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Assessment of Genetic Diversity of Moroccan Cultivated Almond (*Prunus dulcis* Mill. DA Webb) in Its Area of Extreme Diffusion, Using Nuclear Microsatellites

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ABSTRACT

Assessment of genetic diversity of Moroccan cultivated almond (*Prunus dulcis* Mill.) grown from seed and cultivated at four eco-geographical regions was performed using 16 nuclear SSRs. 238 alleles were detected with an average of 14.88 alleles per locus, ranging from 4 (locus BPPCT027) to 24 (locus CPSCT018). The size of alleles ranged from 84 bp (locus UDP96-003) to 253 bp (locus UDP96-018). A high genetic diversity of the local almonds is apparent and structured into three major clusters (Oasis cluster, High and Anti Atlas cluster, and Middle Atlas cluster). Compared to the Mediterranean genetic pools, from the East to West, the genetic diversity tends to be limited in Morocco which is the area of its extreme diffusion.

Keywords: Almond; Genetic Diversity; Polymorphism; Spatial Genetic Structure; *Prunus dulcis*; Microsatellites; SSR

1. Introduction

The almond [*Prunus dulcis* (Miller) DA Webb, syn. *Prunusamygdalus* Batsch] is a widely grown fruit tree that is commercially important throughout the world. It is native to mountainous regions of Central Asia [1,2] and is probably the oldest domesticated fruit tree in the third millennium BC [3]. The almond tree was spread from its origin through the Mediterranean by the Phoenicians, Greeks and Romans in three main dispersion routes: the north route, the southern route and the route through the seas [4,5].

The cultivated almond tree was introduced in the Mediterranean region during the second millennium BC [6,7] with a broad exchange of almond in the fourth century BC [8]. It led to the differentiation between two groups, the Mediterranean species and species of Central Asia [2]. It evolved slowly by seeding to the nineteenth century [1] and its culture, in the region, is often associated with seedling populations with selection of local varieties in some countries [9]. This mode of propagation by seeding generated a great variability in local genotypes. Therefore, the Mediterranean region is regarded as a second source of domestication of the almond [5,10,11].

Morocco is an area of extreme diffusion of the almond tree. It was cultured by the Carthaginians in the fourth century [12] as well as by the Arabs in the sixth century [10]. Almond culture is currently about 146,000 ha [13] of which less than half (about 4 to 5 million trees) consists of populations grown from seed, localized mainly in the south [14]. This sexual propagation has led to high genetic diversity and the country is now considered as a secondary center of almond diversity [15]. Several works on collection and morphological characterization were performed on these populations [16-19]. This traditional plant material, resulting from many centuries of adaptation, may provide a basis for an almond breeding program. A collection that grouped individuals from different regions of Morocco was installed at the experimental field of INRA in Aïn Taoujdate [19] for evaluation efforts. This collection possesses a genetic basis necessary for any breeding program. Genetic characterization of plant material is necessary for the identification of potential genitors and their value in a breeding program. For the optimization of crossing schemes, the molecular characterization of this plant material is essential.

Morphological characters were used in phenotypic observations to characterize the genetic diversity of almond species, but their interactions with the environment and the small number of characters [20-22] prompted the use of other more discriminating techniques. Currently, DNA markers are widely used in studies of genetic diversity

and the clarification of certain research questions, among others, those concerning their genetic origin [9]. These tools have evolved over time and the initial studies were based on isozymes [23-28], RFLP [28], RAPD [29-34], ISSR and AFLP [32,33]. The relatively recent use of microsatellites (simple sequence repeat: SSR) in the characterization of *Prunus* species and other perennial fruit species showed their power of discrimination [9,35-40]. These tools have proven well suited for a wide genetic characterization [41]. They are multi-allelic, co-dominant and highly repeatable and are therefore particularly suitable for phylogenetic studies because of their high polymorphism and abundance [42].

The objective of this work concerns the characterization of genetic variation, using nuclear SSRs, of Moroccan almond plants, quantification of allelic richness, the study of its genetic structure in Morocco and the selection of microsatellite markers developed recently adapted to the characterization of this plant material.

2. Material and Methods

2.1. Plant Material

Collection of plant materials, the object of the present work, consisted of 127 accessions (**Table 1**) of almond [*Prunus dulcis* (Miller) DA Webb, syn. *Prunusamygdalus* Batsch] from different regions of Morocco (**Figure 1**). The areas sampledare grouped into four broad geographic regions (**Figure 1**, **Table 1**) based on climatic conditions, including altitude and type of climate.

2.2. Methods

2.2.1. DNA Extraction

DNA was extracted from young leaves harvested after flowering, following the method described by [43]. The leaves (30 mg) were ground manually with mortar and extracted with Cetyl-Trimethyl Ammonium Bromide (CTAB) hot extraction buffer [100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 4% (w/v) PVP (polyvinyl pyrrolidone), 10 mM β-mercaptoethanol and sodium bisulfite ($NaHSO_3$)]. The mixture was incubated at 65℃ for 1 h, then mixed with 500 µl of chloroform/isoamyl alcohol (24:1) and centrifuged at 13,000 rpm for 15 min. The supernatant was recovered and mixed with 2/3 volume of isopropanol at room temperature (30 min). The resulting pellet was washed in 1 ml of ethanol (76%), dried and then suspended in 100 µl of TE buffer [10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0]. The DNA was quantified by spectrophotometer and stored at 4℃.

Table 1. Plant material collection grouped according to the geographical origin.

Regions (population)	Main areas sampled	Number of accessions collected	Accession No.
Oasis	Errachidia, Sakkoura, Draâ, Tiliwine, Ghris, Goulmima, Tinghir, KelaâtMaggouna et Ouarzazate	72	from 1 to 72
High Atlas	Imi-N-Tanoute, Aït Ourir et Asni	26	from 73 to 98
Middle Atlas	Azilal	10	from 99 to 108
Anti Atlas	Tafraout	9	from 109 to 117
Genotypes to local names		10	from 118 to 127

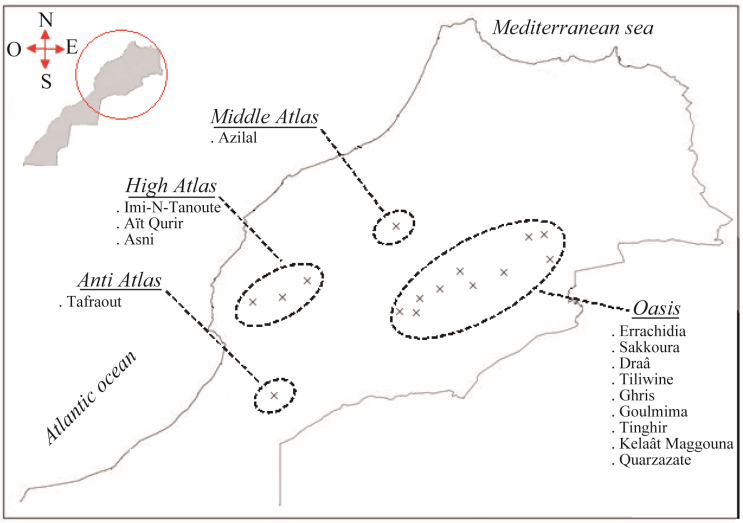

Figure 1. Location map of geographic regions sampled.

2.2.2. PCR Amplification and Electrophoresis

The extracted DNA was amplified by PCR. The sixteen-pairs of primers flanking SSR sequences used in this work were cloned and sequenced in peach [44-48]. The multiplex PCR reactions were carried out with the Type I Microsatellite PCR Kit® (Qiagen) in a final volume of 10 µl, containing 1× of Qiagen Master Mix, 2 µM of each primer and 2 ng/µl of template DNA. The PCR program included: an initial denaturation at 95°C for 5 min, 35 cycles of 30 sec at 95°C, 1 min at the annealing temperature and 1 min 72°C, followed by a terminal phase of 7 min at 72°C. PCR reactions were carried out in an Eppendrof Mastercycler Gradient thermocycler. Samples were prepared by 3 µl diluted PCR products to 14.803 µl formamide and 0.197 GeneScan™ 500 LIZ®Size Standard (Applied Biosystems, USA). The PCR products were detected by ABI 3130 XL 16-capillary sequencer (ABI Prism Applied Biosystems, Foster City, CA, USA).

2.2.3. Data Analysis

Reading the sizes of alleles (bp) was accomplished using Gene Mapper 4.0 software (Applied Biosystems). The number of alleles per locus (Na), observed heterozygosity (Ho) and expected heterozygosity (He) were calculated using the Genetix 4.03 software [49]. The level of polymorphism was estimated by calculating the polymorphism information content (PIC) described by [50] and modified by [51] using the formula $PIC = 1 - \sum p_i^2$, where p_i is the frequency of the ith allele, by the powerof discrimination (PD, [52]), according to the formula above, for which the allele frequency was replaced by the frequency of the genotype and the probability of identity ($PI = 1 - \sum p_i^2 + \sum\sum \left(2p_i p_j\right)^2$, where p_i and p_j are the frequency of the ith and jth alleles, respectively)for which two individuals sharing the same genetic profile by chance [53], calculated using the identity 4.0 [54].

Genetic differentiation was assessed by calculation of Wright's fixation index (Fis) according to [55] and Fst values between each pair of populations using the Genepop 4.1 software [56]. The genetic relationships among genotypes based on the similarity matrix using the proportions of alleles [57] were studied. A dendrogram was prepared based on the unweighted pair group method with arithmetic averages (UPGMA) using the Ntsys-pc 2.02i program [58] (Rholf, 1998). A factorial correspondence analysis (FCA) was carried out also in our work using Genetix 4.03 software [49].

3. Results

The use of 16 SSR loci in the almond trees revealed a total of 238 different alleles, ranging from 4 to 24 alleles per locus. The average number of alleles per locus was 14.88. The size of these alleles varied from 84bp to 253 bp. The observed heterozygosity (Ho) varied between 0.045 (Locus BPPCT027) and 0.916 (Locus CPSCT018) with an average of 0.596. The expected heterozygosity (He) ranged from 0.043 (Locus BPPCT027) to 0.884 (Locus CPSCT018) with an average of 0.699. The calculated values of the probability of identity (PI) and the power of discrimination (PD) showed that the locus CPSCT018 is the most informative with values of 0.012 and 0.979, respectively. This locus has the highest value of polymorphism information content PIC (0.921) relative to other loci. Thus, the least informative locus is BPPCT027 with (PI = 0.817, PD = 0.162 and PIC = 0.098). The averages of PI, PD and PIC for all loci were, respectively, 0.119, 0.868 and 0.763 (**Table 2**).

The comparison of almonds belonging to 4 major geographic regions showed that the number of alleles observed differ from one geographic area to another. The almond trees in the oasis region is characterized by the highest number of alleles (192 alleles) while the lowest number characterized almond trees native to the Anti-Atlas. Thus, the number of alleles per locus (N_A) according to geographical origin, follows the same order with the highest value obtained at the oasis (N_A = 12) and the smallest value in the Anti Atlas (N_A = 5.38). The observed heterozygosity (**Table 3**) is similar in all four geographic areas studied (Ho = 0.600).

The comparison of pairwise Fst values of populations shows that the values vary between 0.00726 and 0.04354 (**Table 4**). Genetic distances are low for the almond trees that come from three geographic regions of the Atlas (High, Middle and Anti Atlas). Fst values of these are not significant. However, the difference is significant between the Oasis almonds and those of the Atlas. The dendrogram was constructed using the UPGMA method and is based on similarity data of 127 genotypes, revealing the existence of a very significant level of genetic diversity among genotypes. Thus, positioning arbitrarily at a level of 27% similarity, three homogeneous groups are distinguished (**Figure 2**). The first group consists of accessions from the region of the Oasis and most of the accessions of the Middle Atlas, the second group is composed mainly of those from the regions of High and Anti Atlas and the third group includes, in addition to genotypes to local names, a mixture of genotypes from regions of the Oasis and High Atlas. A more advanced structure was obtained by three-dimensional factorial correspondence analysis (FCA). The three axes explain, respectively, 48.49%, 32.22% and 19.29% of the variance and allow the distinction of three homogeneous clusters (**Figure 3**). Cluster A contains mostly the accessions of Oasis, cluster B consists of genotypes of High and Anti Atlas and the last cluster C is composed only of

Table 2. Observed alleles and diversity parameters obtained with the 16 SSR loci among almond genotypes.

Locus	Reference	Motif	Sequence (5' - 3')	N	Size (bp)	Ho	He	PI	PD	PIC
BPPCT001	Dirlewanger *et al.* (2002)	$(GA)_{27}$	AATTCCCAAAGGATGTGTATGAG	20	122 - 163	0.227	0.825	0.035	0.898	0.858
			CGAAACCGAGTAAGTGGAC							
BPPCT007	Dirlewanger *et al.* (2002)	$(AG)_{22}(CG)_2(AG)_4$	TCATTGCTCGTCATCAGC	15	130 - 164	0.810	0.818	0.024	0.968	0.882
			ATGGCGATTGAAGTCTTTAGAC							
BPPCT017	Dirlewanger *et al.* (2002)	$(GA)_{28}$	TTAAGAGTTTGTGATGGGAACC	12	138 - 177	0.788	0.789	0.031	0.959	0.870
			CGAACCAATACGATTTAATACGAA							
BPPCT025	Dirlewanger *et al.* (2002)	$(GA)_{29}$	TCCTGCGTAGAAGAAGGTAGC	19	156 - 195	0.729	0.802	0.034	0.963	0.843
			CGGTAAACCTGTAAATACAGC							
BPPCT027	Dirlewanger *et al.* (2002)	$(GA)_{11}$	CTCTCAAGCATCATGGGC	4	238 - 248	0.045	0.043	0.817	0.162	0.098
			CTATAATGTTGGCCCGTTGT							
BPPCT036	Dirlewanger *et al.* (2002)	$(AG)_{11}$	AAGCAAAGTCCATAAAAAACGC	5	244 - 252	0.238	0.391	0.291	0.655	0.509
			TTACCTCGCAGAAGCGGA							
CPSCT018	Aranzana *et al.* (2002)	$(GAA)_2(GA)_8$	AGGACATGTGGTCCAACCTC	24	130 - 183	0.916	0.884	0.012	0.979	0.921
			TACTTTCATTGCCCCTTGGG							
CPDCT045	Aranzana *et al.* (2002)	$(GA)_{21}$	TGGGATCAAGAAAGAGAACCA	16	143 - 189	0.459	0.805	0.023	0.944	0.888
			TTTGTACACGTTCGTGTGGA							
pchgms1	Sosinski *et al.* (2000)	$(AC)_{12}(AT)_6$	GGGTAAATATGCCCATTGTGCAATC	17	183 - 229	0.630	0.790	0.029	0.962	0.873
			CTCCTAACTGCATCAAGTTACTAGG							
pchgms3	Sosinski *et al.* (2000)	$(CT)_{14}$	ACGGTATGTCCGTACACTCTCCATG	18	173 - 219	0.774	0.797	0.051	0.942	0.822
			CAAATTATCCTCGTTAGTGTCCAAC							
UDP96-001	Cipriani *et al.* (1999)	$(CA)_{17}$	AGTTTGATTTTCTGATGCATCC	8	100 - 124	0.535	0.547	0.176	0.891	0.652
			GTTATGGCCAGGAATACCGT							
UDP96-018	Cipriani *et al.* (1999)	$(AC)_{21}$	TTCTAATCTGGGCTATGGCG	7	230 - 253	0.460	0.469	0.237	0.754	0.573
			GGGACAGCATTTACACTTGAAG							
UDP96-003	Cipriani *et al.* (1999)	$(CT)_{11}(CA)_{28}$	TTGCTCAAAAGTGTCGTTGC	17	84 - 129	0.809	0.811	0.038	0.959	0.847
			CGGTCACAACGTGATGCACA							
UDP97-401	Cipriani *et al.* (1999)	$(GA)_{19}$	TAAGAGGATCATTTTTGCCTTG	20	106 - 153	0.759	0.797	0.023	0.968	0.884
			TGGGAGTCAGGAGGTCCC							
UDP98-408	Testolin *et al.* (2000)	$(CT)_{14}$	ACAGGCTTGTTGAGCATGTG	18	91 - 137	0.836	0.832	0.031	0.964	0.867
			AGTTTAAAAGGGTGCTCCC							
UDP98-409	Testolin *et al.* (2000)	$(AG)_{19}$	GCTGATGGGTTTTATGGTTTTC	18	119 - 173	0.521	0.791	0.048	0.926	0.827
			ACAACTATCTCCTATTCTCAGGC							

N: number of alleles, Ho: observed heterozygosity, He: expected heterozygosity, PI: probability of identity, PD: power of discrimination, PIC: polymorphism information content

Table 3. Genetic diversity in the geographical areas.

Population	Genotypes	N	N_A	Ho	He	Fis
Oasis	72	192	12.00	0.597	0.742	0.180
High Atlas	26	136	08.50	0.599	0.711	0.174
Middle Atlas	10	107	06.69	0.598	0.683	0.169
Anti Atlas	09	086	05.38	0.590	0.661	0.167

N: total number of alleles detected in each zone, N_A: average number of alleles per locus, Ho: observed heterozygosity, He: expected heterozygosity, Fis: fixation index intra-population.

Table 4. Fst pairwise values between different geographical origins.

Pop	Oasis	High Atlas	Middle Atlas
High Atlas	0.03899**		
Middle Atlas	0.03843*	0.03239ns	
Anti Atlas	0.04354**	0.00726ns	0.03823ns

ns: non-significant; *P < 0.05; **P < 0.001.

genotypes from the Middle Atlas.

4. Discussion

The present work is the first study which characterizes the diversity of almond grown in Morocco using microsatellite markers as molecular tools. The plant material analyzed comes from seedlings carrying no specific name but it is known locally, by farmers, under the "Louzbeldi" name. Farmers continue to maintain these almond accessions and to further exploit the germplasm by traditional planting of seedlings to establish new orchards.

The SSR loci used in this study were selected, from a set of primer pairs developed in peach, on the basis of their rate of allelic polymorphism [44-48]. The results obtained in our study are consistent with Sosinski et *al*. [45] and Xie *et al*. [59] data, which confirmed the interspecific usefulness of microsatellite markers in *Prunus* species. The three genetic parameters (PI, PD and PIC) have shown therefore that all SSR loci may be recommended for future studies of genetic diversity of almond except BPPCT027locus, which is not very discriminating.

The number of alleles obtained for each locus is high in general but with differences between sub-populations located in the regions. The differences in the number of alleles detected in the oasis population (192 alleles) and that of the Anti-Atlas (86 alleles) could be due to the number of genotypes analyzed at each site. The level of genetic diversity is quite important because of traditional multiplication mode (by seeds) for a crop formerly introduced in Morocco [10,12]. A spatial genetic structure appears to be demonstrated by the parameters of Fst. This differentiation is most notable between the Oasis genotypeson the one hand and the provenances from the High Atlas, Middle Atlas and Anti Atlas on the other hand. These latter are genetically similar and Fst values are not significant (**Table 4**).

The three homogeneous clusters, emerged by FCA, maybe the cause of an exchange of plant material as seeds between regions of High Atlas and those of Middle and Anti Atlas which constitute a geographical continuum. This exchange seems to be limited (or non-existent) with the Oasis probably because of the remoteness and geographical isolation of the Oasis zones.

By comparing the Moroccan population with other genetic pools, since the center of origin of this species in the east to west through the different distribution centers around the Mediterranean, the average number of alleles per locus (14.88) found at the national level is high. This number is higher than that of the almond trees from Iran; countries belonging to the center of origin of the species since the values reported by Shiran *et al*. [37] and by Kadkhodaei *et al*. [60] are 6.64 and 12.86, respectively. For those authors who have characterized a small number of known cultivars (39 and 53, respectively), the average value of observed heterozygosity (0.50) and (0.54) remains low compared to the Moroccan population. This result is due to the bursting of the species diversity following to its old propagation by seeds and the self-incompatibility characterization of the almond compared to the other *Prunus* species. These high values may also be due to differences of SSR primer pairs used, the large number of genotypes analyzed in our study and that any work on pre-selected genotypes (case of works of Shiran *et al*. [37] and Kadkhodaei *et al*. [60]) may limit the genetic diversity.

The average value of the polymorphism information content PIC (0.76), which provides an estimate of the

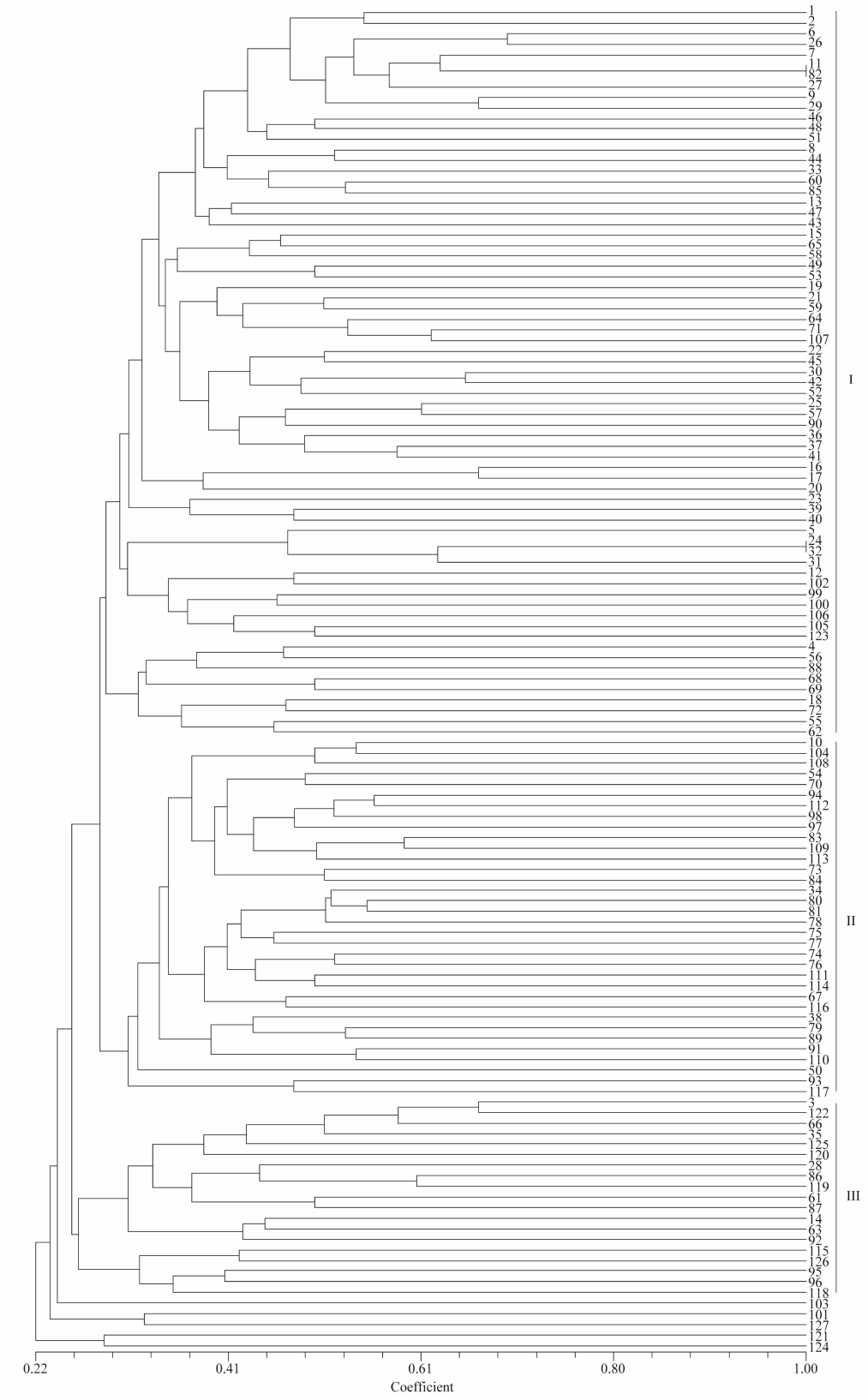

Figure 2. Genetic relationships among genotypes of Moroccan almonds. The dendrogram is based on the Dice similarity coefficient and UPGMA algorithm.

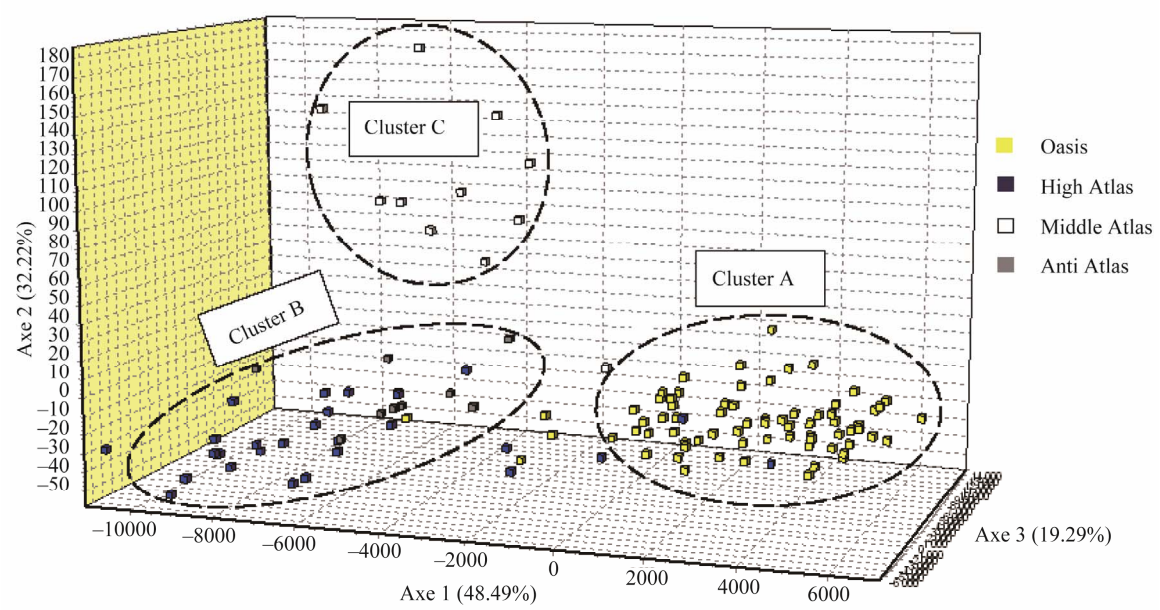

Figure 3. Factorial Correspondence Analysis (FCA) of 127 genotypes belonging to four geographic regions. The analysis allows the distinction of three homogeneous clusters A, B and C.

power of a marker in the discrimination taking into account not only the number of alleles at a locus but also the frequency on these alleles, is sufficiently high and is slightly low compared to the value (0.89) reported by Kadkhodaei *et al*. [60]. For the average value of power of discrimination PD (0.87), it is superior to that obtained by Shiran *et al*. ([37]; 0.78) in the characterization of 39 almond cultivars with 18 primer pairs of which 8 are similar to those used in our work. These high values of PIC and PD can be explained by the fact that we selected microsatellite markers specific presenting the highest values of these parameters rather than in the previous similar studies.

Compared to the Tunisiangene pools, genetic diversity parameters (number of alleles per locus 15.9, Ho = 0.68, PIC = 0.84 and PD = 0.84) reported by Gouta *et al*. [61] are more important. This diversity is also higher in Spain where Fernándezi Martí *et al*. [62] found a number of alleles per locus largest (17.21) and Ho = 0.72. The increase in the diversification parameters of the almond trees in these countries (Tunisia and Spain) is observed compared to Morocco. This slight decrease in diversity towards the West of the Mediterranean is consistent with studies previously established on the species. Using the RAPD method, Mir Ali and Nabulsi [63], found that genetic diversity of Syrian almonds exceeds that found by Bartolozzi *et al*. [31] who reported a low variability in RAPD markers among California almond cultivars. In the same direction, Martins *et al*. [32,33] found considerable polymorphism in the Portuguese almond collection. Therefore, the genetic diversity of the almond trees tends to be more restricted toward the Western Mediterranean as compared to the East, as reported by Delplancke *et al*. [5].

This work documented the existence of a wide diversity of almond trees in different regions of Morocco as revealed by SSRs, a powerful tool used to characterize genetic diversity. This diversity provides a basis for a national program of genetic improvement.

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