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Assessment of Genetic Diversity of *Argania spinosa* L. Growing in Arid and Semi-arid Areas of Morocco as Revealed by Inter-Simple Sequence Repeats

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Abstract: *Argania spinosa* L. (Sapotaceae family), endemic to Morocco, is a multipurpose tree with an important ecological and socio-economical interest. Nevertheless, the sustainability of the agro-forestry system is now threatened by overgrazing and over-exploitation leading to an alarming decline in the number of trees. In the present study, inter-simple sequence repeats (ISSR) markers were used to evaluate the genetic variation and to assess the genetic diversity distribution within and among 17 argan populations growing naturally under semi-arid and arid climate. Thus, a total of seven primers generated 260 well-defined bands, with an average of 37.14 bands per primer. Studied populations showed a relatively high level of genetic diversity at species level and low level of genetic diversity at population level. The percentage of polymorphic bands, Nei's gene diversity and Shannon's information index at population and species level were 30.15%, 0.164, 0.217 and 98.8%, 0.172, 0.293, respectively. A relative low level of genetic differentiation (Gst = 0.39) was in agreement with the results obtained from the analysis of molecular variance (AMOVA). Estimated gene flow was Nm = 0.781 among populations. Mantel test revealed a non-significant correlation between genetic and geographic distance (r = 0.065, P < 0.05), suggesting that the geographic distribution is not the major factor that shaped the present population genetic structure. The results can help preserve *A. spinosa* L. tree as an endangered species, however, refining these finding using co-dominant markers is recommended in order to establish conservation strategies.

Key words: *A. spinosa* L., ISSR, genetic diversity, genetic differentiation, gene flow.

1. Introduction

The argan tree (*Argania spinosa* (L.) Skeels, aka, *Argania sideroxylon* Roem. & Schult.), unique representative of the Sapotaceae family in Morocco, is endemic to the country [1]. It is widely distributed in the Southwest region, and three relics populations of small sizes are in the North at Oued Grou (near Rabat), Northeast at Beni Snassen and South at Goulimime [2, 3] covering over 900,000 ha [4].

This multipurpose tree is known for its agro-economic importance due to its drought resistance characteristics and powerful role against desertification [3]. In addition, the argan tree is considered as a livelihood, because its seeds are used to produce the argan oil, largely used for cooking

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purpose and thought to have various cosmetic and medicinal properties (e.g., skin revitalization, decreasing cholesterol level, stimulation of vascular circulation). Argan wood is used especially by local population as fodder to produce charcoal, fuel or in some house constructions [5]. Nevertheless, the over-exploitation and deterioration of its habitats decrease the number of trees annually and rapidly [6].

Genetic collapse, which is the characteristic of species with small populations sizes and a history of fragmented populations, is thought to have a powerful impact on the capacity of a species to survive in environmental changes [7]. Also, genetic diversity has been shown to be related to fitness, and a loss of genetic diversity reduces the capacity of populations to evolve to face environmental changes [8, 9]. Thus, the establishment of levels of genetic variation within rare and endangered plant species is considered to be crucial for their long-term survival [10] and important for the assessment of effective conservation strategies [11, 12]. Although the genetic diversity of argan tree has been studied using agro-morphological [13-16] and biochemical markers [17, 18], only few studies used molecular markers. Thus, Bani-Aameur and Benlahbil [15] used random amplified polymorphic DNA (RAPD) markers, Majourhat et al. [19] utilized simple sequence repeats (SSR) markers to characterize the most common morphotypes and El-Mousadik and Petit [17] identified the haplotypes of this endangered species using PCR-restriction fragment length polymorphism (RFLP) of chloroplast DNA. Nevertheless, the limited number of samples was a limiting factor in the analysis of genetic diversity in those studies. A larger number of samples are required, if a more comprehensive study is to be undertaken into the genetic diversity present in this species.

Out of the various molecular markers, the inter-simple sequence repeats (ISSR) remains a reliable DNA markers system [20] and a simple tool widely used in genetic diversity studies. Therefore, in this study, ISSR markers were used to investigate the genetic diversity of *A. spinosa* L. and to assess the genetic variation within and among its populations in Morocco.

2. Materials and Methods

2.1 Plant Material

Based on the homogenous area card established by the Moroccan High Commission for Water, Forest and Desertification (HCEFLCD) [4], three bioclimatic regions, mainly under semi-arid and arid climate, were selected, namely, North atlasic plains and plateaus, the argan region and the plains and plateaus of the Oriental (Fig. 1, Table 1). Over these regions, 17 populations with a total of 340 individuals were sampled (20 individuals from each population). Fresh leaves were collected from each sampled individual, labeled and stored at -80 °C for DNA further use.

2.2 DNA Extraction

Total genomic DNA was extracted from 50 mg dried leaf tissue, using the isolate plant DNA mini kit (Bioline, USA) according to the manufacturer's protocol. The DNA was then suspended in TE buffer. Two DNA preparations were performed separately for each sample.

DNA concentration and quality were determined by spectrophotometry (ND-2000, NanoDrop, USA) and evaluated by electrophoresis on 1% agarose gel. The DNA concentrations were adjusted to 50 ng/μL for all samples and then stored at -20 °C for subsequent use.

2.3 DNA Amplification by ISSR

In this study, 22 ISSR primers were used for initial screening of 10 randomly sampled argan trees. Out of these, seven primers giving consistent informative patterns from two DNA preparations were selected for further analysis (Table 2).

The polymerase chain reaction (PCR) was run according to the modified protocol of Zietkiewicz et al. [20] in a total volume of 25 μL, containing 1× PCR

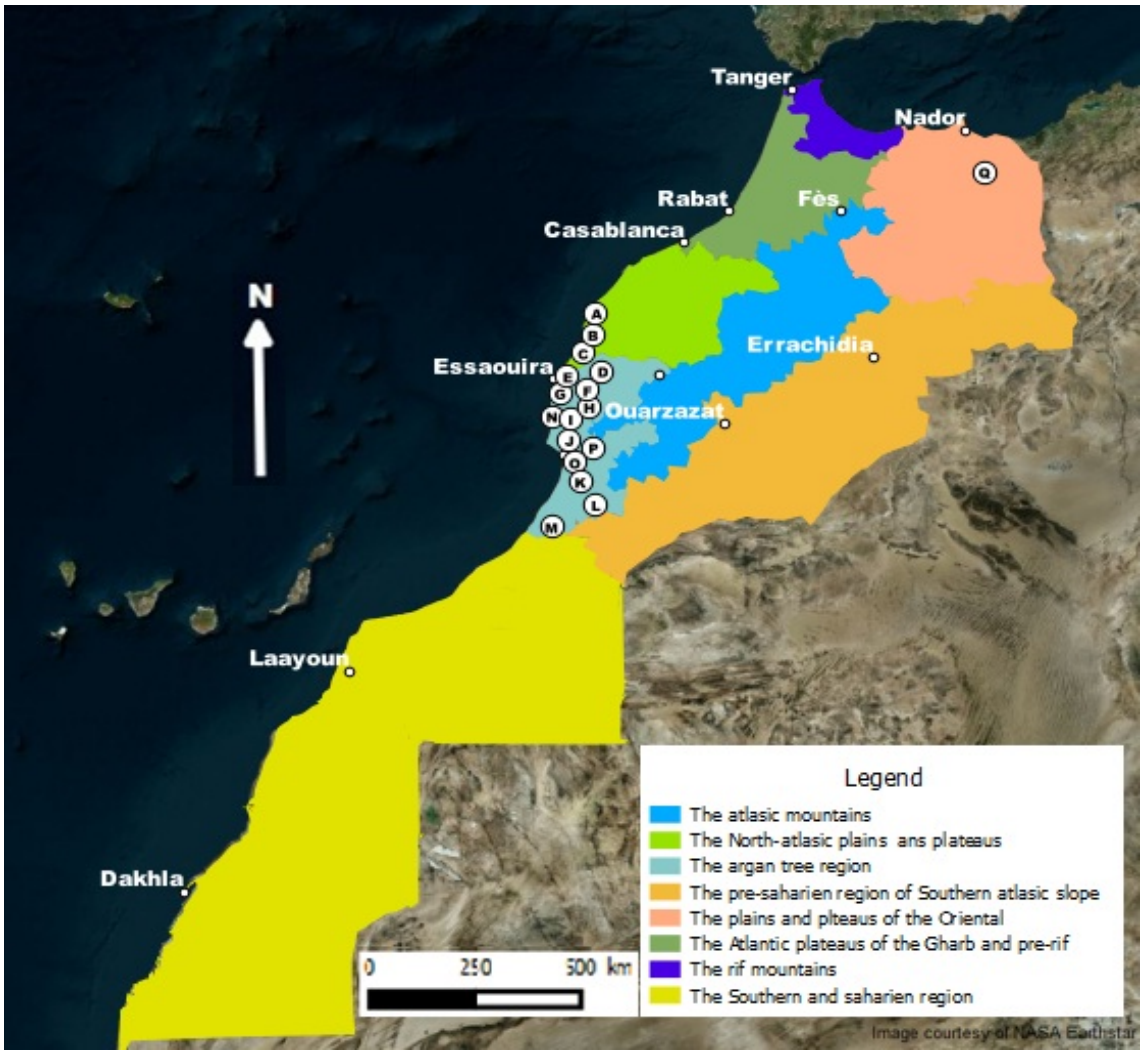

Fig. 1 Geographic repartition of bioclimatic regions with the distribution of 17 population of *A. spinosa* L. sampled.
For population codes, see Table 1 for details.

buffer, 1.5 mM of $MgCl_2$, 1 U/μL Taq DNA polymerase (Promega), 1.5 mM aliquots of dATP, dCTP, dGTP, and dTTP (Promega), 1.5 mM ISSR primer and 75 ng of total genomic DNA. Amplification was performed using GenAmp® thermal cycler (Applied Biosystem, CA, USA) after initial denaturation at 94 °C for 7 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at the specific annealing temperature (Table 2) for 45 s and extension at 72 °C for 2 min. An additional cycle comprising 7 min extension at 72 °C was included as a final step.

The ISSR-PCR products were then separated by electrophoresis in 2.8% agarose gel in 1× TAE buffer at constant voltage of 90 V for 3 h. The gel was then stained with ethidium bromide and photographed under UV light using Enduro™ GDS (Labnet, USA). Band size was estimated using 100 bp ladder molecular size standards (Bioline, USA).

2.4 Data Analysis

The amplified fragments, with the same mobility according to their molecular weight (bp), were scored in terms of a binary code as present (1) or absent (0). Only distinct, reproducible fragments were scored for each population, then matrix for the ISSR phenotypes was gathered using POPGENE version 1.32 [21]. Assuming Hardy-Weinberg equilibrium, the percentage of polymorphic bands (*PB*%), Nei's gene

Table 1 Bioclimatic regions and sampling details of argan populations investigated in the study.

Regions/superficies	Provenance	Code on the card	Abbreviation	Bioclimate	Altitude (m)	Longitude	Latitude
North atlasic plains and plateau 3,921,628 ha	Retmana	A	RT	Semi-arid	58	9°19′24.2″	32°02′05.4″
	Ouled Lhaj	B	OH		120	9°24′11.7″	31°56′13.7″
	Jbel Kourati	C	JK		360	9°24′03.0″	31°47′22.4″
The argan region (Chiadma-Haha-Souss Massa) 3,437,004 ha	Mramer	D	MR	Semi-arid	396	9°10′02.03″	31°38′23.9″
	Rbaï	E	RB		269	9°28′34.4 ″	31°32′07.8″
	Tamsrourt	F	TS		540	9°22′58.0″	31°21′34.9″
	Timzgida Ouftass	N	TO		231	9°48′09.02″	31°00′20.2″
	Neknafa	G	NK		242	9°33′56.1″	31°19′20.6″
	Ait Issi	H	AI		980	09°22′32.4″	31°02′13.5″
	Tamanar North	I	TM		576	9°37′20.3″	31°00′41.5″
	Immouzar	J	IZ		1,100	9°30′54″	30°39′00.9″
	Ait Baha	K	AB	Arid	490	9°13′32.6″	30°06′27.1″
	Tafraoute	L	TA		900	9°03′21.5″	29°42′47.2″
	Lakhessass	M	LA		954	9°43′50.3″	29°24′12.4″
	Admine forest	O	AD		83	9°21′38.0″	30°19′53.1″
	Menizla	P	MZ		256	9°05′39.2″	30°33′44.8″
The plains and plateaus of the Oriental 7,062,472 ha	Beni Snassen	BS	BS	Arid	195	2°35′38.3″	34°51′09.5″

20 individuals were sampled from each population.

Table 2 ISSR primers used for ISSR analysis in the study.

No.	ISSR primer	Sequence of primers (5'—3')	Tm	Size bands (bp)	Total bands amplified	Polymorphic bands (PB)	Polymorphism (%)
1	FL2	$(AC)_8CG$	59.9	150-1,500	36	36	100
2	FL3	$(AC)_8CT$	57.6	200-2,000	33	33	100
3	FL4	$(AG)_8CC$	51.7	100-1,500	24	24	100
4	FL6	$(CA)_8AG$	53.7	200-2,000	29	29	100
5	FL8	$(GA)_8CC$	56.0	200-2,000	29	29	100
6	FL9	$(GA)_8CG$	56.0	150-1,500	56	56	100
7	FL10	$(GA)_8CT$	57.6	200-2,000	53	53	100
Total					260	260	100
Average			56.0	150-2,000	37.14	37.14	100

Tm: melting temperature.

diversity (*H*) [22], Shannon's index (I) [23], observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's genetic distance [24], Nei's genetic differentiation index (*Gst*) and gene flow (*Nm*) were calculated to describe the genetic variation at intra- and inter-populations levels.

Cluster analysis was assessed using NTSYS-pc 2.10 software to construct a dendrogram through the unweighted pair group method with arithmetic mean (UPGMA) in order to examine genetic relationships among populations [25].

The analysis of molecular variance (AMOVA) was also measured using GenALEx 6.5 [26] to estimate the partition of genetic variance among and within populations. In addition, the Mantel test of genetic and geographic distances was carried out to evaluate a potential association between both data matrices.

3. Results and Discussion

3.1 Genetic Diversity

Due to their ability to detect low levels of genetic diversity, ISSR markers have been used to study the genetic variation of many plant species [20]. They are also potentially effective in analyzing bio-geographical patterns among populations within species [27]. Hence, seven ISSR markers were used to study the genetic diversity of endemic plant *A. spinosa* L. and to amplify a total of 340 individuals. Samples were collected from 17 argan populations distributed in three major bioclimatic regions under arid and semi-arid climate (Table 1).

Amplified DNA produced 260 bright and identifiable polymorphic fragments ranging from 150 bp to 2,000 bp. Fragments' number for each primer varied from 24 (FL4) to 56 (FL9) with an average of 37.14 per primer (Table 2).

Percentage of polymorphic bands (*PB*%) ranged respectively from 21.15% (BS) to 38.46% (RB) with an average of 30.15% at population level and 98.8% at specie level. The average effective number of alleles per locus (*Ne*), assuming Hardy-Weinberg equilibrium, was 1.175, ranging from 1.123 to 1.218, while the mean observed number of alleles (*Na*) ranged from 1.211 to 1.384 with an average 1.302. Nei's genetic diversity (*H*) varied from 0.074 to 1.114 with an average of 0.164 (Table 3).

Previous studies have demonstrated that endangered and rare species tend to have low level of genetic diversity based on ISSR data [28, 29], while some investigations have lately proved that some endemic species have high level of genetic diversity [30, 31]. The authors' results showed that the genetic diversity of argan tree is relatively higher (*PB*% = 98.8% and *H* = 0.172) contrary to what has been reported by other studies on the same species (allozymes analysis [18] and RAPD [15, 19]).

However, the level of genetic diversity that has been measured in argan was in agreement with the level found in some other endemic species with low level of genetic diversity at species level and high genetic diversity at population level, for instance, *Heptacodium miconioides* (Caprifoliaceae), a rare endemic endangered plant of China [32] and *Lactoris fernandeziana* (Lactoridaceas) endemic of the Juan Fernandez Archipelago, Chile [33]. These two species respectively showed *PB*% = 78% and *PB*% = 73% at species level and *PB*% = 27.22%, *PB*% = 27% at population level. On the other hand, Shannon's information index and Nei's gene diversity also showed low genetic variation at population level (0.164 and 0.217, respectively) and high genetic variation at species level (0.172 and 0.293, respectively) (Tables 3 and 4), suggesting that argan populations are endangered not as a result of a lack in the genetic variation but due to other deteriorating factors, such as increasing clearing, overgrazing and over-exploitation.

3.2 Genetic Differentiation and the Gene Flow

The total genetic diversity and Nei's gene diversity within populations were *Ht* = 0.172 and *Hs* = 0.105, respectively. The estimated coefficient of genetic variation (*Gst*) over all polymorphic loci was estimated to 0.3902 (Table 4). Consequently, 39.02% of the genetic variation occurred within population, suggesting that the genetic diversity of argan mainly occurs among rather than within population.

At present, natural distribution of *A. spinosa* L. in Morocco is limited on a specific area. Furthermore, the genetic differentiation level of the argan tree was high compared to perennial woody endemic species (*Gst* = 0.179) [34], especially when compared to other tropical trees, such as *Madhuca hainanensis* [35], *Vouacapoua americana* [36] and even the same family endemic tropical tree (Sapotaceae) *Vitellaria paradoxa* (*Gst* = 0.047) [37]. The high level of genetic differentiation can be explained by many factors, such as the geographic distribution, the breeding system and the genetic drift.

Table 3 Genetic diversity within argan populations.

Parameter	RT	OL	JK	MR	RB	TS	NK	AI	TM	TO	AD	MN	IZ	AB	TA	LA	BS	Mean[1]	Mean[2]
Na	1.261	1.303	1.342	1.334	1.384	1.319	1.292	1.315	1.300	1.296	1.338	1.315	1.292	1.303	1.288	1.226	1.211	1.302	0.198
H	0.093	0.110	0.114	0.107	0.132	1.114	0.104	0.105	0.097	0.112	0.121	0.112	0.099	0.102	0.102	0.085	0.074	0.164	0.172
I	0.139	0.164	1.173	0.163	0.200	0.171	0.152	0.159	0.148	0.166	0.182	0.168	0.149	0.155	0.153	0.126	0.112	0.217	0.293
Ne	1.155	1.188	1.187	1.175	1.218	1.190	1.164	1.174	1.158	1.193	1.204	1.186	1.166	1.171	1.172	1.146	1.123	1.175	1.243
NPB	68	79	89	87	100	83	76	82	78	77	88	82	76	79	75	59	55	78.41	255
PB%	26.15	30.38	34.23	33.46	38.46	31.92	29.23	31.54	30.00	29.62	33.85	31.54	29.23	30.38	28.85	22.69	21.15	30.15	98.8

Means of *Na*, *Ne*, *H* and *I* are all over loci of 17 populations; mean[1]: values at population level; mean[2]: values at specie level.
Na: observed number of alleles; *H*: Nei's (1973) gene diversity; *I*: Shannon's information index; *Ne*: Effective number of alleles; NPB: number of polymorphic bands.

Table 4 Genetic structure of argan populations.

PB%	*I*	*Ht*	*Hs*	*Gst*	*Nm*
98.8	0.158	0.172	0.105	0.3902	0.7814

PB%: percentage of polymorphic bands; *Ht*: total genetic diversity; *Hs*: genetic diversity within populations; *Gst*: coefficient of genetic differentiation.

Table 5 AMOVA for argan populations.

Source	DF	SS	MS	VC	% VC	*P* value
Among regions	2	1,070.150	535.075	3.885	12%	< 0.001
Among populations	14	3,972.121	283.723	13.376	40%	< 0.001
Within populations	323	5,232.800	16.201	16.201	48%	< 0.001
Total	339	1,0275.071		33.461	100%	< 0.001

DF: degree of freedom; SS: sum of squares; MS: mean of squares; VC: variation compounds.

The AMOVA confirmed those finding by revealing a highly significant genetic differences ($P < 0.001$) within the 17 argan populations (Table 5). Most genetic diversity was observed within populations (48%), while the revealed variability was 40% among populations and only 12% among regions. Furthermore, the level of gene flow (*Nm*) was measured to be 0.7814 individual per generation between populations, suggesting that gene exchange between populations was very low.

Nei's genetic distance and genetic identities were evaluated to further elucidate the genetic differentiation between populations (Table 6). The average genetic distances between the 17 populations is 0.96. It ranged from 0.017 (RT-OL) to 0.986 (RB-IZ) and the genetic identity was between 0.883 (BS-AB) and 0.994 (IZ-AD).

A dendrogram was generated to establish the relationship among the 17 populations, based on the UPGMA method. Four main clusters were obtained in Fig. 2. The first cluster consists of populations RT, OH, JK, MR and RB from the North atlasic plains and plateaus. The three other clusters included the remaining populations from the argan region.

The authors noticed that populations with a large geographic distance are genetically linked. Thus BS from the plains and plateaus of the Oriental in the North of the country is related to the argan region in the Southeast of the country. This was further confirmed by the Mantel test, which showed that there was no significant correlation between geographical distance and pairwise genetic distance ($r = 0.065$; $P = 0.05$). This result was never reported before, because the genetic diversity of BS population was never included in previous investigations.

Principal coordinate analysis (PCoA) was run to provide spatial representation of the relative genetic distance among populations and the obtained results were in agreement with the UPGMA clustering. The first two compounds explained 25.66% and 21.63% of

Table 6 Nei's unbiased measures of genetic distance (1978) and genetic identity between argan populations.

Population	RT	OL	JK	MR	RB	TS	NK	AI	TM	TO	AD	MZ	IZ	AB	TA	LA	BS
RT	****	0.9830	0.9685	0.9246	0.9069	0.9090	0.8870	0.8982	0.9093	0.9180	0.9273	0.9021	0.9065	0.9014	0.9212	0.9221	0.8974
OL	0.0171	****	0.9775	0.9311	0.9165	0.9128	0.8975	0.9097	0.9174	0.9270	0.9356	0.9101	0.9105	0.9129	0.9342	0.9300	0.9072
JK	0.0320	0.0228	****	0.9527	0.9379	0.9331	0.9194	0.9282	0.9328	0.9326	0.9470	0.9159	0.9250	0.9215	0.9382	0.9382	0.9189
MR	0.0784	0.0714	0.0485	****	0.9660	0.9213	0.9092	0.9243	0.9240	0.8937	0.9136	0.8937	0.9081	0.9057	0.9358	0.9319	0.8961
RB	0.0977	0.0872	0.0641	0.0346	****	0.9351	0.9202	0.9361	0.9375	0.9150	0.9264	0.9070	0.9173	0.9125	0.9406	0.9350	0.9013
TS	0.0954	0.0913	0.0692	0.0820	0.0671	****	0.9739	0.9532	0.9331	0.9199	0.9309	0.9028	0.9217	0.9074	0.9197	0.9135	0.9097
NK	0.1200	0.1081	0.0840	0.0952	0.0831	0.0640	****	0.9513	0.9233	0.9087	0.9200	0.8896	0.9142	0.8959	0.9068	0.9051	0.8943
AI	0.1073	0.0947	0.0746	0.0787	0.0660	0.0479	0.0499	****	0.9473	0.9253	0.9360	0.9044	0.9357	0.9080	0.9231	0.9201	0.9123
TM	0.0951	0.0862	0.0696	0.0790	0.0646	0.0693	0.0798	0.0542	****	0.9403	0.9404	0.9227	0.9457	0.9068	0.9298	0.9254	0.9080
TO	0.0856	0.0758	0.0698	0.1124	0.0888	0.0835	0.0958	0.0776	0.0615	****	0.9534	0.9168	0.9311	0.9033	0.9185	0.9149	0.9170
AD	0.0754	0.6660	0.0544	0.0904	0.0764	0.0717	0.0833	0.0661	0.0615	0.0478	****	0.9571	0.9949	0.9169	0.9344	0.9340	0.9313
MZ	0.1030	0.0942	0.0879	0.1123	0.0976	0.1022	0.1170	0.1005	0.0804	0.0868	0.0439	****	0.9391	0.9098	0.9230	0.9237	0.9171
IZ	0.0982	0.0938	0.0779	0.0964	0.9863	0.0816	0.0897	0.0665	0.0559	0.0714	0.0519	0.0628	****	0.9104	0.9254	0.9277	0.9193
AB	0.1038	0.0911	0.0817	0.0991	0.0915	0.0972	0.1100	0.0965	0.0978	0.1017	0.0868	0.0945	0.0938	****	0.9621	0.9512	0.8834
TA	0.0820	0.0681	0.0638	0.0664	0.0613	0.0837	0.0978	0.0800	0.0728	0.0850	0.0679	0.0801	0.7750	0.0386	****	0.9739	0.9127
LA	0.0811	0.0726	0.0638	0.0705	0.0672	0.0905	0.0998	0.0832	0.0775	0.0889	0.0683	0.0794	0.0750	0.0501	0.0265	****	0.9044
BS	0.1082	0.0974	0.0846	0.1097	0.1039	0.0946	0.1117	0.0917	0.0965	0.0867	0.0711	0.0865	0.0842	0.1240	0.0913	0.1004	****

The values below diagonal were Nei's unbiased measures of genetic distance (1978) and the values above diagonal were genetic identity.

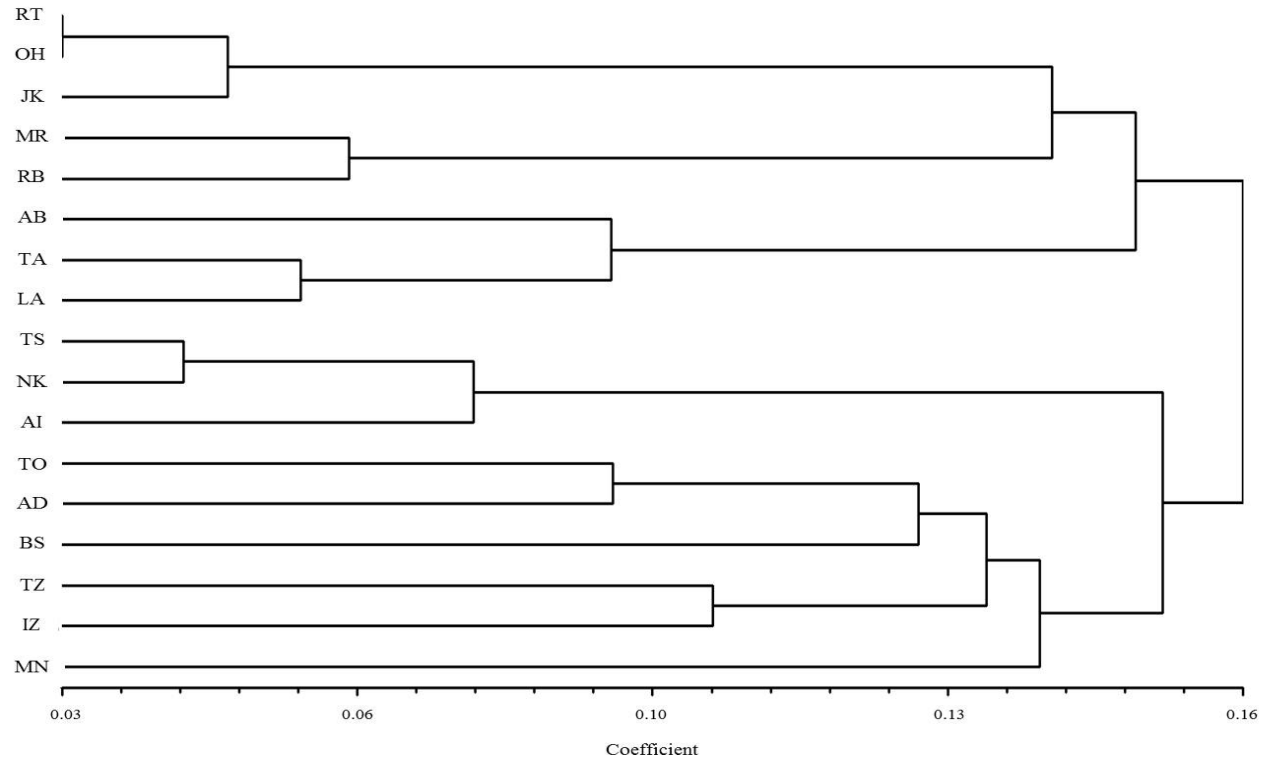

Fig. 2 Dendrogram based Nei's (1978) genetic distance of populations of *A. spinosa* L. using UPGMA method.

total variation, respectively (Fig. 3).

Pfeifer and Jetschke [38] identified geographical isolation as main factor influencing genetic differentiation by limiting the amount of gene flow via pollen and seeds. The sampled argan populations in the present study covered a large area of the argan forest distribution. Therefore, the largest inter-population geographic distance is 1,088.52 km

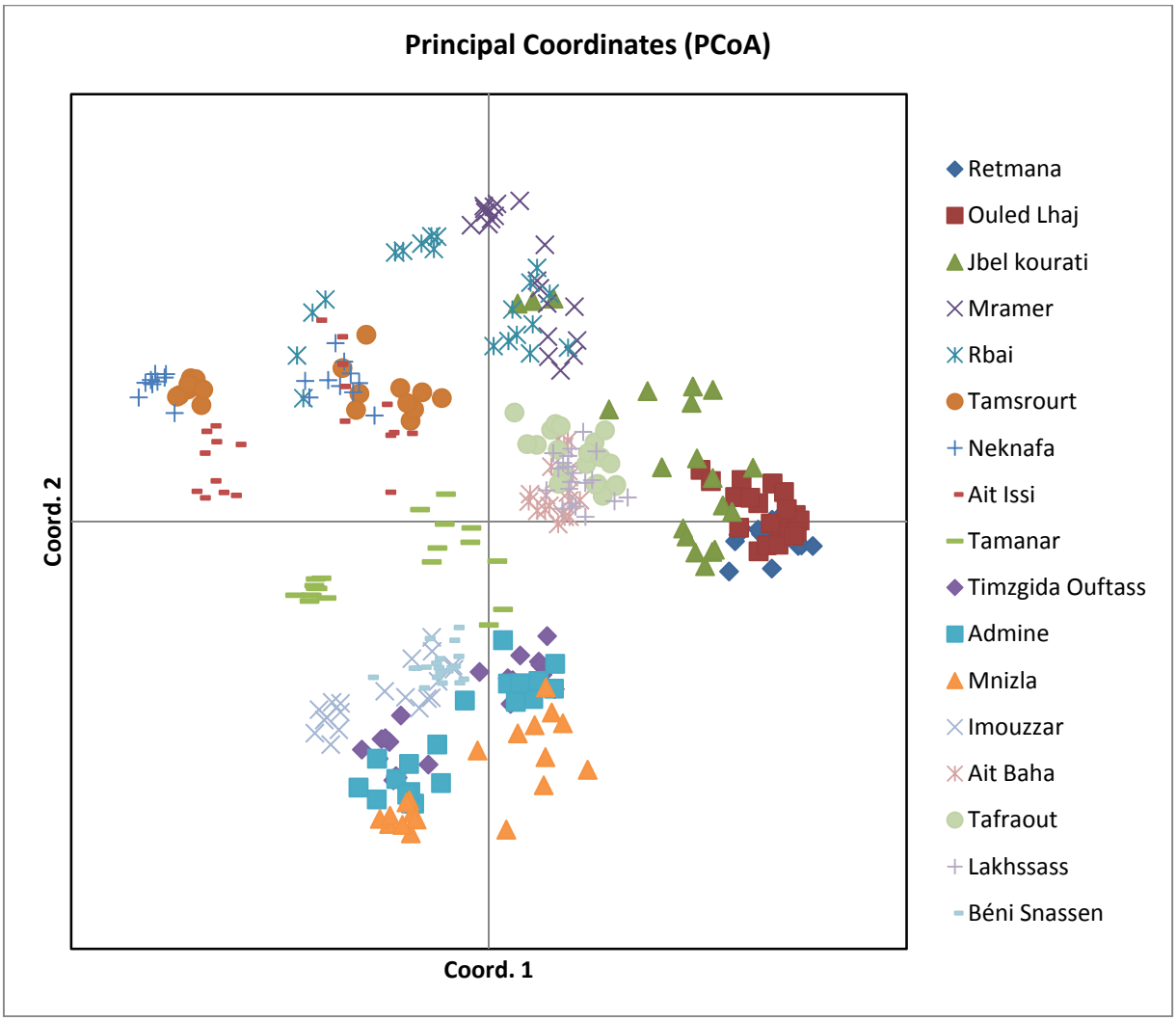

Fig. 3 Two-dimensional representation of the first two axes of the principal component analysis from the matrix of genetic distances of 340 samples from 17 populations.

Percentage of variance accumulates on the first axes was 47.29% (axis 1 = 25.66%, axis 2 = 21.63%).

(IZ vs. BS). The argan tree is outcrossing and its pollens are dispersed mostly by wind and insects [39]. Hence, the different geographic barriers and characteristics of the three studied regions could hinder the gene flow via seed and pollen dispersal among populations. Also, it is known that outcrossing species maintain a strong level of genetic variation within population and a relatively low variation among populations [40]. Moreover, if $Nm < 1$, genetic drift becomes the main cause of genetic variation in a population, on the contrary, if $Nm > 1$, then gene flow neutralizes the variance in genes caused by genetic drift [41]. In this study, *Nm* value (0.781) is lower than those reported for other forest trees with a large distribution [41], which indicates that the gene flow among argan populations with discontinuous distributions was limited and might raise the genetic differentiation among populations. However, the non-significant correlation found between genetic distance and geographic distance ($r = 0.065$; $P < 0.05$) indicates that the genetic or geographic isolation might not be the main factor conditioning genetic difference.

The preservation of genetic variability is one of the main objectives in conserving endangered species [42]. The molecular tools, such as ISSR markers, can be an

effective tool for analyzing gene polymorphism particularly in *A. spinosa* L.. It has however some disadvantages, mainly marks dominant gene and does not distinguish homozygote from heterozygote, thus limiting the obtained genetic information. More specific molecular technology is required to study the genetic diversity and gene flow and to provide essential information in the formulation of appropriate management strategies for the conservation of this species.

4. Conclusions

A. spinosa L. is an endangered tree endemic to Morocco. Genetic variability was studied using seven ISSR markers to amplify a total of 340 individuals originating from 17 populations growing under arid and semi-arid climate. They generated 260 well-defined bands, with an average of 37.14 per primer. The populations revealed a relatively high level of genetic diversity at the species level and low level of genetic diversity at population level. A relative low level of genetic differentiation ($Gst = 0.39$) was in agreement with the results obtained by the AMOVA, which was further confirmed by a limited gene flow ($Nm = 0.781$) among populations. Mantel test showed no significant correlation between genetic and geographic distance, indicating that the geographic distribution is not the major factor shaping the present population genetic structure. However, preserving *A. spinosa* L. tree as an endangered species requires refining these finding using co-dominant markers in order to establish effective conservation strategies.

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