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Assessment of genetic diversity of Moroccan *Pistacia lentiscus* L. populations using ISSR markers

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Abstract

Pistacia lentiscus L. is an indigenous species of considerable ecological and economic importance. An understanding of the patterns of genetic variation within and between Moroccan populations of this species is essential to design optimal genetic management strategies for its conservation. Here, inter simple sequence repeat (ISSR) technique was used to study genetic variation of 11 populations sampled from different regions in Morocco. The 13 primers used produced 121 bands which 110 bands (90.90 %) were polymorphic. The mean values of PIC, RP, I and Ht were 0.79; 4.89; 0.47 and 0.31 respectively, implying the important genetic variability between the studied populations. Moreover, AMOVA analysis showed that 58% of total genetic variability is accounted within population and 42% between populations. The UPGMA dendrogram and Bayesian model-based clustering approach identified five gene pools structured independently from the geographical origin of populations. These results indicate that the ISSRs markers used represent an efficient and important tool for the genetic analysis of the lentisk and the existence of a large genetic variability in this species in Morocco.

Keywords: *Pistacia lentiscus;* wild populations; ISSR markers; genetic differentiation.
Abbreviations: ISSR_Inter Simple Sequence Repeats; Hs_The diversity within population; Ht_ The total gene diversity; GST_ The coefficient of gene differentiation.

Introduction

Pistacia lentiscus L. (mastic tree) is a dioecious shrub with high drought resistance, native in entire Mediterranean Basin (Cristiano et al., 2016; Zohary, 1952). Its distribution around the Mediterranean basin extends to North and Eastern Africa and Madeira Island (Padulosi et al., 1996). It is well known in Mediterranean countries for its resin, mastic gum, used since antiquity for incense, as a chewing gum for pleasant breath, for spicing liqueurs and jam, and in the cosmetic industry (Browicz, 1987). It has been reported that almost of the parts of the plant, such as fruits, galls, resin and leaves are used in the traditional medicine as analgesic, antibacterial, antifungal, antioxidant, stimulating, diuretic and spasmolytic characteristics (Charef et al., 2008; Dedoussis et al., 2004; ElHaouari et al., 2018; Fazeli-Nasab and Fooladvand, 2014; Mahmoudi and Ebrahimzadeh, 2010). *P. lentiscus* is an evergreen shrub, 2-3 m in height, or sometimes a small tree up 3-4 m in the height. It is characterized by flowers of a more or less brown color grouped in racemes, and its fruits consist of small black drupes (AL-Saghir et al., 2012).

In Morocco, the mastic tree is an indigenous species spreads naturally in the whole country and grows in several zones such as the Atlas and Rif mountains and Atlantic and Mediterranean coast (Fennane et al., 2007). This species has the ability to resist in different climate and adapt to several conditions, because of its resilience and minor nutrient demands (Bammou et al., 2015). The description of Moroccan mastic tree genetic resources may help to identify different genotypes and rationalize conservative treatments. However, few genetic studies were carried out on this species. To the best of our knowledge, the diversity present in the Moroccan *Pistacia lentiscus* has been analyzed, only, on the basis of morphological traits (Bouta et al., 2020). However, no information is available on the genetic characterization of this species in Morocco. For this, it becomes necessary to find more discriminating markers, which could provide information about the variability within and among populations of this species and investigating new resources of variation which might be used for specific conservation programs. The present work provides the first data on the genetic diversity of Moroccan mastic tree based on molecular markers. Our objectives were to give a preliminary estimation of the genetic diversity of this species and the identification of genetically similar groups of populations collected from different regions in Morocco.

Results

ISSR polymorphism and genetic diversity

A total of 13 primers were screened for their ability to generate consistently amplified bands patterns and to assess polymorphism in the tested populations. These primers gave

a total of 121 amplification products with 110 polymorphic (Table 1). In fact, these mentioned primers generated multiple banding 5-14 amplified DNA bands with an average of 9.3 bands per primer. Hence, we may assume that a high degree of genetic diversity at the DNA level characterizes the Moroccan mastic tree.

The PIC, measured as the presence of polymorphic fragments for all primers, was high, and varied within a relatively narrow range of 0.23 (for primer UBC 828) to 0.91 (for primer UBC 868), with an average of 0.79. About the resolving power Rp, the lowest value (2.18) was recorded using UBC 807 primer and the highest (9.53) by UBC 868. The ISSR primers (UBC 861 and UBC 867) seem to be the most informative primers for distinguishing the studied populations, since they presented relatively high Rp rates. Regarding the effective multiplex ratio (EMR) and the marker index (MI), UBC 861 primer produced the highest means values of EMR (13) and MI (11.7) and lowest means values are shown by UBC 828 (EMR = 2) and (MI = 0.46).

In addition, the highest value of the total genetic diversity Ht (0.38) and the genetic diversity within population Hs (0.25) was observed in UBC 868 (Table 2). However, the lowest values for the mentioned parameters were obtained in UBC 840 (0.16) and UBC 836 (0.10), respectively. The Shannon information index (I) showed a minimum value (0.26) for UBC840 and a maximum value (0.55) for UBC841 and UBC861 with a mean of 0.47. Nei's coefficient of genetic differentiation (Gst) showed the highest value (0.55) for primer UBC841 and the lowest (0.22) was observed for primers UBC840, with an average of 0.43.

The result of AMOVA analysis revealed that the differentiation among populations accounted for 42% of the total genetic variability (F_{ST} = 0.42), while that within populations accounted for 58% (Table 3). The high level of genetic differentiation between the 11 populations is justified by the low gene flow (N_m = 0.95). According to the geographic distribution, the percentage of variation among geographic group was positive (3.76 %), with the high variation was observed among populations (56.46 %) while among populations variation within groups accounted 39.78%. In contrast, the percentage of bioclimatic distribution among groups was negative and accounted – 0.94 %, and the high variation was obtained among populations (57.6%).

Cluster analyses

The dendrogram of genetic relationship among populations are presented in Fig 1. Five mains groups are identified and labeled as I, II, III, IV and V. Group I consisted of all trees of population Lekbab (L1-L2-L3-L4-L5) and most trees of populations Modj (M1-M2-M3-M5), Immioudar (IM1-IM3-IM4-IM5), Isseksi (I1-I2-I3-I4), Tighssaline II (TGII1-TGII3-TGII4-TGII5), M'rirt (MR2-MR3-MR4-MR5), Tighssaline I (TGI1-TGI3-TGI4), Tifrdine (T1-T3-T4) and one tree of population Ayt Yahya Oussad (A1). Group II included only one tree from Tifridine population (T5). Group III composed of all trees of populations Bin El Ouidane (BO1-BO2-BO3-BO4-BO5) and Azilal (AZ1-AZ2-AZ3-AZ4-AZ5), four trees from population Ayt yahya oussad (A2-A3-A4-A5) and one tree of each population Modj (M4), Isseksi (I5), Tifridine (T2), Tighssaline II (TGI2) and Tighssaline I (TGI5). Similarly to group II, group IV included only one tree from M'rirt population (MR1). The last group V comprised two trees one from population ImiOuddare (IM2) and one from population Tighssaline II (TGII2). The 55 trees of analyzed were revealed to belong to 55 different ISSR haplotypes, reflecting high intra-population diversity.

The genetic structure of Moroccan mastic tree was examined according to the model with two to nine clusters (K = 2 and 9, respectively). The ad-hoc quantity based on the second order rate of change of the likelihood function (ΔK) (Evanno et al. 2005) revealed a first level of clustering at K = 3 and showed an accurate representation of K = 5 (ΔK =9.33) (Fig. 2). According to this model, mastic trees were assigned to five genetically different clusters. The first one (red) is composed of all individuals from population of Lekbab (L1-L2-L3-L4-L5) and most trees of populations Modj (M1-M2-M3-M5) Immioudar (IM1-IM3-IM4-IM5), Isseksi (I1-I2-I3-I4),Tighssaline II (TGII1-TGII3-TGII4-TGII5), M'rirt (MR2-MR3-MR4-MR5), Tighssaline I (TGI1- TGI3-TGI4), Tifrdine (T1-T3-T4) and one tree of population Ayt Yahya Oussad (A1).The second cluster (the purple one) included only one tree from Tifridine population (T5). The third Cluster (green) contained all trees of populations Bin El Ouidane (BO1-BO2-BO3-BO4-BO5), Azilal (AZ1-AZ2-AZ3-AZ4-AZ5), four trees from population Ayt yahya oussad (A2-A3-A4-A5) and one tree of each population Modj
(M4), Isseksi (I5), Tifridine (T2), Tighssaline II (TGI2) and Tighssaline I (TGI5). Similarly, to cluster 2, cluster 4 (yellow) included only one tree from M'rirt population (MR1). The last cluster 5 (blue) comprised two trees one from population ImiOuddare (IM2) and one from population Tighssaline II (TGII2). The 55 trees of analyzed were revealed to belong to 55 different ISSR haplotypes, reflecting high intra-population diversity.

Discussion

Assessment of genetic variation of *P. lentiscus* is crucial for conservation strategies and efficient use of these resources in breeding programs. This study described the use of ISSR markers to characterize and survey molecular polymorphism of Moroccan mastic tree populations for the first time. ISSRs markers were successfully used to evaluate the genetic diversity in many tree species (Reddy et al., 2002), including mastic tree (Kostas et al., 2021; Turhan and Özcan, 2018) (Turhan-Serttas and Ozcan, 2018; Kartas et al., 2021).

In this preliminary study a high level of genetic diversity was found in *P. lentiscus* populations as measured by ISSRs markers. The average PBP (90.9 %) indicates a high rate of polymorphism and the effectiveness of ISSR markers at detecting genetic diversity in this species. Our results are consistent with those previously reported using ISSR markers for mastic tree in Turkey and Greece (Kostas et al., 2021; Turhan and Özcan, 2018). The average PIC value obtained in this study was 0.79, considered highly informative in detecting the genetic variation among tested populations. According to Guo and Elston (1999) , the values above 0.5 are highly informative. Also, higher values of Rp, EMR and MI were obtained (9.53, 13 and 11.7, respectively). Additionally, the high multi-locus value of Ht (= 0.31) suggests the presence of a high level of polymorphism of Moroccan *P. lentiscus* populations. This high genetic diversity is in agreement with the general tendency of perennial woody species (Ht = 0.28 obtained from 195 entries) and angiosperms species (Ht = 0.28 obtained from 73 entries) (Hamrick et al., 1992).

On the other hand, the result obtained using molecular variance (AMOVA) showed that 58% of the variation was distributed within populations while 42% (Fst = 0.42) of the diversity remained between populations. This high genetic

Table 1. Characteristics of the tested ISSR primers and statistical parameters: polymorphism information content, resolving powers, effective multiplex ratio and marker index.

Primer name	Primer Sequence	Ta (°C)	Range (bp)	NB	PB	PBP (%)	PIC	Rp	EMR	MI
UBC 807	(AG) 8T	45,1	1500-400	5	5	100.00	0.74	2.18	5.00	3.70
UBC 810	(GA) 8T	44,1	1800	7	7	100.00	0.83	4.18	7.00	5.81
UBC 811	GA(AG) 7C	46,1	1500-450	8	7	87.50	0.84	2.87	6.13	5.15
UBC 827	(AC) 8G	47,9	2036-396	9	9	100.00	0.83	3.40	9.00	7.47
UBC 828	(TG) 8A	52,8	1800-506,5	8	4	50.00	0.23	4.36	2.00	0.46
UBC 834	(AG) 8YT	47,6	1800-298	10	10	100.00	0.81	3.63	10.00	8.10
UBC 836	(AG) 8YA	48,3	1400-300	9	9	100.00	0.83	4.54	9.00	7.47
UBC 840	(GA)YT	46,5	1400-300	8	8	100.00	0.80	2.80	8.00	6.40
UBC 841	(GA) 8YC	47,1	1800-310	9	9	100.00	0.86	5.85	9.00	7.74
UBC 855	(AC) 8YT	45,5	1636	12	8	66.67	0.88	6.65	5.33	4.69
UBC 861	(ACC) 6	52	1700-310	13	13	100.00	0.90	8.11	13.00	11.70
UBC 868	(GGA) 6	50	1600-350	14	12	85.71	0.91	9.53	10.29	9.36
UBC 889	BDB(AC) 7	34,3	1500-450	9	9	100.00	0.86	5.52	9.00	7.74
Average				9.3	8.46	90.90	0.79	4.89	7.90	6.60
Total				121	110	-	-	-	-	-

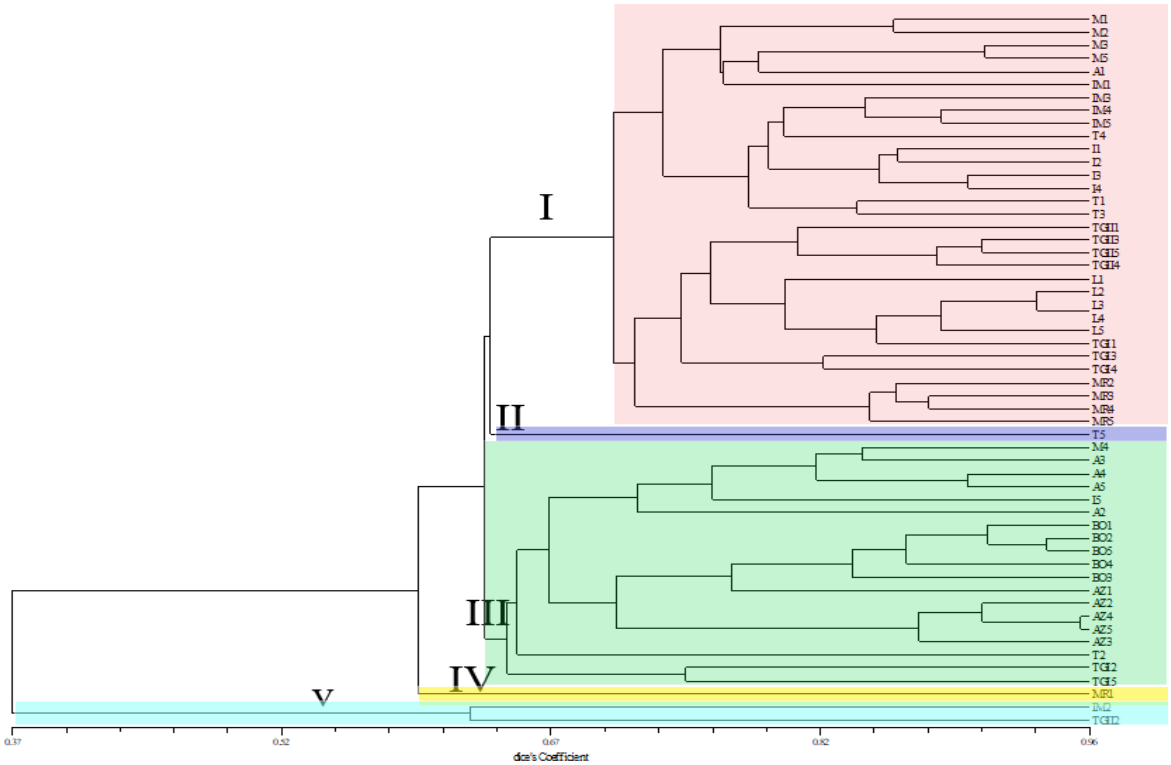

Fig 1. UPGMA Cluster analysis of 55 Moroccan *P. lentiscus* trees based on ISSR markers.

diversity within Moroccan lentisk trees populations could be explained by the out-crossing system. Cross-pollination reproduction due to the dioecy of species significantly influences the high variation within populations and explains the level of polymorphism detected. Our results are consistent with those previously reported in Israeli mastic tree populations (Werner et al., 2002).

On the other hand, our results indicate the presence of genetic differentiation among the populations

Of *P. lentiscus* (Fst = 0.42 > 0.25). This value is clearly higher than the value generally observed in

outbreeding species, indicating the high degree of separation between populations. Similar result is obtained in Israeli mastic tree populations (Werner et al., 2002). Several factors such as discontinuous distribution of populations, limited pollinator movements and low rate of seed migration could be an efficient obstacle to the gene flow and the origin of the high value of differentiation among Moroccan *P. lentiscus* populations. The UPGMA dendrogram classified the studied populations in five groups independently from their geographic and bioclimatic origins. Structure analysis reinforced the results obtained by the UPGMA dendrogram, showing the identification of five clusters and presented similar grouping of the genotypes with some minor disagreements. Also, the obtained clusters were not in accordance with the dendrogram.

Table 2. Genetic diversity analysis of 11 populations of Moroccan *P. lentiscus* based on ISRR markers.

Locus	Gst	Ht	Hs	I
UBC 807	0.44	0.33	0.19	0.50
UBC 810	0.53	0.37	0.17	0.54
UBC 811	0.50	0.28	0.13	0.43
UBC 827	0.35	0.28	0.17	0.46
UBC 828	0.42	0.30	0.18	0.47
UBC 834	0.30	0.22	0.19	0.34
UBC 836	0.50	0.28	0.10	0.42
UBC 840	0.22	0.16	0.13	0.26
UBC 841	0.55	0.37	0.22	0.55
UBC 855	0.38	0.34	0.16	0.50
UBC 861	0.47	0.34	0.15	0.55
UBC 868	0.43	0.38	0.25	0.54
UBC 889	0.52	0.36	0.20	0.53
Average	0.43	0.31	0.17	0.47

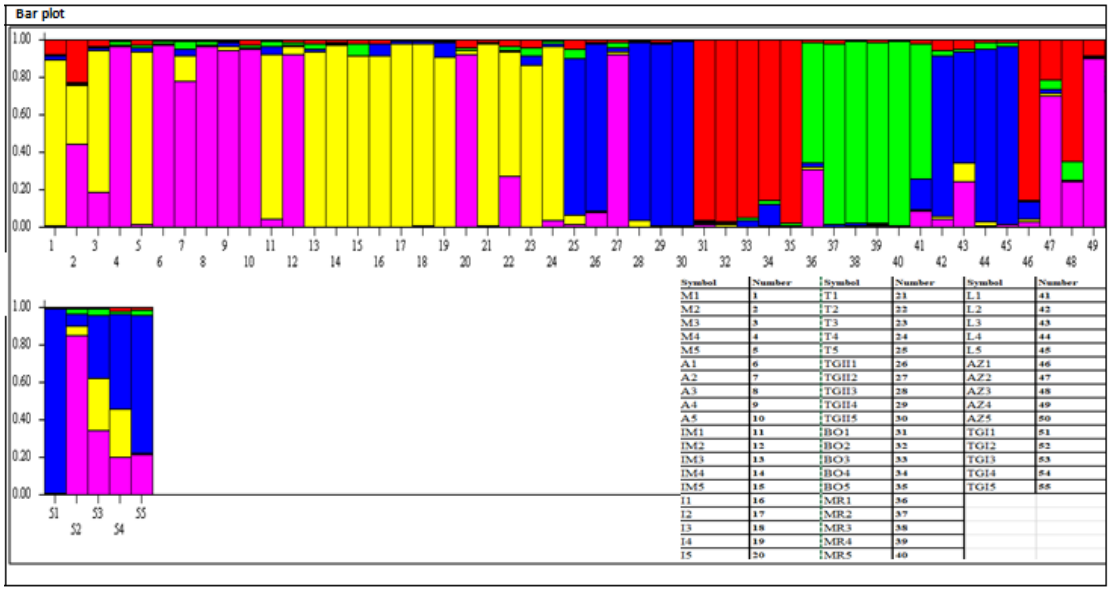

Fig 2. Genetic clustering obtained from the STRUCTURE analysis (N=55). Each individual is presented by a single vertical column, divided into K colored segments that represented the individual's estimated proportion of membership of that genetic cluster.

Table 3. AMOVA analysis of the ISSR variation of *P. lentiscus* populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	F- Statistique
		Total			
Among populations	10	156.455	2.46818 va	42.76	
Within populations	44	145.4	3.30455 vb	57.24	F_{ST}= 0.42
Hierarchical					
Among geographic group	2	36.895	0.22014 va	3.76	F_{CT}= 0.03761
Among populations within groups	8	119.56	2.32809 vb	39.78	F_{SC}= 0.41332
Among populations	44	145.4	3.30455 vc	56.46	F_{ST}= 0.43539
Among bioclimatic group	2	30.543	-0.05409 va	-0.94	F_{CT}=-0.0943
Among populations within group	8	125.911	2.48687 vb	43.35	F_{SC}=0.42941
Among populations	44	145.4	3.30455 vc	57.6	F_{ST}= 0.42403
Total	54	301.854	5.73		

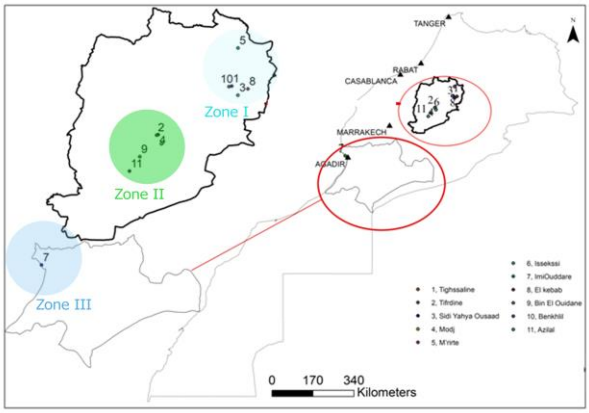

Fig 3. Map of Morocco showing locations of the ***P. lentiscus*** populations analyzed. ((•) stands).

Table 4. Geographic and meteorological conditions of eco-regions of *P. Lentiscus* populations used in this study.

Station name	Abbreviations	Zone	Altitude (m)	Latitude north	Longitude west	Rainfall mm/year	Annual average temperature
Tighssaline I	TGI	Middle Atlas	1344 m	32°76	5°63	635 mm	16.1 °C
M'rirt	MR	Middle Atlas	1111m	33°19	5°54	717 mm	14.7 °C
Lkbab	L	Middle Atlas	1401 m	32°71	5°57	616 mm	14.1 °C
Ayt yahya oussad	A	Middle Atlas	1451m	32°68	5°54	620 mm	13.3 °C
Tighssaline II	TGII	Middle Atlas	1245 m	32°76	5°65	635 mm	16.1 °C
Modj	M	High Atlas	1200 m	32°30	6°30	594 mm	14.7 °C
Isseksi	I	High Atlas	1427 m	32°22	6°27	594 mm	14.7 °C
Azilal	AZ	High Atlas	1354 m	32°01	6°56	563 mm	15.7 °C
Tifrdine	T	High Atlas	805 m	32°32	6°32	493 mm	18.3 °C
Bin el ouidane	BO	High Atlas	866 m	32°10	6°46	490 mm	17.6 °C
Imiouddare	IM	Souss	298m	30°59	9°74	290.6 mm	18.3°C

Materials and Methods

Plant material and DNA extraction

Eleven natural populations of *Pistacia lentiscus* were collected from different geographic sites in Morocco. They correspond to 5 sites in High Atlas, 5 sites in Middle Atlas and 1 coastal locality on Sous region. For each site, leaves were randomly collected from five trees. Geographical characteristics and ecological parameters of all sites studies are presented in Fig. 3 and Table 4. DNA was extracted from young leaves using CTAB procedure as described by Doyle and Doyle (J.Doyle and L.Doyle 1990). The quantity and quality of isolated total genomic DNA were estimated using the spectrophotometer method.

ISSR analysis

A total of 13 ISSR primers were used for the study of the genetic relations among mastic tree populations (Table 1). The reaction mixture was carried out in a final volume of 12.5µl, containing 40 ng of DNA template, 2.5 mM MgCl2, 0.8 mM dNTP, 1x reaction buffer, 0.8 µM primer and 0.625 U of Taq DNA polymerase. PCR amplifications were carried out in a thermocycler (Multigene gradient, Labnet, NJ. USA) through 30 cycles, each consisting of : denaturation at 94°C for 45s, annealing at determined temperature for 45s, extension at 72°C for 2 min and final extension at 72°C for 5 min. The optimum annealing temperature for each primer was determined by using the gradient PCR. The amplification products were separated by electrophoresis on 1.8 % agarose gel submerged in 0.5x TBE buffer and then stained with 1 ug/ml of ethidium bromide. The DNAs were visualized under UV light using the Gel Doc system (EnduroTM GDS, Labnet). The sizes of amplification products were estimated using DNA marker (1 Kb, Invitrogen).

Evaluation of diversity and Data analyses

Since ISSR primers are dominant markers, amplified bands were scored 1 for presence or 0 for absence. For each primer, the total number of bands and the polymorphic one were calculated. The ability of the most informative to differentiate populations was assessed using various parameters : polymorphism information content (PIC) according to Lynch and Walsh (1998), resolving power (Rp) as described by Prevost and Wilkinson (1999), effective multiplex ratio (EMR) and marker index (MI) according to Powell et al. (1996). The POPGENE 1.32 software (Francis et al., 1997) was used to determine diversity within populations (Hs), total genetic diversity (Ht), coefficient of gene differentiation (Gst) and Shannon's Information index (I).

The analysis of molecular variance (AMOVA) was performed to estimate variance components fo ISSR data, partitioning the variation within and among populations and also to research the amount of variation partitioned between geographic and bioclimatic groups FCT and within groups (FSC) (Excoffier et al., 2005). The pairwise genetic differentiations (FST) among the populations were also generated by AMOVA and the gene flow (Nm) can be approximated through Wright's island model: Nm = 0.25 (1 / FST - 1) (Slatkin and Barton, 1989). The genetic distance matrix between 55 studied mastic trees, based on Euclidean distance, was used to construct a dendrogram in MEGA version 3.1 software (Kumar et al., 2004) using the UPGMA (Unweighted Pair Group Mean with Arithmetic Average) method. Populations structure was inferred using Bayesing clustering methods implemented in STRUCTURE version 2.3.4 (Pritchard et al., 2000).

Conclusion

This preliminary study on mastic tree in Morocco showed a high level of genetic diversity of the studied populations. This high degree of variability suggests the adaptability of mastic tree populations to contrasting climatic and edaphic conditions. These results are considered as the first step of genetic diversity studies which will be helpful to determine a suitable and sustainable method of conservation in the face of genetic erosion of this species. Furthermore, and in order to provide better understanding of the genetic diversity of this species, high number of populations and other molecular markers should be further investigated.

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