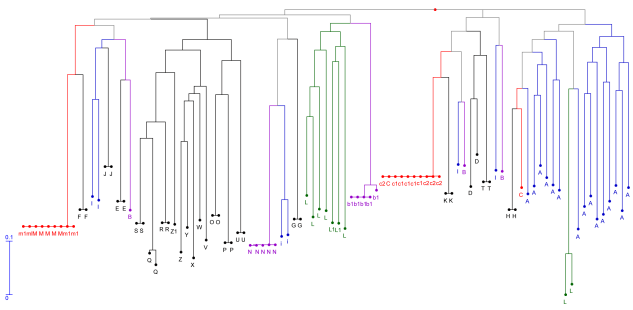

Figure 4: Dendrogram of genetic similarity of Moroccan cultivars.

A: Tgharas **B:** Tadmant (Figuig) **C:** Boufeggous (Figuig)

D: Boufeggous gharas (Figuig) **E:** Assiane **F:** Aziza bouzid

G: Taâbdount **H:** Aziza manzou **I:** Admam,

J: Afroukh n'tijent **K:** Mejhoul **L:** Mâles (Figuig)

b1: Tadmant (Zagora) **c1:** Boufeggous (Zagora) **M:** Jihel2

N: Aguelid **O:** Oum nehl **P:** Iklane

Q: Bousthammie noire **R:** Bouzgagh **S:** Meh Albayed

T: Ahardane **c2:** Boufeggous (Tata) **m1:** Jihel1 (Tata)

i: Adamam (Tata) **U:** Bouskri **V:** Bouhassa

W: Bouyatoub **X:** Sayer layalat **Y:** Amanane

Z: Taghamint **Z1:** Tiguemi n'tanount **L1:** Mâles (Tata)

Discussion

MPdCIR044, MPdCIR048 and MPdCIR063 markers had a low or a lack of amplification of DNA samples. Similar observations have been reported by Zehdi et al. [18]; Billotte et al. [37] and Bodian et al. [23,24] for the first two markers.

The number of alleles found per locus ranging from 4 to 11 is the same as that found by Bodian et al. [24] who used the cultivars of Figuig only with the same markers. It is comparable to that found by Zehdi et al. [18] (ranging between 4 and 10) who recognized 7.14 alleles per locus when examining 46 Tunisian date palm accessions using 14 microsatellite loci. It is also comparable to Elmeer et al. [22] results who found between 4 and 12 alleles per locus. However it is lower to that found by Billotte et al. [37] (ranging between 5 and 18) and it is very high compared to Ahmed and Al-Qaradawi [42] studies who marked 40 different alleles with a mean of 4 alleles per locus by examining 15 Qatari date palm cultivars.

An excess of heterozygosity manifested by negative Fis values was observed. The average value of Fst equal to 0.363 means that the index of genetic differentiation was very high among all cultivars. This result is obtained by Bodian et al. [24] when examined only Figuig cultivars.

Statistical analysis showed that the genetic similarities between cultivars are fairly variable (ranging from 0.146 to 0.745). These values suggest that there are cultivars that are genetically very close and others that are very far. These genetic similarities are comparable to those found by Ahmed and Al-Qaradawi [42] (ranging between 0.00 and 0.75) and by Zehdi et al. [18] (ranging between 0.3008 and 0.7885) and by Bodian et al. [24]. The highest similarity value observed between "Boufeggous" and "Boufeggous gharas" means that they are genetically the closest. While Aguelid and Mejhoul had the smallest similarity value, that means they are the most genetically distant. "Mejhoul" and "Boufeggous" have also a high genetic similarity (0.702).

The analysis of molecular variance (64% of variability among cultivars), the average value of Fst and the values of genetic similarity suggest a variable polymorphism among Moroccan cultivars.

The dendrogram showed that "Jihel" individuals of Tata and those of Zagora were in the same subgroup and in the same level. So this cultivar was the same of one oasis to another and individuals did not show any genetic variability. This was the case of "Boufeggous" individuals of Figuig, Tata and Zagora. So "Jihel" of Tata was the same that "Jihel" of Zagora and "Boufeggous" of Figuig was also the same that "Boufeggous" of Tata and Zagora. However "Tadmant" individuals of Figuig were not in the same group with those of Zagora. Moreover, "Tadmant" individuals of Figuig showed variability while those of Zagora were identical. That could mean that "Tadmant" of Figuig was different that "Tadmant" of Zagora. They were two cultivars which had the same name but were genetically different: they could be homonyms.

All males were in the same subgroup except two males from Figuig. Similar results were observed when Figuig cultivars only were analyzed [24]. Males from Tata and those from Figuig were in the same subgroup. Males were not clustered according to their geographical origin.

This study revealed the existence of genetic variation among Moroccan cultivars. So genetic differentiation was high and an excess of heterozygosity was observed. In general, cultivars were identical from one oasis to another ("Boufeggous", "Jihel"). But, one case of homonymy was noticed. In fact "Tadmant" of Figuig was genetically different from "Tadmant" of Tata and Zagora.

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