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Molecular variance of the Tunisian almond germplasm assessed by simple sequence repeat (SSR) markers

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The genetic variance analysis of 82 almond (*Prunus dulcis* Mill.) genotypes was performed using ten genomic simple sequence repeats (SSRs). A total of 50 genotypes from Tunisia including local landraces identified while prospecting the different sites of Bizerte and Sidi Bouzid (Northern and central parts) which are the most important locations of almond diversity in Tunisia were included. Analysis of molecular variance (AMOVA) was performed for 11 populations from these different regions and foreign countries to examine the distribution of genetic variation of the accessions studied. Results show that the major variation occurred within populations in each geographic site. Additionally, this analysis demonstrates that the genetic diversity within local almond cultivars was important, with a clear geographic distinction between the Northern and the Southern Tunisian cultivars. The value of prospecting new sites, preserving genetic diversity and encouraging on farmers almond collections is also discussed.

Key words: *Prunus dulcis* Mill., Genetic resources, AMOVA, local ecotypes, geographic origin, Tunisia.

INTRODUCTION

In Tunisia, the almond *Prunus dulcis* (Miller) D.A. Webb, syn. *P. amygdalus* Batsch sector, plays an important social economic role with approximately 22 millions of trees dispersed on more than 200,000 ha (FAOSTAT, 2010). In fact, the Tunisian almond plantations located throughout all the country under different climatic conditions offered stable incomes for rural farmers. About 90% of the land devoted to this fruit crop is in the Central and Southern agricultural area of the country under arid and semi-arid conditions. Bizerte (37°16'N, 9°52'E), Sidi Bouzid (35°04'N, 9°49'E) and Sfax (34°44'N, 10°46'E) are the main producing regions (Figure 1).

While preserving genetic resources of fruit trees in gene banks is generally difficult to handle and might not be exhaustive, precise identification of landraces in farm is highly recommended. Furthermore, the correct evaluation of relatedness is capital for efficient genetic resource management and for maintaining enough variability for breeding programs. In the last five years, more than three million of almond trees were lost because of the long period of drought. This has increased the need to preserve as much as possible the Tunisian almond genetic

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Abbreviations: UPGMA, Unweighted pair group method with arithmetic mean; **AMOVA,** analysis of molecular variance; **SSR,** simple sequence repeat.

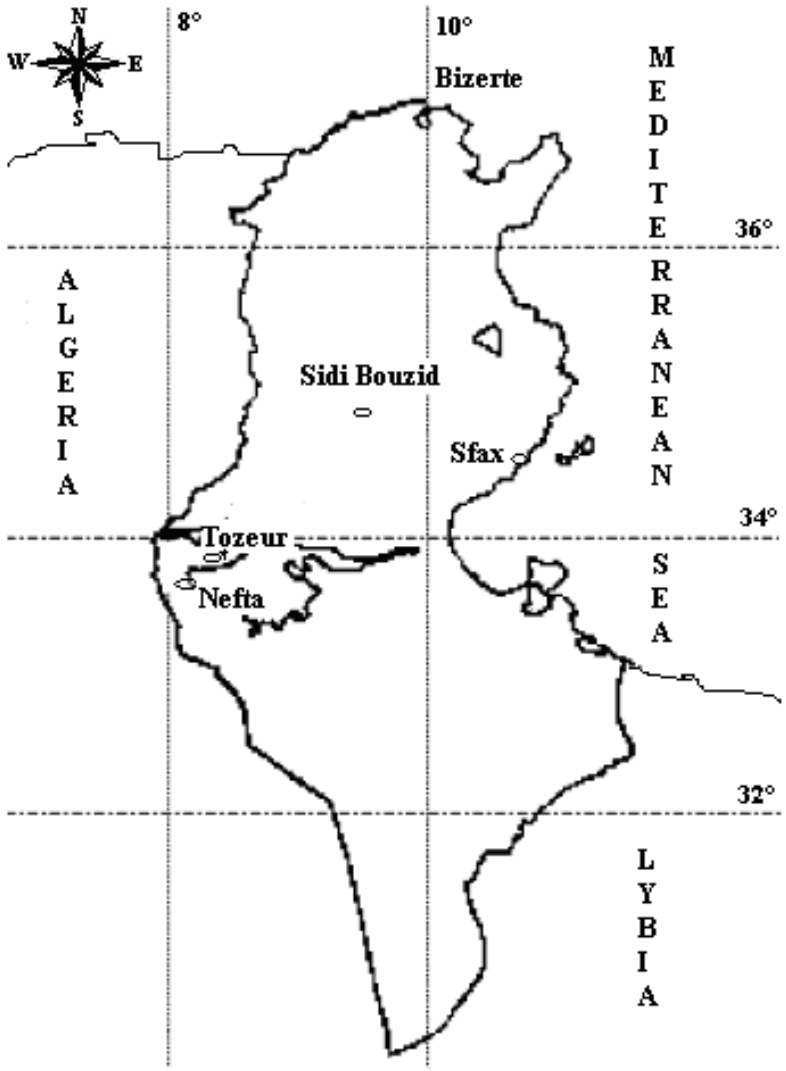

Figure 1. Location of the different geographic sites in Tunisia cited in our study: Bizerte, Sidi Bouzid, Sfax, Tozeur and Nefta.

diversity, in order to reduce genetic erosion. For this, a prospecting effort was carried out during these last few years through the Northern and Central part of Tunisia and an important genetic diversity resulting from chance seedlings or human selections was found.

Recently, various molecular markers such as sequence related amplified polymorphism (SRAP), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNP) have been used for almond genetic diversity assessment (Jing et al., 2013; Sorkheh et al., 2007; Wu et al., 2008). Microsatellites or simple sequence repeat markers (SSRs) are the preferred marker for a wide range of applications in genetics and plant breeding. These markers have been used for the molecular characterization and estimation of genetic diversity among different *Prunus* species, including peach (Aranzana et al., 2002; Bouhadida et al., 2007, 2011; Dirlewanger et al., 2002 and Martínez-Gómez et al., 2003a), sweet cherry (Wunsch and Hormaza, 2002) and apricot cultivars (Hormaza, 2002; Maghuly et al., 2005). Moreover, SSRs are currently being employed for molecular characterization, estimation of genetic diversity and genetic relationships among almond cultivars (Gouta et al., 2010) and other related species (Martínez-Gómez et al., 2003b; Sánchez-Pérez et al., 2006; Shiran et al., 2007; Xu et al., 2004; Zeinalabedini et al., 2008), but to our knowledge, no population identification of the Tunisian almond germplasm was carried out and molecular variance (AMOVA) analysis is still lacking.

As few information is available about the genetic origin of the existing diversity for the Tunisian almond cultivars and their relationship with almonds from the northern border of the Mediterranean, the aims of this work were to identify the SSR analysis of the population arrangement of the accessions collected directly from different sites of the country (Sidi Bouzid and Bizerte), to deter-

mine their relatedness to European and American cultivars and to examine the distribution of the genetic variability.

MATERIALS AND METHODS

Eighty-two (82) almond accessions from different origins (Table 1) were analyzed in this study. Most of them originated from Tunisia (50), the others maintained in the National Collection were from Morocco (1), Spain (8), France (9), Italy (7), USA (3), or were of unknown origin (4).

The 50 Tunisian local genotypes were from the region of Bizerte, Nefta, Sfax and Tozeur that are either preserved in the National Collection of Ettaous, or originated from recent prospection undertaken in the regions of Sidi Bouzid and Bizerte (Figure 1). All the local genotypes assayed were early flowering, self incompatible and had sweet kernels.

Genomic DNA extraction

From all accessions, young leaves were collected for DNA extraction. Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987). DNA concentration and dilutions for polymerase chain reaction (PCR) amplification were carried out as described by Bouhadida et al. (2007).

DNA amplification

DNA was amplified by PCR using 10 primer pairs of microsatellite (Table 2), nine pairs derived from a library enriched for AG/TC motifs, constructed with the almond cultivar 'Texas' (Mnejja et al., 2004) and one pair previously cited by Joobeur et al. (2000).

Amplification reactions were carried out in a final volume of 15 µl containing 10 ng of template DNA and PCR reagents as described in Gouta et al. (2010) with a Gene Amp 2700™ thermocycler (Applied Biosystems, CA, USA) using the following temperature cycles: 1 cycle of 3 min at 95°C; 35 cycles of 1 min at 94°C, 45 s at the corresponding annealing temperature (Table 2) and 1 min at 72°C. The last cycle was followed by a final incubation for 7 min at 72°C and the PCR products were stored at 4°C before analysis. Two independent SSR reactions were performed for each DNA sample. PCR products were loaded on 5% polyacrylamide sequencing gels, silver-stained according to the protocol described by Bassam et al. (1983). Fragment sizes were estimated using 30-330 bp AFLP ladder (Invitrogen, Carlsbad, CA, USA) DNA sizing markers, and analyzed by the quantity one program (Bio Rad, Hercules, CA, USA).

Diversity parameters

Allelic composition of each accession and total number of alleles were scored for each SSR locus from gel profile analysis. Putative alleles were indicated by the estimated size, in bp. Wright's fixation index [F (1/1 – Ho/He) (Wright, 1951)], was calculated for accessions with one or two bands per microsatellite where (Ho) represented the observed heterozygosity, and (He) the expected heterozygosity.

Significant deviations ($P < 0.01$) from Hardy–Weinberg equilibrium (HWE) at individual loci were tested using a Markov chain method by ARLEQUIN, version 3.01 (Excoffier et al., 2005).

Geographic distribution

Distribution of genetic diversity was studied for all accessions through an analysis of molecular variance (AMOVA) using the program ARLEQUIN, version 3.01 (Excoffier et al., 2005). For this, all the cultivars were separated according to their origin into three groups (G1, G2 and G3) including 11 populations as follow: G1, including 6 populations; P1, with genotypes from Sfax (13); P2, South (Tozeur (3) and Nefta (1)); P3, Ben Aoun (14); P4, Regueb (5); P5, Ouled Haffouz (8) and P6, unknown origin (4); G2:P1, North cultivars (Bizerte) (6) and P2, American cultivars (3); G3, European cultivars, P1, Spain (8); P2, France (9) and P3, Italy (7). The cultivar 'Ramlet' originating from Morocco which is phenotypically similar and probably synonyms to the Spanish 'Ramillete' was added to the population from Spain in this analysis.

F statistics relative to each component (that is, F_{CT} among groups, F_{SC} among populations within groups, F_{ST} within populations) were computed. Pairwise F_{ST} values between regions were used to construct an unweighted pair group method with arithmetic mean (UPGMA) dendrogram using NTSYSpc 2.11 (Rohlf, 2000).

RESULTS

The fixation index average (F=0.13) shows a deficit of heterozygosity and a significant divergence over Hardy-Weinberg expectation ($P < 0.01$) for nine of the 10 loci studied (Table 3).

AMOVA calculations were performed (Table 4) with 11 populations grouped by their geographic origin into three groups as described in the material and methods and divided into 11 populations: The G1 with local cultivars from South (Tozeur and Nefta), Center (Sidi Bouzid): Ben Aoun, Ouled Haffouz and Regueb; G2 grouped the American cultivars in addition to the Tunisians from Bizerte that were also added to this group according to the results of the dendrogram (Figure 2), which surprisingly allocated this population in the same cluster with the American cultivars and G3 including cultivars from France, Italy, Spain.

The results of the AMOVA analysis (Table 4) showed that although the great majority of the variation (90.54%) estimated with the ten SSR markers occurred within populations, a small but significant proportion was attributed to differences among groups (6.48%) and among populations within groups (2.98 %). F values at different levels were significant (F_{CT} = 0.06484, F_{SC} = 0.03187, F_{ST} = 0.09464) with $P < 0.001$. Similar variation percentage within populations (88.7%) was also noted by Bouhadida et al. (2011) when studying genetic variability of introduced and local Spanish peach cultivars.

The dendrogram (Figure 2) based on population pair wise genetic distance (F_{ST}) between regions, showing the distribution of genetic diversity for all accessions, differentiates two main groups (A and B). Group A includes the foreign populations and cultivars from North of Tunisia (Bizerte) while in group B are included the rest of the Tunisian populations from the central (Sidi Bouzid) and Southern (Sfax, Tozeur and Nefta) part of Tunisia.

DISCUSSION

In this work, we studied with 10 SSRs, the main Tunisian almond cultivars preserved in the National Collection

Table 1. List of origin, location and description of the 82 almond genotypes studied.

Cultivar	Origin and Location	Description
Abiodh Ras Djebel	Bizerte (Tunisia) - E. C.	Old local cultivar
Faggoussi	Bizerte (Tunisia) - E. C.	Old local cultivar
Khoukhi	Bizerte (Tunisia) - E. C.	Old local cultivar
Harth Nefta	Nefta (Tunisia) - E. C.	Seedling selection
Achaak M.	Sfax (Tunisia) - E. C.	Seedling of Achaak
Abiodh de Sfax	Sfax (Tunisia) - E. C.	Old local cultivar
Achaak	Sfax (Tunisia) - E. C.	Old local cultivar
Elloumi	Sfax (Tunisia) - E. C.	Old local cultivar
Fekhfekh	Sfax (Tunisia) - E. C.	Old local cultivar
Grosse Tendre de Sfax	Sfax (Tunisia) - E. C.	Old local cultivar
Guerneghzel	Sfax (Tunisia) - E. C.	Old local cultivar
Guerneghzel CH.	Sfax (Tunisia) - E. C.	Old local cultivar
Ksontini B	Sfax (Tunisia) - E. C.	Old local cultivar
Mahsouna	Sfax (Tunisia) - E. C.	Old local cultivar
Sahnoun CH.	Sfax (Tunisia) - E. C.	Old local cultivar
Triki	Sfax (Tunisia) - E. C.	Old local cultivar
Zahaaf	Sfax (Tunisia) - E. C.	Old local cultivar
Tozeur 1	Tozeur (Tunisia) - E. C.	Seedling selection
Tozeur 2	Tozeur (Tunisia) - E. C.	Seedling selection
Tozeur 4	Tozeur (Tunisia) - E. C.	Seedling selection
B200	Unknown - E. C.	Unknown origin
B202	Unknown - E. C.	Unknown origin
B203	Unknown - E. C.	Unknown origin
B204	Unknown - E. C.	Unknown origin
Forme en Boule	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Forme en Poire	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Houcine B.N. 2	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Lakhdhar	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Port retombant	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 1	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 2	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 3	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 4	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 5	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 6	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 7	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 8	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Tlili 9	Ben Aoun (Sidi Bouzid - Tunisia)	Chance seedling
Belgacem N.2	Regueb (Sidi Bouzid - Tunisia)	Chance seedling
Guernghzel B.N.	Regueb (Sidi Bouzid - Tunisia)	Chance seedling
Cheikh Sadok 1	Regueb (Sidi Bouzid - Tunisia)	Unknown origin
Cheikh Sadok 3	Regueb (Sidi Bouzid - Tunisia)	Unknown origin
Cheikh Sadok 4	Regueb (Sidi Bouzid - Tunisia)	Unknown origin
Ancetre 1	Ouled Haffouz (Sidi Bouzid - Tunisia)	Unknown origin
Bouchouka B.S.	Ouled Haffouz (Sidi Bouzid - Tunisia)	Unknown origin
Bouchouka. K.F.	Ouled Haffouz (Sidi Bouzid - Tunisia)	Unknown origin
K.F.3	Ouled Haffouz (Sidi Bouzid - Tunisia)	Chance seedling
K.F.4	Ouled Haffouz (Sidi Bouzid - Tunisia)	Chance seedling
Merghad H.1	Ouled Haffouz (Sidi Bouzid - Tunisia)	Chance seedling
Nabil F.	Ouled Haffouz (Sidi Bouzid - Tunisia)	Chance seedling
Porto Farina*	Ouled Haffouz (Sidi Bouzid - Tunisia)	Unknown origin cultivar

Table 1. Contd.

Blanco	Bizerte (Tunisia)	Old local cultivar
Dillou	Bizerte (Tunisia)	Unknown origin cultivar
Khoukhi Bizerte	Bizerte (Tunisia)	Old local cultivar
Bruantine	France - E. C.	Old local cultivar
Doree	France - E. C.	Old local cultivar
Ferraduel	France - E. C.	Cristomorto x Aï ♣
Ferragness	France - E. C.	Cristomorto x Aï ♣
Fournat de Breznaud	France - E. C.	Marie (1901) ♣
Languedoc	France - E. C.	Old local cultivar
Lauranne	France - E. C.	Ferragness x Tuono ♣
Pointue d'Aureille	France - E. C.	Old local cultivar
Soucaret	France - E. C.	Old local cultivar
Avola	Italy - E. C.	Old local cultivar
Cristomorto	Italy - E. C.	Unknown origin
Fasciuneddu	Italy - E. C.	Unknown origin
Genco	Italy - E. C.	Genco G. (1910)♣
Mazetto syn. Tuono	Italy - E. C.	Old local cultivar
Pizzuta	Italy - E. C.	Old local cultivar
Super Nova	Italy - E. C.	Induced mutation from Fascionello♣
Desmayo Larguetta.	Spain - E. C.	Old local cultivar
Desmayo Rojo	Spain - E. C.	Unknown origin
Guara	Spain - E. C.	Old local cultivar
Malagueña	Spain - E. C.	Old local cultivar
Marcona	Spain - E. C.	Old local cultivar
Mas Bovera	Spain - E. C.	Primorskiy x Cristomorto ♣
Moncayo	Spain - E. C.	Tardive de la verdiere x Tuono
Tarragona	Spain - E. C	Unknown origin
Nec Plus Ultra	USA - E. C.	Hatch A.T. (1884)♣
Non Pareil	USA - E. C.	Hatch A.T. (1884)♣
Peerless	USA - E. C.	Unknown origin

♣A.J. Felipe (2000). *This cultivar was identified in Sidi Bouzid but Porto Farina is the native name of a city (actually Ghar El Melh) in Bizerte. E.C: Accessions are from Ettaous National Collection.

together with some European and American genotypes, as well as some genotypes collected directly from the field of the region of Sidi Bouzid, which is one of the most important area of almond diversity in Tunisia.

The deficit of heterozygosis showed by H_0 and H_e values, the fixation index average (F=0.13) and the significant divergence over Hardy-Weinberg expectation ($P < 0.01$) for nine of the 10 loci could be explained by the population structure and (or) inbreeding like effect. In fact the need of cross pollination for the majority of almond cultivars as auto-incompatible and the historical origin of almond along the both shores of the Mediterranean are strong statements in favor of these hypothesis.

Regarding the analysis with AMOVA of the geographic distribution of genetic diversity for the genotypes studied, its was concluded that more than 90% of the variability detected for all the genotypes studied occurred within populations and less than 10% among groups and among populations within groups (Table 4). This could be probably due to gene flow among population and among groups. On the other hand, the easy germination and transportation of almond seeds could also allow significant gene flow and transfer among different regions and countries. This partition of genetic variation with high level of inter-population gene flow was also pointed out by Escribano et al. (2007) for cherimoya (*Annona cherimola* Mill.) who stressed that it was probably due to the easy germination of seeds of this species. Others author pointed out that environmental factors and geographical distance can affect the genetic structure and lead to higher differentiation among populations (Peleg et al., 2008; Wu et al., 2010). Omirshat et al (2009) studying the population structure of the Chinese wild almond supported our findings and concluded that the exceedingly high genetic differentiation within populations observed

Table 2. SSRs used to study the 82 almond genotypes.

Locus/GenBank accession number	Primer sequence (5'–3')	Motif	Size (bp)	Reference
CPDCT022/AY862459	F: GATCGGCGTCTCCTTTATC R: AAAGCAAGCAGGCAAATGAA	$(CT)_{17}$	133–161	Mnejja et al., 2004
CPDCT025/AY862462	F: GACCTCATCAGCATCACCAA R: TTCCCTAACGTCCCTGACAC	$(CT)_{10}$	172–194	Mnejja et al., 2004
CPDCT027/AY862464	F: TGAGGAGAGCACTGGAGGAG R: CAACCGATCCCTCTAGACCA	$(CT)_{19}$	156–176	Mnejja et al., 2004
CPDCT033/AY862470	F: CAAAACACAAAAACCCACCA R: ATTCGGGGAGTCAATCAGG	$(CT)_{18}$	126–150	Mnejja et al., 2004
CPDCT038/AY862475	F: ATCACAGGTGAAGGCTGTGG R: CAGATTCATTGGCCCATCTT	$(GA)_{25}$	149–181	Mnejja et al., 2004
CPDCT040/AY862477	F: TGATGAGGCCTAGAAATTGGA R: CACAGCAATCAGCAAAAAGC	$(GA)_{24}$	138–170	Mnejja et al., 2004
CPDCT042/AY862479	F: ACGCGTTACAAGTGAGATGC R: TGAAAAATCTTGATGGACGTG	$(GA)_{27}$	164–186	Mnejja et al., 2004
CPDCT044/AY862481	F: ACATGCCGGGTAATTAGCAA R: AAAATGCACGTTTCGTCTCC	$(GA)_{21}$	163–185	Mnejja et al., 2004
CPDCT047/AY862437	F: TCAAAAACACCCATTATTGAA R: AAACATTTAGGGCTTGTTTGG	$(CT)_{10}$	182–204	Mnejja et al., 2004
PS9f8	F: GGTTCTTGGTTATTATGA R: ACATTTCTATGCAGAGTA	-	156	Joobeur et al., 2000

Table 3. Locus name, size range of the amplified fragments, expected heterozygotie (He), observed heterozygotie (Ho), Wright's fixation index (F) and probability test for divergence from Hardy–Weinberg equilibrium (HWE) calculated for 10 SSRs markers in 82 almond cultivars.

Locus	Range size (bp)	He	Ho	F	Divergence from HWE
CPDCT022	133-175	0.83	0.59	0.29	**
CPDCT025	162-200	0.90	0.73	0.19	**
CPDCT027	156-202	0.83	0.87	-0.05	0.032
CPDCT033	116-150	0.85	0.72	0.15	**
CPDCT038	147-197	0.82	0.55	0.33	**
CPDCT040	138-174	0.84	0.61	0.27	**
CPDCT042	160-212	0.92	0.7	0.24	**
CPDCT044	161-227	0.81	0.49	0.40	**
CPDCT047	170-218	0.89	0.73	0.18	**
PS9f8	126-178	0.88	0.81	0.08	**
Mean		0.86	0.68	0.13	

**Significant at $P \leq 0.01$.

for many fruit trees is mainly due to their self incompatibility characteristic.

As a consequence, the UPGMA dendrogram (Figure 2) based on population pairwise genetic distance (F_{ST}) between regions, clearly differentiates two main groups (A and B). Group A included all the European and North American populations in addition to cultivars from Bizerte (North of Tunisia). Group B which includes all the rest of

Table 4. Analysis of molecular variance (AMOVA) partitioning genetic variability within and among 11 populations and three groups after amplification of 82 almond genotypes using 10 SSRs.

Source of variation	df	Sum of squares	Variance component	Proportion of variation (%)	*F*	*P*
Among groups	2	38.357	0.28815	6.48	0.06484	< 0.001
Among populations within groups	8	47.608	0.13244	2.98	0.03187	< 0.001
Within populations	153	615.632	4.02374	90.54	0.09464	< 0.001
Total	**163**	**701.598**	**4.44433**			

Probabilities were derived from 10,100 permutations tests and represent the probability of observing larger variance components at random.

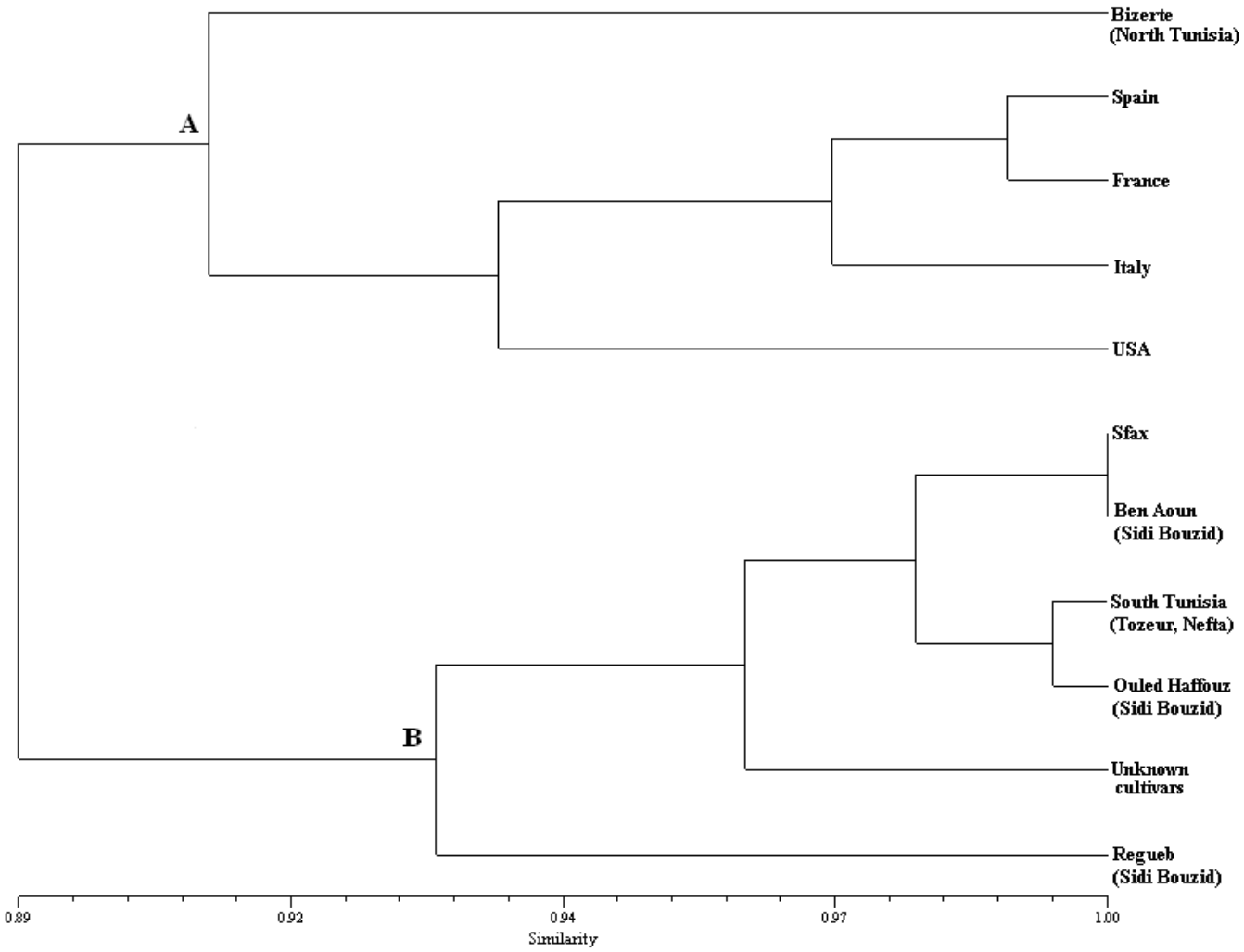

Figure 2. UPGMA dendrogram of population pairwise genetic distances (F_{ST}) among regions derived from AMOVA calculations after amplification with 10 SSRs of 82 almond genotypes.

the Tunisian populations with the four of unknown origin reveals further information. In fact the different populations of Sidi Bouzid (Ben Aoun, Ouled Haffouz and Regueb) seemed to cluster differently. The most distant is the population of Regueb, followed by Ouled Haffouz which is very close to extreme southern (Tozeur and Nefta) while Ben Aoun population is almost identical to Sfax. In fact this can be explained by the easy exchange of almond seeds by farmers and the habitual trade between Sfax (as the 'capital' of the South of Tunisia) and the other southern parts. However, it should be noted that the unknown cultivars population is represented with

a low number of individuals; consequently its position within this group should be taken cautiously. The clear distinction between the northern from one side and the central and southern populations from the other side is definitely due to the natural selection.

These results, reveal the high diversity and the distinct origin of the Tunisian almond germplasm and can be considered as another statement in favor of the hypothesis advanced by Gouta et al. (2010, 2012) regarding a distinct parental and origin of our local cultivars. Thus, prospecting new sites and helping farmers to preserve on farm large diversity will guaranty a sustainable and valueble source of traits for future breeding goals at an international level especially with the actual threats of global warming and its negative effects on biodiversity.

In conclusion, SSRs and AMOVA analyses have been successfully used to examine the crop origin, the degree of parentage and the population distribution of the main Tunisian landraces. In fact, the Northern landraces from Bizerte were genetically related to the European and American cultivars, in the second position were those from Regueb (Sidi Bouzid) while all the others were the most distant. As these last have proved a good adaptation to severe agroecological conditions, they can provide potential new genes for drought tolerance which is of great interest for developing new cultivars.

In summary, the great diversity found inside the Tunisian almond germplasm supports the idea that Tunisia has a valuable source of almond genes to be preserved and exploited in further international breeding programs, although further investigation have to be done for population structure and pedigree analyses

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