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Genetic diversity and structure of natural populations of *Arbutus unedo* across altitude, bioclimate and geographical origin as revealed by ISSR markers

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Abstract

In order to assess the genetic diversity and structure of *Arbutus unedo* species, 66 individuals' trees from 11 natural populations representing its main geographical area in Morocco and belonging to different bioclimates and altitude were analyzed using 14 ISSR primers. A total of 142 bands were obtained, of which 134 were polymorphic with a polymorphism percentage of 94.06% and a polymorphism information content (PIC) of =0.47. The use of 134 markers revealed a high level of genetic variation within and between populations. The multi-locus values of Hs and Ht were 0.24 and 0.34, respectively. The overall AMOVA analysis showed that 79.30% of the total genetic variability was within populations and only 20.70% was between populations. A large genetic differentiation between populations was detected (FST=0.206) which could be attributed to restricted gene flow (Nm=0.97). The results of the hierarchical AMOVA, revealed very little genetic differentiation between altitudinal, bioclimatic and geographic groups (FCT=3.20%, FCT= 2.30% and FCT=2.10% respectively). Furthermore, geographic distances were revealed not correlated with genetic distances between populations (r=0.05, Mantel t-test=0.38, P=0.64) suggesting that isolation by distance has not played an important role in shaping the genetic structure of this species. The NJ dendrogram and Bayesian model-based clustering approach identified four groups of *A. unedo* independently of their bioclimate, geographic origin and altitude. The data obtained in this study could play a crucial role in establish efficient strategies for genetic resources conservation and to work out the scheme of breeding programs of *A. unedo*.

Keywords: *Arbutus unedo* L.; ISSR markers; genetic diversity; genetic structure; natural populations; Morocco.
Abbreviations: GST- The coefficient of gene differentiation; Hs-The diversity within group; Ht- The total gene diversity; ISSR-Inter Simple Sequence Repeats.

Introduction

Arbutus unedo L. is a fruit tree belonging to *Ericaceae* family and often called strawberry tree. It is found in western, central and southern Europe, northeastern Africa, the Canary Islands and western Asia. It can also be found distributed in countries in North America, and on the Atlantic coast such as Ireland and Macaronesia (Canary Islands) (Celikel et al., 2008; Rodriguez et al., 2011; Abbas, 2015). In the Mediterranean, it is present in Portugal, Spain, France, Italy, Albania, Croatia, Bosnia, Montenegro, Grec, Turkey and the Mediterranean islands (Pedro, 1994; Torres et al., 2002; Rodriguez et al., 2011). It's a tree growing in acidic and calcareous soils and in rocky areas, and can reach up to 12 meters (Krussmann, 1982; Rodriga de Sà, 2010). In Morocco, the *A. unedo* is growing spontaneously in semi-arid, subhumid and humid bioclimates with average annual temperatures and precipitation, respectively, of 12.4°C to 18.4°C and 337 to 1115 mm on silty-clay, clayey-limestone, marly limestone and clayey soils. It is mainly associated with *Quercus ilex* and sometimes with *Pinus halepensis* and *Tetraclinis articulata*. Its altitudinal range is between 150 m and 1613 m in different biogeographic regions that could contribute to such genetic diversity (Faida et al., 2019; Faida et al., 2021). The species is diploid (2n=26), reproduces sexually via seeds and is capable of vegetative spreading through root suckers. It is believed as a long-distance dispersal species, and seeds are dispersed by frugivores mainly birds and mammals (Debussche and Isenmann, 1989; Aparicio et al., 2008). The autogamy is the most frequent mode of pollination, but the anemogamy and entomogamy may little occur (Hagerup, 1957). In folk medicine, the species is used for its antiseptic, diuretic laxative and vascular properties (Pallauf et al., 2008). Fruits are processed into jam, wine, distillates and liqueurs (Ayaz et al., 2000; Alarcao-E-Silva et al., 2001). From an ecological perspective, *A. unedo* is also an interesting plant. It contributes to maintain the biodiversity of the fauna, helps to stabilize soils avoiding erosion, has a strong regeneration capacity following fire, and survives quite well in poor soils (Gomes and Canhoto, 2009). Today, uses of the *A. unedo* are a result

of traditional habits rather than of economics because of heterogeneity of its plants, the lack of selection and varietal identification (Jaradat, 1995). The breeding programs to obtain *A. unedo* cultivars with high fruit quality have rarely been attempted. Improvement works were mainly made in China (Songlin et al., 1995; Cai-Huang, 1997; Jihua et al., 1997), in Italy (Mulas and Deidda, 1998), in Turkey (Karadeniz et al., 1996; Gozlekci et al., 2003; Seker et al., 2004) and in Greece (Smiris et al., 2006). In Morocco, the investigations aiming at the efficient valorization, conservation and improvement of this species, to meet socio-economic and environmental imperatives remain very limited and often fragmentary. Nevertheless, the studies on the status of the distribution and ecology, as well as the morphological variability of the *A. unedo* in Morocco have been carried out (Faida et al., 2019; Faida et al., 2021). However, morphological characterization is not sufficient to evaluate and differentiate genotypes of the same species. The Molecular markers have replaced or complemented morphological characterization of species, as they are virtually unlimited, allow direct access to an individual's genome, not influenced by the environment, and take less time for characterization. The analysis of genetic variability within and among populations over the geographical range of the species, based on molecular markers, can minimize future risk of genetic erosion, establish forms of rational economic exploitation, and assist in the development of pertinent conservation and genetic improvement strategies (Reis, 1996). Studies of population genetic structure provide windows to the roles that the fundamental evolutionary forces of selection, gene flow, and drift play in processes such as local adaptation and speciation (Foster et al., 1998; Slatkin, 1994). In this context, several molecular markers have been used to assess the diversity and genetic relationships of the *A.unedo* in Tunisia and Portugal, including RAPD (Random Amplified Polymorphic DNA) (Takrouni et Boussaid, 2010 ; Rodriga de Sà et al., 2011 ; Gomes et al., 2012 ; Lopes et al., 2012), Isozymes (Takrouni et al., 2012), ISSR (Inter-Simple Sequence Repeats) (Rodriga de Sà et al., 2011), SNPs (Single Nucleotide Polymorphic) (Fazenda and Miguel, 2013), and SSR (Simple Sequence Repeats) (Gomes et al., 2012 ; Ribeiro et al., 2017 ; Fazenda et al., 2019). ISSRs markers have proven to be a valuable tool for assessing genetic diversity in many species including *A. unedo* due to their simplicity and applicability (Rodriga de Sà, 2010). Then, we used 14 ISSR primers in eleven natural populations of *A. unedo* from different bioclimates, altitudes and geographical origins. To best of our knowledge, in Morocco no genetic diversity study of arbutus had been performed before. The main objective of this work is to evaluate the genetic diversity and structure of Moroccan *A. unedo* populations. This information will be useful to define conservation strategies and improvement programs for this species.

Results

ISSR polymorphism

The 14 ISSR primers used for all populations generated 142 discernible and reproducible DNA fragments, of which 134 (94.06%) were polymorphic. Bands size ranged from 300 to 5000 bp. The total number of bands per primer ranged from 6 (UBC818) to 15 (UBC845) with an average of 10.00. The percentage of polymorphic bands per primer (P%), ranged from 83.33% (UBC818, UBC841) to 100% (UBC807, UBC810, UBC 814, UBC 835 and UBC 835) with an average of 94.06% (Table 2).

According to (Roldán-Ruiz et al., 2000), the polymorphism information content (PIC) value of each marker is represented by the probability that the marker is present or absent in two random individuals in the population, and dominant markers, such as ISSR, with values close to 0.50 are the best. The PIC value of the markers used in this study ranged from 0.40 for the UBC 814 primer to 0.50 for the UBC 807, UBC 834, UBC 835, UBC 841, and UBC 844 primers, with an average of 0.47. For resolving power (Rp), used to determine the ability of primers to differentiate populations, the values presented in this study range from 1.55(UBC 818) to 8.70(UBC 844) with an average of 5.78.

Genetic diversity

The defined parameters (I, Hs, Ht, Gst and Nm) can describe, up to a certain level, the genetic diversity of populations as well as that of the individuals that compose them. The results of the ISSR analysis presented in Table 3, showed that the Shannon information index (I) ranged from 0.36 (UBC818) to 0.58 (UBC836) with an average of 0.51, while the total genetic diversity (Ht) varied from 0, 22 (UBC818) to 0.39 (UBC836) with a mean of 0.34. for the genetic diversity within populations (Hs), it changed from 0.16 for UBC818 to 0.30 for UBC836 with a mean of 0.24.

Nei's coefficient of genetic differentiation (Gst) was 0.28, meaning that 72% of the total genetic variability exist within populations and only 28% occur between populations. The lowest value of Gst was noted with UBC814 (0.14) and the highest with the UBC 807 primer (0.36). This result was confirmed by FST value, which revealed that 20.70% of the entire genetic variation is presented among populations, and within populations variability accounted for 79.30% (Table 4).The great level of genetic differentiation of populations (FST= 0.207) was in accordance with the low value of gene flow estimated (Nm=0.97) which provides information on amount of migration between populations studied.

In addition, a hierarchical AMOVA (Table 4) was used to identify the source of genetic variation among populations based on altitudinal, bioclimate, and geographic groups. The results revealed very little genetic differentiation between altitudinal, bioclimatic, and geographic groups (FCT=0.032; FCT=-0.023 and FCT=0.021 respectively), which suggests that there is no sign of local adaptations of studied *A. unedo* populations, by using ISSR markers.

Genetic distances between *Arbutus unedo* populations were estimated using FST values to search for genetic variation between them. The pairwise FST values and geographic distances between the 11 populations are presented in Table 5. From a total of 66, we note that 60 pairwise genetic distances (FST) between populations were significant ($P < 0.05$). It varied from 0.020 (DB/KS, 58.41 km) to 0.325 (GT/BZ, 415.16 km), indicating that DB and KS populations were the closest to each other genetically, and that the populations BZ and GT were the most genetically distant. The matrix of 66 pairwise genetic distances (FST) among the 11 populations was not significantly correlated to that of corresponding geographical distances after Mantel test execution ($r = 0.055$; $t = 0.38$; $p = 0.65$). The results of intra-population variation of *A. unedo* populations showed a low level (0.27) for the IK populations from the Central Rif and OB from the High Atlas (Table 5). However, the greatest variability was recorded in the DB (0.37) population from the Western Rif and KS (0.36) from the North West.

Clustering analysis

The neighbour-joining dendrogram, based on the SM coefficient between individuals, resulted in four main groups of *A. unedo* individuals trees (Fig. 2). The first (I) (the largest group) was formed by 29 trees and is divided into two subgroups namely: I.1 and I.2. The first I.1, included trees (BZ1, BZ2, BZ3, BZ6, KS1, KS2, OS2, OS5, OS6, OL1 and ZZ5) of the subhumid zones, (DB1, DB2, DB5) of the humid bioclimatic stage and (AT2 and TZ6) originating from the semi-arid. The second I.2 was composed of trees (BZ4, BZ5, KS3, KS4, KS5, KS6) of the subhumid, (DB4, DB3 and DB6) of the humid and (TZ1, TZ2, TZ3 and TZ5) all belonging to the semi-arid zones. The second group (II) is bifurcated also into two distinct subgroups. The first II.1 is composed of the trees: OL2, OL3, OL4 and OL5 from very low altitude, GT1 and GT2 from low altitude and OB4, OB5 and OB6 from moderate altitude group. While OL6 from very low altitude, GT3 from low altitude and OB1, OB2 and OB3 from moderate altitude constitute the second group (II.2). The third group (III) includes three trees GT4, GT5 and GT5 from the High Atlas which constitute the first subgroup (III.1), while the second (III.2) is composed of the following trees: IK3, IK4, IK5, IK6, ZZ1, ZZ2, ZZ3, ZZ4 and ZZ6 from the Rif, TZ4 from the High Atlas and AT6 from the Central Plateau. The fourth group contained the remaining trees analyzed, in which two subgroups could be identified. The 66 arbutus trees analyzed have generated 65 ISSR haplotypes, reflecting a high intra-population variability. It is worth mentioning that most individuals in a given population tend to cluster together and are therefore more genetically similar than individuals from other populations.

The genetic structure of natural populations of *A. unedo* was further analyzed with a model-based Bayesian cluster approach (Structure software). The ad-hoc quantity based on the second-order rate of change of the likelihood function (ΔK) (Evanno et al., 2005) showed that the accurate representation of the genetic structure of Moroccan *A. unedo* was observed for K= 4 (ΔK = 19.44) (Fig 3.). Based on the permuted average Q-matrix generated by Clumpak for the 10 Structure runs, the highest H' was also observed for K= 4 (H'=0.99). This model was considered the best to depict the genetic structure of in Morocco. Individuals with a membership coefficient less than 0.8 were considered as admixed; 36 individuals among the 66 studied (54.54%) were assigned to one of the model's defined groups. According to the K = 4 model, the Moroccan *Arbutus* individuals were assigned to four genetically different groups (Fig 4.), which appeared in line with the NJ dendrogram. The percentages of pure individuals in groups I (red), II (green), III (blue) and IV (yellow) were 58.82%, 56.26%, 45% and 61.53% respectively. The first group (red) consists of trees: TZ1, TZ4 from the semi-arid, and : ZZ1, ZZ2, ZZ4, ZZ6, IK3, IK4, IK5 and IK6 from the sub-humid with an assignment coefficient of 0.856 to 0.979, while the other trees: AT6, ZZ3, TZ2, AT5, AT1, TZ3 and TZ5 considered as admixed and present a membership coefficient between 0.425 and 0.662. While the second group (green) is composed of: BZ4, BZ5, KS1, KS3, KS4, KS5 and KS6 originating from the North West and DB6 originating from the Central Rif having a coefficient of assignment oscillating between 0.869 and 0.965, the rest of this group contains: trees OS1, OS3 and OS4 from the Middle Atlas, IK1, IK2 and BZ1 from the Rif and DB2 from the North West, which could be considered as admixture (coefficients of 0.516 and 0.781). The third group (blue) includes nine individuals belonging to the different altitude groups such as: OL1, OL3, OL6, GT1, GT2 and GT3 from low altitudes and OB2, OB5, OB6 from middle altitudes with an assignment coefficient ranging from 0.846 to 0.961, while the rest of the trees constituting this group from low altitudes (GT4, GT5, GT6, OL2, OL4, and OL5), moderate altitude (OB1, OB3, and OB4), and very low altitude group (AT2 and KS2) with an assignment coefficient ranging from 0.540 to 0.777 are reported as admixture.

The last and fourth group contains the individuals: BZ6 from the North West, DB1 from the Western Rif, TZ6 from the High Atlas, ZZ5 from the Pre-Rif, AT3 and AT4 from the Central Plateau, OS2, OS5 and OS6 from the Middle Atlas, with a membership coefficient of 0.823 to 0.977; the remaining trees: DB3, DB4 and DB5 from the Western-Rif and BZ2, BZ3 from the North-West have a probability of assignment lower than 0.80%. Therefore, the genetic structure of the studied *Arbutus unedo* trees in four main groups was not been operated neither with their altitude and bioclimate nor with geographical origin.

Discussion

Evaluation of genetic diversity among and within *Arbutus unedo* populations could play a crucial role to minimize genetic erosion and to establish efficient strategies for genetic resources conservation in ex-situ and in-situ conditions, as well as to work out the scheme of breeding programs for weight and quality of fruit and to evaluate the adaptation level of plants to different climatic contexts which are modified through the changes of environmental factors (Santiso et al., 2015). In this study, we examined the genetic diversity and populations' structure of *A. unedo* on a large scale using a panel of 134 ISSR markers. In general, the use of ISSR markers was found to be very efficient and yielded a high polymorphism rate. Indeed, the percentage of polymorphic bands obtained in the present study (94.06%) was higher than that reported in Portuguese *A. unedo* by Rodriga de Sà (2010) using RAPD (83.9%) and ISSR (86.7%) markers and by Gomes et al. (2012) based on RAPD (57.3%) and SSR markers (80%), and in Tunisian *A.unedo* by Takrouni and Boussaid (2010) using RAPD (65%) and Takrouni et al. (2012) using isozymes (63.33%). In addition, the mean value of PIC (0.47) indicated that ISSR markers presented a high performance in the genetic exploration of this species. This value is lower than the one obtained by Gomes et al. (2012) in the germplasm of Portuguese *A. unedo* using SSR markers (0.71).

The high multi-locus value of Ht (0.34) suggests the presence of a high level of studied *A. unedo* polymorphism. This value was higher than that obtained by Rodriga de Sà (2010) (Ht=0.329) in the same species in Portuguese using RAPD and ISSR markers. Nevertheless, it was similar to that revealed by Kabiri et al.(2022, Ht= 0.33) in *juglans regia* using SSR markers. Indeed, the recorded polymorphism is confirmed by the Shannon index value (0.51). The high genetic diversity obtained in Moroccan *A.unedo* is in agreement with the general trend for all plant species (Ht=0.30), long-lived woody perennial species (Ht=0.28) and for angiosperm species (Ht=0.28) (Hamrick et al., 1992). This high diversity of Moroccan *A. unedo* genotypes could be explained by seed and pollen migration between populations (Mesléard and Lepart, 1991). The coefficient of genetic differentiation between populations was significant (GST=0.28), indicating that Moroccan *A. unedo* populations were largely differentiated.

Table 1. Environmental characteristics of eleven *Arbutus unedo* populations sampled. T (ºC): Average annual temperature in ºC; Pr (mm): Average annual precipitation in mm; VLA: Very Low Altitude; LA: Low Altitude, MA: Moderate Altitude.

Populations	Code	Distances geographies	Geographic region	Latitude N	Longitude W	Altitude (m)	T (°C)	Pr (mm)	Bioclimat and altitude groups
Brikcha-Zoumi	BZ	35.38 Km N.E of Ouezzane	North West	34°48’	5°29’	422	17.90	889	Sub-humid (VLA)
Khmiss Essahel	KS	41.17 Km N.E of Laarache	North West	35°15’	6°02’	150	17.90	700	Subhumid (VLA)
Dardara-Bab Taza	DB	26.11Km S.E of Chefchaouen	Western Rif	35°01’	5°09’	745	15.30	1135	Humid (VLA)
Ikaouen, Ketama	IK	149.36 Km SW of El Hoceima	Rif Central	34°43’	4°37’	654	12.40	831	Sub-humid (VLA)
Zrizer	ZZ	20.43 Km N of Taounate	Pre-Rif	34°37’	4°35’	645	18.20	613	Sub-humid (VLA)
Sources Oum Errabia	OS	34.28 Km N.E of Khénifra	Middle Atlas	33°00’	5°29’	1613	13.20	702	Sub-humid (MA)
Ouelmès	OL	88.13 Km S.E of El Khmissat	Central Plateau	33°28’	6°09’	983	13.60	756	Sub-humid (LA)
Aïn Tizgha	AT	26.94 km N.E of Benslimane	Central Plateau	33°39’	7°02’	275	17.40	463	Semi-arid (VLA)
Ghmate, Ourika	GT	76.61 Km S.E of Marrakech	High Atlas	31°33’	7°42’	967	17.70	337	Semi-arid (LA)
Tazart	TZ	67.53 Km E of Marrakech	High Atlas	31°29’	7°24’	867	18.00	360	Semi-arid (VLA)
Bin El Ouidane	OB	8.87 Km N of Azilal	High Atlas	32°05’	6°29’	1313	17.60	490	Semi-arid (MA)

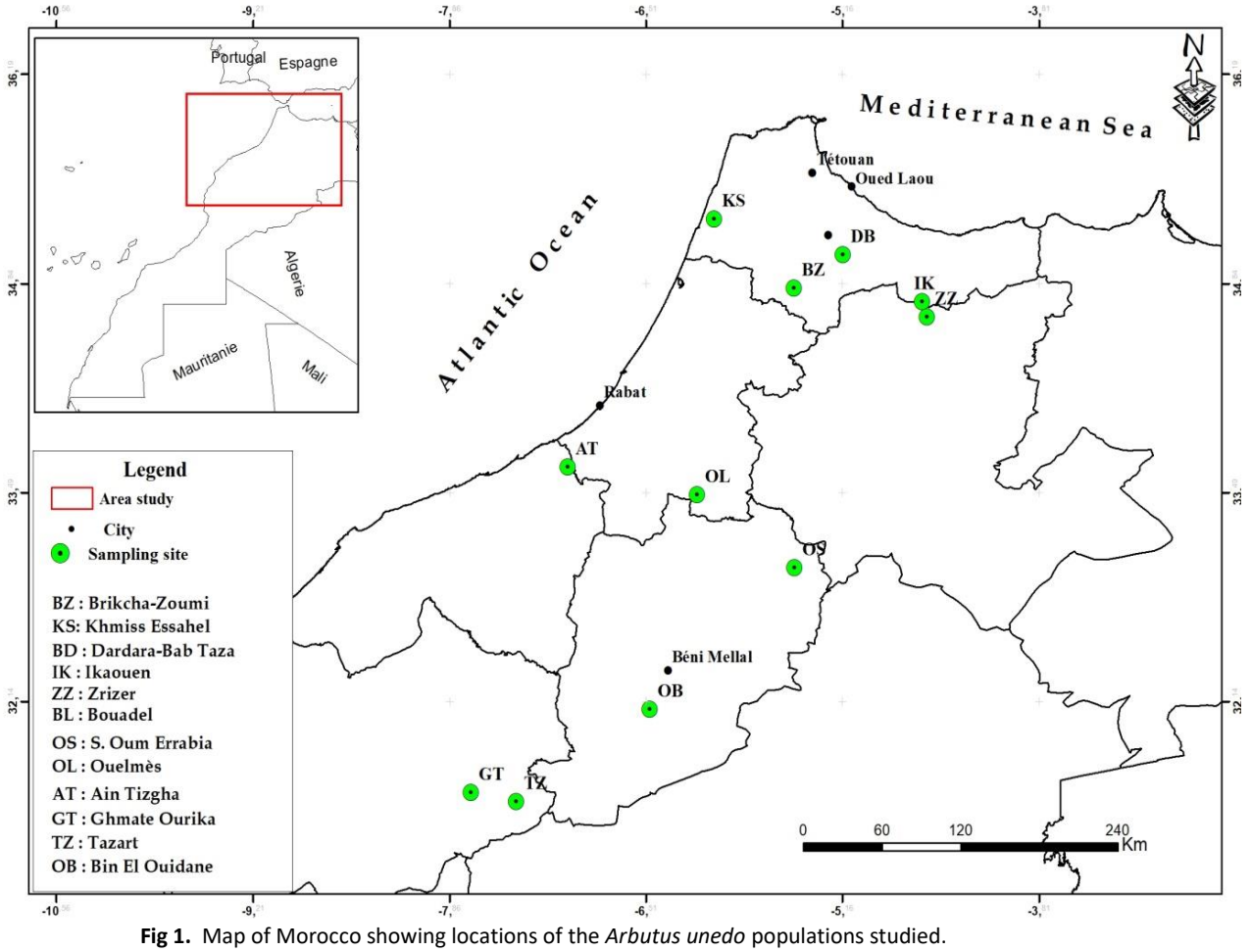

Fig 1. Map of Morocco showing locations of the *Arbutus unedo* populations studied.

Table 2. Properties of 14 ISSR primers used.

Primer	Sequence (5'-3')	AT°C	TN	PB	%P	PIC	RP
UBC 807	(AG)8T	46.1	10	10	100	0.5	5.67
UBC 810	(GA)8T	44.8	11	11	100	0.49	7.45
UBC 814	(CT)8A	44.3	8	8	100	0.4	4.21
UBC 815	(CT)8G	45.1	10	9	90	0.49	5.3
UBC 818	(CA)8G	46.5	6	5	83.33	0.42	1.55
UBC 834	(AG)8YT	49.5	9	8	88.88	0.5	6.67
UBC 835	(AG)8YC	55.9	13	13	100	0.5	6.76
UBC 836	(AG)8YA	49.1	7	6	85.71	0.47	3.61
UBC 841	(GA)8YC	47.7	12	10	83.33	0.5	6.55
UBC 844	(CT)8RC	47.3	13	12	92.3	0.5	8.7
UBC 845	(CT)8RG	49	15	14	93.33	0.47	7.57
UBC 853	(TC)8RT	49.1	7	7	100	0.46	4.36
UBC 855	(AC)8YT	46.4	9	9	100	0.48	6.03
UBC 857	(AC)8YG	49.3	12	12	100	0.44	6.76
Average	-	-	10	-	94.06	0.47	5.8

AT°C = Annealing temperature in C°; TN = total number of amplified bands; PB= Polymorphic bands; %P = percentage of polymorphism; PIC = polymorphic information content; Rp = resolving power; Y = (C,T); R = (A, G).

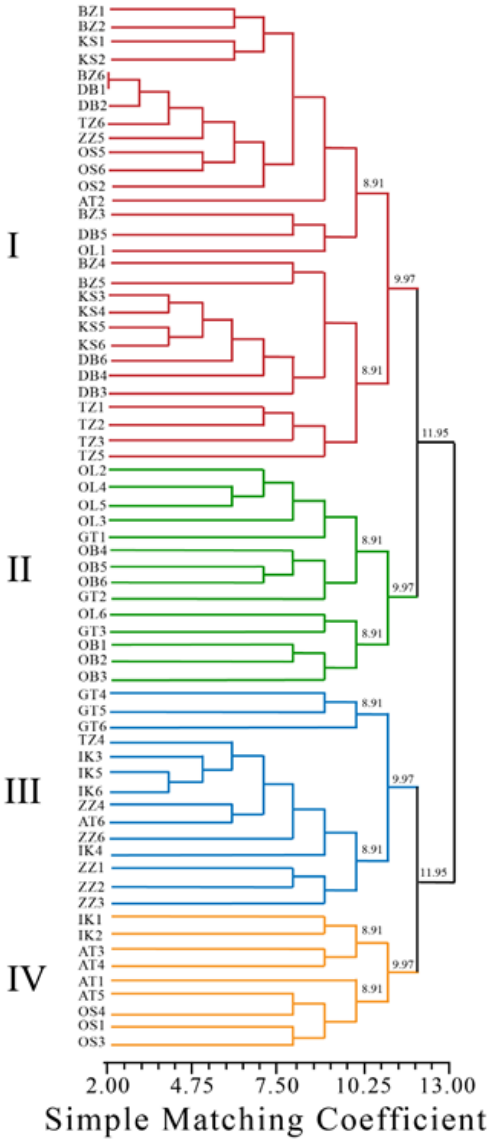

Fig 2. NJ dendrogram of 66 Moroccan *Arbutus unedo* trees based on ISSR markers.

Table 3. Genetic diversity analysis of 11 Moroccan *Arbutus unedo* populations based on ISSR markers.

Primer	Simple size	I	Ht	Hs	Gst
UBC 807	66	0.52	0.35	0.21	0.35
UBC 810	66	0.54	0.37	0.24	0.31
UBC 814	66	0.41	0.25	0.22	0.14
UBC 815	66	0.49	0.32	0.24	0.23
UBC 818	66	0.36	0.22	0.16	0.24
UBC 834	66	0.47	0.30	0.27	0.30
UBC 835	66	0.50	0.33	0.25	0.21
UBC 836	66	0.58	0.39	0.30	0.24
UBC 841	66	0.48	0.32	0.21	0.34
UBC 844	66	0.48	0.32	0.28	0.32
UBC 845	66	0.48	0.31	0.24	0.22
UBC 853	66	0.50	0.33	0.25	0.23
UBC 855	66	0.52	0.34	0.27	0.20
UBC 857	66	0.46	0.29	0.24	0.19
Average	-	0.51	0.34	0.24	0.28

I= Shannon Index, **Ht** = Total genetic diversity; **Hs** = Genetic diversity within group; Gst = Genetic differentiation coefficient.

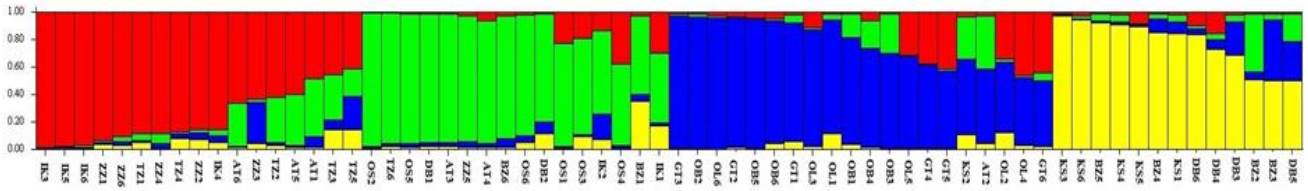

Fig 3. Genetic clustering obtained from the STRUCTURE analysis (N = 66, K=4, ΔK=19.44, H'=0.99). Each individual is represented by a single vertical column, divided into K colored segments that represent the individual's estimated proportion of membership to that genetic cluster. Thin lines separate individuals of different *Arbutus unedo* populations. Individuals are labeled below the Figure

Table 4. Analysis of molecular variance (AMOVA) of 11 populations of *Arbutus unedo* using ISSR markers.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	F-statistics
Global					
Among populations	10	571.485	5.813	20.7	
Within populations	55	1225	22.273	79.3	FST =0.207
Hiéarchical					
Among altitudinal groups	2	139.485	0.902	3.17	FCT= 0.032
Among populations within groups	8	432	5.288	18.58	FSC= 0.192
Within populations	55	1225	22.273	78.25	FST= 0.217
Among bioclimatic groups	2	95.679	-0.627	2.25	FCT=-0.023
Among populations within groups	8	475.806	6.200	22.27	FSC=0.218
Within populations	55	1225	22.273	79.99	FST=0.200
Among geographic groups	4	247.096	0.601	2.13	FCT= 0.021
Among populations within groups	6	324.389	5.299	18.81	FSC= 0.192
Within populations	55	1225.000	22.273	79.06	FST= 0.209
Total	65	1796.485	28.085		

Table 5. Matrix of pairwise FST values (under diagonal) and corresponding geographic distances in km (above diagonal) for 11 populations of *Arbutus unedo* obtained from 14 ISSR markers, and intrapopulational diversity calculated by the average gene diversity over loci derived from the ISSR Data.

	BZ	KS	DB	IK	ZZ	OS	OL	AT	GT	TZ	OB
BZ	0.000	69.41	24.49	80.57	86.01	200.64	160.13+	192.07	415.16	475.17	316.04
KS	0.099NS	0.000	58.41	141.75	149.76	251.71	197.21	200.29	438.04	498.06	353.68
DB	0.037NS	0.020NS	0.000	86.13	92.92	213.71	171.12	198.73	424.95	484.96	327.62
IK	0.253*	0.236*	0.222*	0.000	38.4	206.86	198.09	252.92	453.36	513.33	340.18
ZZ	0.196*	0.245*	0.173*	0.137*	0.000	198.12	193.17	251.13	447.19	507.18	332.6
OS	0.236*	0.213*	0.194*	0.099*	0.102*NS	0.000	82.19	162.17	262.9	262.88	138.79
OL	0.191*	0.220*	0.142*	0.315*	0.232*	0.266*	0.000	84.31	257.27	317.26	157.34
AT	0.265*	0.229*	0.230*	0.230*	0.229*	0.171*	0.138*	0.000	254.65	314.33	181.18
GT	0.325*	0.242*	0.295*	0.264*	0.317*	0.231*	0.263*	0.104NS	0.000	80.29	128.32
TZ	0.197*	0.193*	0.200*	0.191*	0.203*	0.184*	0.233*	0.152*	0.154*	0.000	188.27
OB	0.188*	0.220*	0.154*	0.255*	0.249*	0.254*	0.238*	0.243*	0.272*	0.039NS	0.000
Genetic diversity ±SD	0.29 ±0.17	0.36±0.21	0.37±0.22	0.27±0.16	0.34±0.20	0.30±0.18	0.30±0.18	0.34±0.20	0.30±0.18	0.30±0.18	0.27±0.16

* Significant at p < 0.05.NS: Not significant at p<0.05 level

This result is lower than that found by Takrouni and Boussaid (2010) (GST=0.31) in Tunisian *A. unedo*, and higher than the GST=0.26 value obtained by Rodriga de Sà (2010) in Portuguese *A. unedo*; but it is similar with the one detected by Ribeiro et al., (2017, GST=0.29) using SSRs for Portuguese *A. unedo*. The high level of differentiation between populations was in agreement with the restricted gene flow (0.97) that could be due to presence of geographical barriers. Previous work showed that if gene flow (Nm) <1, genetic drift was the main factor affecting the genetic structure of the populations, while if Nm >1, gene flow was sufficient to counteract the effect of genetic drift, and also to prevent the occurrence of genetic differentiation between populations (Levin, 1984).

The global AMOVA analysis revealed a high genetic diversity within populations (79.30%) than between them (20.70%). Our results are therefore similar to those reported by Takrouni and Boussaid (2010) using RAPDs on nine Tunisian *A. unedo* populations, and indicating that 80.67% of the genetic variation is distributed within populations, moreover our FST=0.206 value is higher than the one found by Takrouni et al. (2012) on fifteen *A. unedo* populations in Tunisia based on isozyme markers (FST=0.031). However, the FST value observed in the current study is in contrast to that expected for autogamous perennial plants where genetic variation between populations can reach 70% (Hovmalm et al., 2004). Plant species differ greatly in how genetic diversity is distributed among populations and among individuals within a population. The distribution pattern is related to the reproductive system and life history characteristics (Hamrick and Godt 1990).

Regarding the result of the hierarchical AMOVA, the differentiation between the altitude, geographical origin and bioclimatic groups of Moroccan *A. unedo* populations was weak (respectively: FCT= 0.032, FCT= 0.021 and FCT=-0.023). The same finding was found by Takrouni and Boussaid (2010) in nine populations of *A. unedo* from three bioclimatic contexts of Tunisia (subhumid, lower humid and upper semi-arid) indicating that the differentiation between these bioclimatic groups was very low (FCT = 0.001). Thus, the bioclimatic type had no effect on the population structure of *A. unedo* and that there was no local adaptation of the studied populations.Furthermore, by analyzing Moroccan walnut populations with SSR markers, Kabiri et al. (2022) found little differentiation of populations grouped according to bioclimatic origin, geographical origin and altitude.

Furthermore, the structuring of the Moroccan *A.unedo* populations into four main groups may be explained by the existence of a broad common genetic basis between the different populations, or the adaptation of these populations to ecologically different habitats is not related to the ISSR markers studied. Genetic distances between populations ranged from 0.02 to 0.32. This high polymorphism reflects high historical genetic variability and is not surprising, as its level is highly dependent on plant life history traits (Hamrick and Godt, 1990; Nybom and Bartish, 2000). Furthermore, the genetic differentiation among *A. unedo* populations were not related to their geographic distances, as shown by Mantel's test (r = 0.055; t = 0.381; p = 0.648). The absence of such association suggests therefore an important role of genetic drift in *A. unedo* and that the genetic differentiation of populations did not follow an isolation model by distance. Similarly, Mantel test performed in the Portuguese study by Ribeiro et al. (2017) for the same species, showed no correlation between genetic and geographic distances. Furthermore, no correlation between geographic and genetic distances was found by Gomes et al. (2012) on Portuguese *A.unedo* (r=0.01, p<0.57). However, another study on *A.unedo* also carried out in Portuguese (Rodriga de Sà, 2010), showed that there was a positive correlation between geographical and genetic distances (r= 0.53, p<0.001) and that the clustering of populations resulting from the UPGMA dendrogram is in agreement with their distant geographical positions.

In general, the differentiation among *A. unedo* populations could result from many factors such as the length of the species' vegetative period, recent fragmentation of a large initial population, and long-distance seed dispersal facilitated by frugivores, primarily birds (Aparicio et al., 2008; Debussche and Isenmann, 1989). Thus, the species should be less sensitive to habitat fragmentation than other autogamous species that lack this ability. Long-distance seed dispersal influences colonization of new habitats, the species' ability to migrate, and the spatial genetic structuring of populations (Aparicio et al., 2008). As with many autogamous species, the differentiation among populations of *A. unedo* in Morocco was not related to geographic distance (Hamrick and Godt, 1990). Thus, genetic structure was not significantly affected by geographic barriers. This feature is still observed for many autogamous species whose seeds are dispersed over long distances (Hamrick and Godt, 1996). Other authors suggest that genetic structure is related to ecological factors such as altitude and rainfall that influence flowering time, regrowth longevity, seed production and germination. Our personal observations suggest that flowering is asynchronous between bioclimatic regions of *A. unedo* and that the number of seeds per fruit differs between populations depending on their provenance (Faida et al., 2021). Aradhya et al. (1993) also reported that the distribution of genetic variation along altitudinal gradients is known to be the result of the interaction between gene flow and genetic drift. Nevertheless, it is possible that there is genetic differentiation between ecological groups of populations that could be not related to ISSR markers.

Material and methods

Plant material

Eleven populations of *A. unedo* representing the natural range of the species in Morocco (North-West, Rif, Central Plateau, Middle and High Atlas) were collected from different bioclimatic zones (semi-arid, sub-humid and humid) and altitudes (very low, low and moderate). The main ecological characteristics and geographical location of the populations are reported in Figure 1 and Table 1. From each population, six individuals were randomly sampled with a distance of more than 10 m between them to avoid collecting several trees from the same parent. On each specimen we have made the collect from the median part by choosing the youngest and healthiest branches. The collected young leaves were stored at -20°C pending DNA extraction.

DNA extraction

Six individuals from each population were randomly taken for DNA extraction. 0.2 g of fresh leaves were ground in liquid nitrogen to a fine powder. Genomic DNA from young leaves was extracted using the method described by Doyle

and Doyle (1990) with some modifications. The quality of the DNA was examined by electrophoresis on 0.7% agarose gels and the amount of DNA was estimated using the spectrophotometer method.

DNA Amplification

14 ISSR primers (UBC) from the Biotechnology Laboratory, the University of British Columbia, Canada, that previously showed reliable and polymorphic band profiles, were used in this study (Kabiri el al., 2019; Ait Bella et al., 2021). PCR amplifications were performed in a 12.5µl volume consisting of 6.25 µl of Green Taq Mix (Vazyme, Nanjing, China), 0.75 µl of d'H2O, 0.4 µM of primer and 30 ng of template DNA and completed with distilled water. PCR amplification was performed in a MultiGene™ thermal cycler (Labnet International, USA) under the following conditions: pre-denaturation at 94 C° for 5min, followed by 35 cycles of denaturation at 94 C° for 30 s, annealing temperature for 30 s and extension at 72 C° for 4 min. Then, a final extension cycle at 72 C° for 7 min was performed. The optimal annealing temperature for each primer was determined using temperature gradient PCR. ISSR products were separated by electrophoresis using a 1.7% agarose gel immersed in 0.5x TBE buffer and then stained with 0.1 µg/µl of ethidium bromide. DNAs were visualized under UV light using the Gel Doc system (EnduroTM GDS, Labnet). A 100 bp Plus II DNA Ladder, TRANS was used for molecular weight estimation of the PCR products.

Data analysis

For ISSR analysis, the presence of an amplified band is coded 1, while its absence is coded 0. The coding of amplified band result in a binary matrix (0/1) that will be used for the calculation of several parameters of genetic diversity, namely: the total number of amplified bands (NT), the percentage of polymorphic bands (%P), the polymorphism information content (PIC) and the resolving power (Rp). PIC and PR were calculated according to Botstein et al. (1980) and Prevost and Wilkinson (1999). The generated binary matrix was analyzed using POPGENE software version 1.32 (1999). The genetic diversity parameters included: Shannon index information (I), genetic diversity within populations (Hs), total genetic diversity (Ht) and Nei's coefficient of genetic differentiation (Gst) were used to estimate the genetic variability of the studied populations. Partition of genetic variability within and between populations was carried out by an analysis of molecular variance (AMOVA). A Second AMOVA was performed to quantify the amount of differentiation between the three altitude groups of populations: very low altitude (populations: BZ, KS, DB, IK, ZZ, AT and TZ), low altitude (populations: OL and GT) and moderate altitude (populations: OB and OIS), the three bioclimatic zones: semi-arid, sub-humid and humid, and between the five geographical regions of the studied populations (North West, Rif, Central Plateau, Middle and High Atlas) (Table 1). Pairwise genetic differentiations (FST) among the 11 populations were also generated by AMOVA. The average gene diversity across loci was calculated to assess intra-population variability (Table 5). The number of significant test permutations was set to 2000 for all analyses. These analyses were performed using the ARLEQUIN version 3.01 software package (Excoffier et al., 2005). Gene flow (number of migrants per generation = Ne m) was estimated through Wright's island model (Slatkin and Barton, 1989) as Nm = 0.25 (1/FST–1). In addition, the generated binary matrix was analyzed by NTSYSpc version 2.02 software (Rohlf, 2001) to perform group analysis using the Neighbor Joining algorithm based on Simple Matching coefficient. A Mantel test (1967) was run to look for significant correlation between matrices of genetic distances (FST) and geographic distances among populations (1000 permutations; routine MXCOMP of the NTSYS-pc; package; Rohlf, 1998). To infer the genetic structure of Moroccan *A. unedo* populations, the model-based clustering approach was used by the STRUCTURE v.2.3.4 software program (Pritchard et al., 2000). The membership of each individual was tested for the range of genetic groups (K) from 1 to 10 with the admixture model, where each simulation was repeated 10 times for each K and each run consisted of a burn-in period of 10 000 and 50 000 iterations. The ideal number of genetic groups (K) was determined by an ad hoc ΔK statistic based on the rate of change of the log likelihood of the data between successive values of K using the Structure Harvester program (Earl, 2012), which implements the Evanno method (Evanno et al., 2005). To obtain optimal alignment of independent runs, the CLUMPP software (Kopelman et al., 2015) was used to calculate the average pairwise similarity (H') of runs.

Conclusion

The evaluation of eleven Moroccan *A. unedo* populations revealed a high level of genetic diversity using SSR markers. These markers are a good tool for estimating genetic variation, which was very high among the studied populations. This result is in agreement with the restricted gene flow. Thus, the analysis of genetic diversity reveals that the genetic structuring of natural populations of *A. unedo* in four different groups was independently of their geographical origin, bioclimatic and altitude. However, populations may develop a potential for adaptation to bioclimatic conditions that cannot be detected by ISSR markers. Therefore, any in situ and/or ex situ conservation strategy should aim to include populations from each bioclimate and ecological group. Thus, should be based primarily on collecting as many individuals as possible within populations rather than between them to capture more genetic diversity. Since *A. unedo* is an autogamous species, sampling must take these criteria into account. To obtain detailed genetic information facilitating the conservation and management of rangelands containing A. *unedo*, wide ranging and fine scale analysis using molecular markers related to the adaptation to habitat conditions will be required in future studies.

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