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Evaluation of genetic diversity and structuration across altitude of walnut (*Juglans regia* L.) accessions from Morocco using SSR markers

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Abstract

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Evaluation of genetic diversity according to altitude level is becoming more and more important in the conservation and improvement programs. In this study, ten walnut accessions were sampled from different regions with different altitude level. By using SSR molecular marker, the genetic diversity and structure of walnut were analyzed. The results showed that the Moroccan walnut had high genetic diversity. The diversity within the accessions (Hs), total gene diversity (H_T) and Shannon's index (I) were 0.21, 033 and 0.49, respectively. Moreover, the total percentage of polymorphic loci (PPB) was 100% for all loci. Moreover, the variation in genetic diversity of all ten studied accessions indicated a great level of genetic diversity of accessions from low altitude (1000-1500) (H_T = 31) than both very-low (500-1000) and moderate altitude (1500-2500) (H_T = 0.25, H_T = 0.29 respectively). AMOVA analysis showed that only 21% of the total genetic variation occurred among accessions, whereas 78% of the variance was within populations, this was in line with the high level of coefficient of genetic differentiation (G_{ST} = 0.37) and the low gene flow (Nm = 0.94). The Cluster and the Structure analyses results indicated that the ten walnut accessions were mainly grouped undependably to the geographic and altitude level into three groups. Based on the available data, it is likely that the altitude factor is enough to significantly influence the walnut genetic diversity, so this factor should be taken into consideration by the walnut crop breeders and users.

Keywords: walnut; altitude; genetic variation; SSR

Introduction

Common or Persian walnut (*Juglans regia* L.), from the *Juglandaceae* family, is a monoecious tree with 2n=32 (Gleeson, 1982). Its native area extends from the Carpathian Mountains of Eastern Europe to the Southern Caucasus, northern Turkey, Iran, to the Tien Shan province of western China to the Himalayan states of India, Sikkim, and Bhutan (Zohary & Hopf, 1993). *Juglans regia* L. is an economically important tree species, cultivated in many semi-arid and temperate regions worldwide for its timber and nutritious nuts (Karimi et al., 2010; Vischi et al., 2017). The major producers of this species are China, USA, Iran and Turkey respectively (FAOSTAT, 2018). The walnut is a traditional fruit crop in North Africa and its first introduction into the Maghreb is attributed to the Romans (Germain, 1992). In Morocco, this species is found in mountainous between 800 and 1800 m of altitude (Lansari et al., 2001) with a total area of 7459 ha and 12637 t of production (FAOSTAT, 2018).

The ecological traits such as the altitude explain an important proportion of the variation among species for the genetic parameters (Hamrick et al., 1991). The altitude levels included an assemblage of environmental variables, which greatly influences the distribution of population genetic variation of plant species (Chen et al., 2008; Hahn et al., 2012). The evaluation of the genetic diversity of plant is

crucial, on the one hand to understand the adaptability and the evolutionary of a population and on the other hand for the conservation and improvement programs. Moreover, the morphological and biochemical analyses often affected by environmental conditions (Kumar, 1999). Therefore, the utilization of molecular markers is recommended to explore the genetic diversity. Various types of molecular markers are used to study the genetic diversity and relationships in walnut, including isozymes (Foroni et al., 2001), RFLP (Fjellstrom & Parfitt, 1994), RAPDs (Fatahi et al., 2010), ISSR (Kabiri et al., 2019), AFLP (Bayazit et al., 2007) and SSRs (Ross-Davis & Woeste, 2008; Pollegioni et al., 2009). This latter marker is considered as the one of the most important molecular markers (Rafalski et al., 1996). The motivation behind this study is to evaluate the genetic diversity and structuration of 10 Moroccan walnut accessions according to their altitude, using SSR markers. The results of this work will provide scientific recommendations about effective management, conservation, and improvement of walnut resources.

Materials and Methods

Plant material

According to the prospecting realized in the main cropping area of walnut in Morocco, 10 accessions grown at different altitudes were sampled (Figure 1, Table 1). The young and fresh leaves were randomly collected from five trees per each accession and stored at -20°C until the DNA extraction.

DNA Extraction

The DNA extraction from young leaves was carried out according to CTAB method (Doyle & Doyle, 1990). The DNA quantity was determined using NanoDrop 8000.

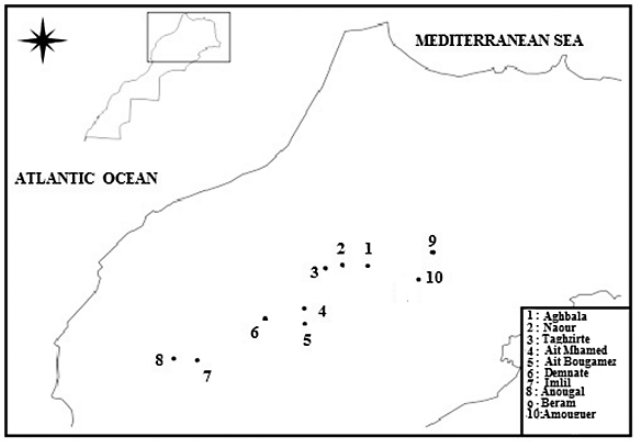

Fig. 1. Sample locations of the Moroccan walnut accessions studied.

Then, the samples were brought to a working concentration of 10 ng/µl.

PCR amplification and electrophoresis

Nine paired primer SSR that were shown to be highly polymorphic, were used to amplify genomic DNA of walnut accessions (Woeste et al., 2002) (Table 2). The PCR reactions were performed in a final volume of 15 µl containing DNA template (100 ng), Ready Mix HS (7.5x), forward (10 µM) and reverse primers (10 µM). The amplifications reactions were realized in "Verity" ABI thermocycler, according to the following procedure: an initial denaturation at 94°C for 4 min, followed by 35 cycles of 30 s at 94°C, 1min at 60°C, and 30 s at 72°C, then a final extension step at 72°C for 5 min.

The PCR products were resolved in 3% agarose gel and stained with ethidium bromide (0.5 µg/m1). DNA bands were visualized under UV light using gel documentation sys-

Table 1. Environmental characteristics of ten sampled walnut accessions.

Accession	Code	Geographic origin	Altitude (m)	Latitude N	Longitude W	Zone	Rainfall average (mm)
Aghbala	AGH	32 Km North east of Aghbala	1673	32°32′	5°39′	Middle Atlas	450
Naour	NAO	Central Naour	1300	32°29′	5°58′	Middle Atlas	600
Taghzirte	TAG	12 Km East of Tagzirte	650	32 26	6° 12′	Middle Atlas	700
Ait Mhamed	AMD	20 Km South east of Azilal	1728	31° 25′	2° 28′	High Atlas	450
Ait Bougamez	ABZ	Ait Bougamez Centre	1996	31°38′	6° 28′	High Atlas	580
Demnate	DEM	3 km South east of Demnate	932	31° 43′	6° 58′	High Atlas	350
Imlil	IML	17 km South of Asni	1763	31° 8′	7° 55′	High Atlas	459
Anougal	ANG	40 km South of Amzmiz	1304	31° 9′	8° 15′	High Atlas	681
Beram	BER	5 km South of Midelt	1540	32° 40′	4° 44′	High Atlas	210
Amouguer	AMG	40 km West of Rich	1569	32° 12′	5° 8′	High Atlas	250

tem (EnduroTM GDS, Labnet). A 50pb DNA ladder (Sigma) was used as the length reference.

Data analysis

The SSR amplified bands were scored as present (1) or absent (0) of band for all the samples. The binary matrix obtained was used for several analyses: The polymorphic information content (PIC) (Rohlf, 1998), resolving power (Rp) (Prevost & Wilkinson, 1999) and marker index (MI) (Prevost & Wilkinson, 1999), which were calculated for each primer to identify the utility and efficacy of these markers. More, the numbers of alleles (Na), effective number of alleles (Ne), The diversity within the accessions (Hs), total gene diversity (Ht), coefficient of gene differentiation (G_{ST}), Shannon's index (I) and Nei's (1978) unbiased genetic distances were measured. Moreover, the variation of genetic diversity according to the altitude level was calculated. All these analyses were carried out using POPGENE version 1.32 software (1999).

The analysis of molecular variance (AMOVA) was applied to estimate on the hand the genetic differentiation among and within accessions and the other hand to research the amount of difference between three altitude groups of accessions: Very low (Demnate and Taghzirte accessions), low (Naour and Anougal accessions) and moderate (Aghbala, Imlil, Ait Mhamed, Ait Bougamez, Amouguer and Beram accessions). These analyses were done using the package ARLEQUIN version 3.01 (Excoffier et al., 2005). From AMOVA *F* statistics, gene flow (Nm) can be approximated through Wright's island model (Slatkin et al., 1989) as Nm = 0.25 (1/FST – 1).

In addition, the data was analyzed by NTSYSpc version 2.02g software to carry out the cluster analysis using Neighbor Joining algorithm based on Dice similarity coefficient method. A Mantel test was used to research any correlation between the genetic and the difference of altitude between the accessions studied using Mx Comp of NTSys-pc software verion 2.02 g (Mantel, 1967). In order to deepen the walnut structuration, the model-based Bayesian clustering algorithm, implemented in STRUCTURE v.2.3.4, was used (Pritchard et al., 2000). STRUCTURE was run independently 10 times for each K value (range 1–10) using 70 000 iterations for burn-in, 10 000 iterations for MCMC (Markov chain Monte Carlo) and the admixture model option with the correlated allele frequencies (Falush et al., 2003). To identify the number of K clusters explaining the observed genetic structure, we used the STRUCTURE Harvester website (Earl & vonHoldt, 2012), which implements the Evanno method (Evanno et al., 2005). A graphical output was generated in CLUMPAK v.1.1 (Kopelman et al., 2015) was used to align the 10 repetitions of the best K.

Results

SSR polymorphism

The nine SSR loci used in this study for characterization of genetic diversity of ten Moroccan walnut were very poly-

Table 2. Properties of nine SSR primers used.

	Sequence (F and R)	Number of alleles	Pic	EMR	MI	RP
WGA1	ATTGGAAGGGAAGGGAAATG CGCGCACATACGTAAATCAC	2	0.23	2.00	0.47	1.52
WGA5	CAGTTTGTCCCACACCTCCT AACCCATGGTGAGAGAGTGAGC	2	0.11	2.00	0.22	1.40
WGA7	ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	5	0.79	6.00	4.74	3.48
WGA8	ACCCATCTTTCACGTGTGTG TGCCTAATTAGCAATTTCCA	2	0.34	2.00	0.68	1.28
WGA9	CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	1	0.00	1.00	0.00	1.40
WGA27	AACCCTACAACGCCTTGATG TGCTCAGGCTCCACTTCC	3	0.40	3.00	1.20	1.96
WGA32	CTCGGTAAGCCACACCAATT ACGGGCAGTGTATGCATGTA	2	0.46	2.00	0.92	1.44
WGA71	ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	2	0.18	2.00	0.37	1.56
WGA89	ACCCATCTTTCACGTGTG TGCCTAATTAGCAATTTCCA	2	0.37	2.00	0.74	1.48
Mean		2,33	0.32	2.44	1.04	1.72
Total		21	2.89	22.00	9.34	15.52

morphic across all ten walnut accessions sampled. Gerald et al. (2005) found a high level of genetic diversity in different cultivars of walnut using these markers. A total of 21 alleles were successfully amplified in 50 trees. The number of alleles varied from 1 for WGA9 to 5 for WGA7 with an average of 2.33. The percentage of polymorphism detected with all these primers was 100%. Moreover, the primers parameters were identified to select the most suitable primer for walnut genetic diversity (Table 2). The PIC value varied from 0.00 (WGA9) to 0.79 (WGA7) with a mean of 0.32. The highest value for the MI (4.74) was registered for WGA7 and the lowest (0.00) was shown by WGA9 primer with an average of 1.04. For the Rp, which present an interesting tool to determine the efficiency of primer to differentiate between accessions, varied from 1.28 (WGA8) to 3.48 (WGA7) with a mean of 1.72.

Genetic diversity and differentiation analyses

Genetic diversity index based on allelic frequencies were presented in Table 3. The number of alleles was fixed in the value of 2 for all loci, while the effective number of alleles varied from 1.41 (WGA27) to 1.94 (WGA9) with a general mean of 1.55 allele per primer. Whereas, the Shannon index ranged from 0.40 for WGA27 to 0.68 for WGAWGA9 with an average of 0.49. With an average of 0.33, the total gene diversity (H_T) showed the highest value (0.49) with WGA9 and the lowest (0.25) with WGA27. Similarly, the gene diversity within accessions (Hs) varied from 0.15 (WGA) to 0.27 (WGA1) with a mean value of 0.21. Moreover, the H_T registered by the accessions from very low altitude (500-1000 m) ($H_T = 0.25 \pm 0.03$) was low of the value showed by the accessions from low (1000-1500 m) and moderate altitude (1500-2500 m) ($H_T = 0.31 \pm 0.04$; $H_T = 0.29 \pm 0.02$ respectively). While the H_S showed a high value in the accessions from very low altitude ($H_S = 0.22 \pm 0.02$) and the low value in the accessions from low and moderate altitude (0.18 ± 0.02, 0.20 ± 0.01, respectively).

For the coefficient of genetic differentiation (G_{ST}) ranged from 0.09 (WGA1) to 0.52 (WGA9) with an average of 0.37. This interesting difference was confirmed by F_{ST} value, which revealed 21.07% of the entire genetic variation among accessions, while the difference within populations accounted for 78.93% (Table 4). The great level of differentiation among accession is in accordance with the low value of gene flow estimated (Nm = 0.94) which provides information on amount of migration between accessions studied. In addition, the hierarchical AMOVA revealed a great difference between the three groups of altitude (FSC = 0.19).

Table 3. Genetic diversity indices of ten walnut accession studied based on SSR markers.

	Na	Ne	I	Ht	Hs	Gst
WGA1	2	1.52	0.44	0.29	0.27	0.09
WGA5	2	1.54	0.53	0.35	0.24	0.33
WGA7	2	1.56	0.54	0.36	0.23	0.35
WGA8	2	1.56	0.48	0.32	0.15	0.40
WGA9	2	1.94	0.68	0.49	0.23	0.52
WGA27	2	1.41	0.40	0.25	0.15	0.35
WGA32	2	1.60	0.54	0.36	0.21	0.38
WGA89	2	1.53	0.45	0.33	0.24	0.27
Mean	2	1.55	0.49	0.33	0.21	0.37

Table 4. Analysis of molecular variance of ten accessions of *Juglans regia* L. using SSR markers

Source of variation	d.f.	Sum of square	Variance components	Percentage of variation	F-Statistic
Global					
Among accessions	9	68.500	0.87022 Va	21.07	FST : 0.21***
Within accessions	40	130.400	3.26000 Vb	78.93	
Hierarchical					
Among altitude group	2	19.133	0.17959 Va	4.28	FSC : 0.18875 ***
Among population within group	7	49.367	0.75848 Vb	18.07	FST : 0.22345***
Within population	40	130.400	3.26000 Vc	77.65	FCT : 0.04278
Total	49	198.900	4.13022		

Significant ($p < 0.05$), *** very highly significant.

Genetic relationship

The genetic distances were calculated for each pair of accessions to estimate the amount of their similarity (Table 5). The result revealed that the accessions with a low difference of altitude (4 m), which are ANG (1304 m) et NAO (1300), have a high value of genetic distance (0.37) in comparison with that revealed between ABZ (1996 m) and TAG (650) (0.21) with a large difference of altitude (1346 m). This finding confirmed by the negative correlation between the genetic distance and difference of altitude between the accessions ($r = -0.13$, $p = 0.20$).

The cluster analysis based on similarity matrix was performed to reveal the relationship among the 50 walnut trees (Figure 2). The dendrogram separates the trees to three groups. The first one composed by BER3, BER4 from an altitude of 1540 m and AMG3 originate from a region of 1569 m. Kknowing that AMG, AMD, DEM TAG and AGH belongs to different altitudes (1569 m, 1728 m, 932 m, 650 m and 1673 m respectively), the second group consisted of AMG5, AMD3, AMD4, DEM3, DEM4, TAG3, TAG2 and AGH5. While the last group was formed by the rest of walnut trees analyzed.

The model-based Bayesian clustering analysis was exploited to deepen the genetic structure of Moroccan walnut. K=3 was proposed by STRUCTURE HARVESTER as the more representative number of groups. Effectively, the STRUCTURE program under admixture and correlated allele frequencies model, the ad-hoc quantity based on

Table 5. Nei's genetic distance and corresponding difference of altitude (in m, above diagonal) for ten accessions of walnut.

	ABZ	NAO	IML	BER	DEM	TAG	AMG	ANG	AGH	AMD
ABZ	0	696	233	456	1064	**1346**	427	692	323	268
NAO	0.1187	0	463	240	368	650	269	**4**	373	428
IML	0.1697	0.1033	0	223	831	1113	194	459	90	35
BER	0.2187	0.2040	0.2055	0	608	890	29	236	133	188
DEM	0.2677	0.1484	0.1610	0.0599	0	282	637	372	741	796
TAG	**0.2112**	0.1638	0.1712	0.0238	0.0741	0	919	654	1023	1078
AMG	0.2618	0.1840	0.1767	0.0138	0.0310	0.0309	0	265	104	159
ANG	0.4928	**0.3798**	0.3499	0.6132	0.4051	0.5895	0.5214	0	369	424
AGH	0.1817	0.1385	0.1656	0.0829	0.0998	0.0606	0.0757	0.381	0	55
AMD	0.2445	0.1895	0.2204	0.0099	0.0512	0.0238	0.0101	0.6358	0.0667	0

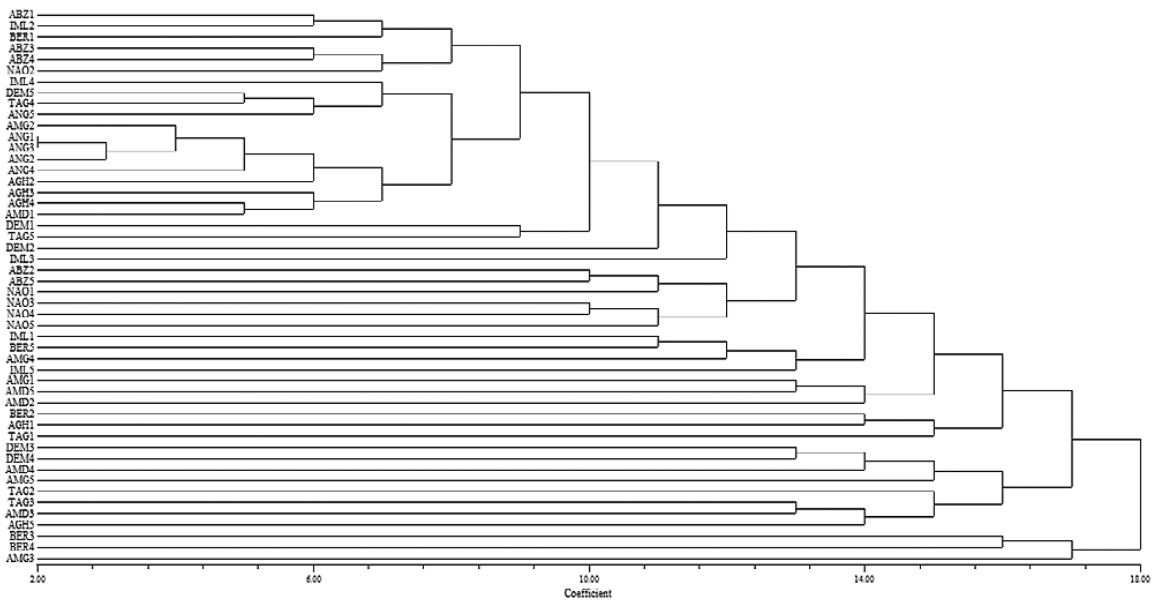

Fig. 2. Neighbor-Joining dendrogram of 50 Moroccan walnut trees based on SSRs markers

the second order rate of change of the likelihood function (ΔK) (Evanno et al., 2005) showed a clear peak at the true value of K = 3, meaning that the accurate representation of Moroccan walnut genetic structure, was observed for K =3 (ΔK = 10.139) (Figure 3). Furthermore, based on the permuted average Q-matrix generated by Clumpak, the highest H′ was observed for K = 3 (H′ = 0.980), indicating the stability of the result for this model. According to the model at K = 3, Moroccan genotypes were distributed on three different groups with a different assigned probability. This grouping was realized independently of their origin geographic and altitude level. The first group formed by the individuals from AGH, DEM, AMG, TAG, AMD and BER, belongs to the altitudes varied from 650 to 1728 m, with a coefficient of assignment from 0.004 to 0.988. While the second composed by the trees originate from BER, AMG, TAG, AMD, ABZ, IML and NAO, with an altitude from 650 to 1996 m, with a coefficient of assignment oscillated between 0.007 to 0.969. Finally, the third group included the individuals belonging to altitudes between 932 to 1996 m, such as ANG, AGH, AMG, BER, DEM, IML, AMD and ABZ with a coefficient of assignment varied from 0.004 to 0.988.

Discussion

In this study the genetic diversity of 50 walnut trees was assessed by SSR markers. According to the results obtained, the SSR present a good tool to identify the genetic diversity of Moroccan walnut. This finding is in agreement with several studies of genetic diversity of walnut with the same markers (Ruiz-Garcia et al., 2011; Ebrahimi et al., 2016; Vischi et al., 2017). All primers used in this work showed a percentage of polymorphism of 100%, which is similar to that revealed by Ahmed et al. (2012) in the genotypes from Jammu and Kashmir in India, but higher of that found by Kabiri et al. (2019) with the ISSR markers using the same accessions (91%). While, they were higher than others percentages obtained by Noor Shah et al. (2016) (89.6%) and Salieh et al. (2013) with SSR marker (88.16%). The average PIC was 0.32 and this value is in accordance with the value showed by Ahmed et al. (2012) and lower than that registered with the same accessions by Kabiri et al. (2019) with ISSR (0.88), Ebrahimi et al. (2016) (0.69) and Mahmoodi et al. (2013) (0.64) using SSR marker. The higher value of PIC, MI and Rp were registered by WGA7 meaning that this primer is the most informative.

In the present study, a wide large of genetic diversity was detected within and among walnut accessions based on 21 loci. This finding was established on the several parameters such as the interesting values of Shannon's index, which is an important criterion to understand the genetic diversity in a population (Ali et al., 2019). Mean Shannon's information index (0.49) was found comparable to the value reported by the study carried out with the same molecular marker in Chinese walnut (0.51) (Yuan et al., 2018) but greater of the result found with ISSR using the same accessions (0.38) (Kabiri et al., 2019). Moreover, the H_T registered a mean value of (0.33), which was higher of the result of ISSR with the same accessions (H_T = 0.25). This later was very important in the accessions from low altitude (H_T = 31), which belong to optimal area of the distribution of this species (800–1800 m). Unfavorable environments conditions and human activities at both very low and moderate altitudinal zones may lead to loss of genetic variation (Chen et al., 2008; Hahn et al., 2012). These results are in conformity with general trend for all plant species (H_T = 0.30 from 584 entries), long-lived woody perennial species (H_T = 0.28 from 195 entries) and for angiosperms species (H_T = 0.28 from 73 entries) (Hamrik et al., 1992). In addition, the walnut is a monoecious plant with separate male and female inflorescences, and a wind-pollinated, which may carry out both geitonogamy and cross pollination, but due to dichogamy, the same tree while self-fertile may not be able to pollinate itself (Aiqing et al., 2014). Therefore, these factors could be the major causes for the high level of diversity on the ten accessions.

According to G_{ST} value (0.37), the studied accessions were revealed much differentiated in congruence with the global AMOVA analysis value (F_{ST} = 0.21). On the one hand, the amount of differentiation observed in this study was higher than the result found by Karimi et al. (2010) (0.12) and Vahdati et al. (2014) (0.08) in Iranian walnut, and by Vishi et al. (2017) (0.02) in Italian walnut. In the other hand, it

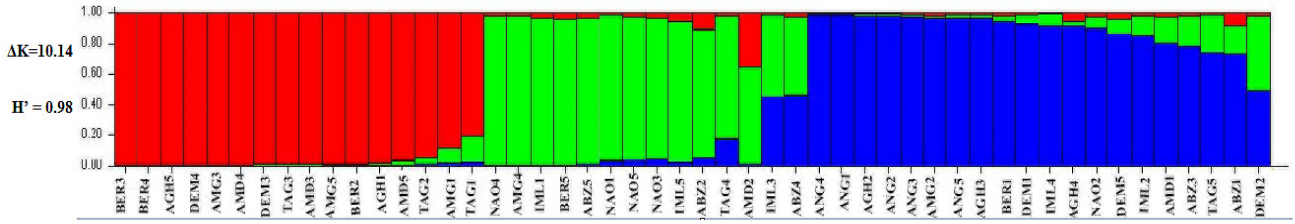

Figure 3. Structure of 10 walnut accessions based on nine SSR markers (K = 3)

is lower of the result showed by Yuan et al. (2018) (0.479) in Chinese walnut and by Ahmed et al. (2012) (0.30) in Indian walnut. The high level of differentiation among accessions, in agreement with the restricted gene flow (0.94), could be due the presence of geographic barriers such mountains.

The Studies have shown that if gene flow is (Nm) <1, genetic drift was the main factors to affect the population genetic structure, while if Nm >1, gene flow was sufficient to counteract the effect of genetic drift, and also to prevent the happening of the population genetic differentiation among populations (Hamrick et al., 1995). Furthermore, the differentiation among the Moroccan walnut accessions showed a high amount of genetic diversity within accessions (79%), which can be caused by the usual method of multiplication by seeding used by farmers. Concerning the result of hierarchical analysis of AMOVA, the differentiation among altitude groups of accessions was large (18.87%), indicating the adaptation of Moroccan walnuts to the local conditions of environment. This result means that the altitude factor has an influence on accessions genetic structuration.

Generally, the results revealed that the regions which have the same altitude are different genetically, while the accessions with the large difference of altitude showed a low value of genetic distance. This result was confirmed by Mantel test, which revealed a negative correlation between the genetic distance and difference of altitude of accessions. The probable reason of the negative correlation may be explained by the wind pollination type.

Moreover, the dendrogram and biased model showed that the ten accessions were congregated into three groups undependably to the geographic and altitude amount. Hahn et al. (2012) suggest that altitude does not affect genetic diversity in the grassland species under study. While, Di et al. (2014) showed that the genetic diversity of eight studied *C. heterophylla* populations varied significantly with changing elevation, with the trend indicating that mid-elevation populations were more genetically diverse than both low-elevation and high-elevation populations.

Conclusion

The evaluation of ten Moroccan walnut accessions revealed a high level of genetic diversity using SSR markers. These markers present a good tool to estimate the genetic variation which was very large among the accessions studied. This result was in accordance with the restricted gene flow. The genetic diversity of low-altitude accessions was more genetically diverse than both very-low-altitude and moderate-altitude accessions. The ten accessions studied were structured in three groups undependably of their origin and altitude level.

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