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Genetic diversity and structure of walnut (*Juglans regia* L.) genotypes from Middle and High Atlas mountains of Morocco as investigated by Inter-Simple Sequence Repeat (ISSR) markers

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Abstract

The genetic diversity and genotypes structure of walnut (*Juglans regia* L.) are essential to understand and manage genetic resources of this species, as well as for further progress in breeding programs. ISSR markers were used to assess the genetic diversity of 66 individuals' trees from 11 accessions, representing the main cropping area of walnut belonging to two ranges types of Mountain in Morocco: middle and high Atlas. Eleven ISSR primers rendered a total of 135 bands (91%) with 0.88 polymorphic information content. The utilization of 123 polymorphic bands revealed a high level of genetic variation within and among the examined accessions. The multi-locus values of Ht and Hs were 0.25 and 0.20, respectively. The AMOVA analysis showed that 71.30% of total genetic variability is accounted within accessions and 28.70% between accessions. This was congruent with the coefficient of genetic differentiation ($G_{ST=}$0.16). Bayesian model-based clustering approach identified three gene pools that were not correlated with mountain range type. This is the first application of ISSR markers for the assessment of genetic diversity in Moroccan germplasm of walnut. This information will be useful to define conservation strategies and improvement programs of this species.

Keywords: Walnut, ISSR, efficiency, genetic differentiation, geographic group; bioclimatic group.
Abbreviations: ISSR_Inter Simple Sequence Repeats; Hs_The diversity within accession; Ht_The total gene diversity; G_{ST}_The coefficient of gene differentiation.

Introduction

Common or Persian walnut (*Juglans regia* L.) is an economically important tree species, cultivated throughout the temperate regions of the world for its timber and nutritious nuts (Amiri et al., 2010; Karimi et al., 2014; Vischi et al., 2017; Shamasbi et al., 2018). Its natural origin is extended from the Carpathian Mountains of Eastern Europe to the Southern Caucasus, northern Turkey, Iran, Tian Shan province of western China, Himalayan states of India, Sikkim, and Bhutan (Zohary and Hopf, 1993; Angmo et al., 2013). The global production of walnut was approximately 3.83 million tones with an area of 1.18 million hectares. China had become the leading world producer with 50.27% of total global production, followed by United States of America with 14.92%, Iran with 9.12% and Turkey with 5.48% (FAOSTAT, 2018). Walnuts are included in FAO list of priority plants because of its nutritive value (Gandev, 2007). They have been a staple food proving energy, protein, essential acids, vitamins and minerals. A wide diversity is available in walnut germplasm throughout the world, which has not been studied yet (Noor Shah et al., 2018). In Morocco, walnut is a traditional fruit crop and its first introduction is attributed to the Romans (Germain, 1992). Walnut tree produces only 12 937 tones with an area of 7459 hectares (FAOSTAT, 2018), widespread in mountainous areas with an altitude between 800 and 1800 m and under different environments (Lansari et al., 2001). The tree can be found in humid and warm conditions in the north of Rif Mountain, in the Atlas chain (High and Middle Atlas Mountains) and in arid regions of Southeastern Morocco. More than half of the plantings resulted from the prevailing way of seed propagation practiced by farmers, since grafting is less adopted (Kodad et al., 2014; Lansari et al., 2001). Recent researches have been conducted on the high nutritional value of walnut (Yerlikaya et al., 2012; Akbari et al., 2014), its beneficial effects on human health and high antioxidant capacity (Carvalho et al., 2010) and high w-3 and w-6 fatty acid concentration (Tapia et al., 2013), mainly due to increase in world demand for walnuts (FAOSTAT, 2018). To comply with this rising demand, the study of genetic diversity of walnuts has gained increasing interest, to provide useful information for the management of genetic resources and rational use of populations in breeding programs, focused on the development of new, high-yielding cultivars which are more adapted to drought conditions (Mahmoodi et al., 2013; Aiqing et al., 2014; Karimi et al., 2014; Vischi et al., 2017; Noor Shah et al., 2018; Shamasbi et al., 2018). Walnut is highly divergent due to its open-pollination and seed propagated method. Therefore, utilization of molecular

markers that are not affected by environment factors is recommended to enhance the exploration of diversity among genetic resources.
Several types of molecular markers are employed for assessment of genetic diversity and relationships in walnut, including Isozymes (Foroni et al. 2001; Vyas et al. 2003), RFLPs (Restriction Fragment Length Polymorphisms) (Fjellstrom and Parfitt, 1995), RAPDs (Randomly Amplified Polymorphic DNAs) (Nicese et al. 1998; Fatahi et al. 2010; Li et al. 2007), AFLP (Amplified Fragment Length Polymorphisms) (Kafkas et al., 2005; Bayazit et al. 2007), SNPs (Single Nucleotide Polymorphic) (Ciarmiello et al., 2011), SSRs (Simple Sequence Repeats) (Ross-Davis and Woeste, 2008; Pollegioni et al. 2009; Karimi et al. 2010) and ISSRs (Inter Simple Sequence Repeats) (Potter et al., 2002; Pollegioni et al 2003; Malvolti et al. 2010; Miltiadis et al. 2010). However, RAPDs have low reproducibility, RFLPs are time-consuming and labour-intensive, SSRs require the knowledge of the flanking regions for the development of species-specific primers and, AFLPs and SNPs have high cost (Reddy et al., 2002). Whilst, the ISSR markers are hypervariable, highly reproducible, fast, inexpensive and do not require any prior sequence information of amplified locus (Zietkiewics et al., 1994; Reddy et al., 2002). ISSR is a kind of DNA sequences confined by two inverted SSR composed of the same motives, which are amplified by a unique PCR primer. ISSR-PCR detects the levels of variation in microsatellite regions and gives multi-locus schemes, which are very iterative, plentiful and polymorphic in plant genomes (Agostini et al., 2008; Yu et al., 2011). Moroccan walnuts have been poorly characterized for their diversity. There is only one study on phenotypic variability of few Southeastern populations conducted by Kodad et al. (2014). Nevertheless, the morphological characteristics often do not result in a clear diagnosis due to effects of different environmental conditions. Therefore, conservation of genetic resource strategies has become imperative to preserve local walnut germplasm. Thus, identification and characterization of the collected genotypes constitutes an attractive task to examine level and distribution of genetic diversity in this crop. In this work, ISSR markers have been used to provide useful information to establish efficient strategies for conservation of genetic resources and improvement programs of *juglans regia.* These markers have been utilized for tree improvement programs and conservation of crop genetic resources (Martins et al., 2003; Kaumar et al., 2009; Ajal et al., 2014, Ben Tamarzizt et al., 2015). To our best knowledge, this is the first report on the use of ISSR markers to examine genetic diversity and structure in Moroccan walnut germplasm, although this technique has previously been applied to the species *juglans regia* L. in other countries (Malvolti et al., 2010, Ebrahimi et al., 201; Aiqing et al., 2014). In the present article, we report the use of some markers in assessing genetic diversity and structure of 11 walnut accessions originating from Middle and High Atlas Mountain in Morocco. Besides, we evaluate the efficiency of ISSR for walnut diversity study.

Results

ISSR markers

The eleven ISSR primers revealed a total of 135 bands ranged from 7 for UBC818 to 19 bands for UBC841 with an average of 12.27 (Table 1), while the number of polymorphic bands per primer varied from 6 (UBC818, UBC855) to 19 (UBC841), with an average of 11.18. The percentage of polymorphism (PPB) of scored fragment ranged from 66% for UBC855 primer to 100% for UBC807, UBC811, UBC836, UBC841 and UBC889, with an average of 91%. PIC value ranged from 0.80 (UBC818) to 0.93 (UBC841) with an average of 0.88. Moreover, the highest value of EMR (19.00) and the lowest (4.00) were observed for primers UBC841 and UBC855, respectively, with an average of 10.35. The MI values varied from 3.45 to 17.63. Primer (UBC841) showed the highest MI value, whereas primer (UBC855) had the lowest, with an average of 9.21. The Rp used to determine the ability of primers to differentiate between accessions, ranged from 0.70 (primer UBC855) to 9.21 (primer UBC841), with an average of 4.91.

Genetic diversity analyses

Estimates of genetic diversity of studied accessions are summarized in Table 2. The number of alleles observed (Na) at each primer ranged from 1.7 (UBC810) to 2.1 (UBC811) with a mean of 1.90 alleles per primer. For all samples, the effective number of alleles (Ne) varied from 1.25 for UBC834 to 1.68 for UBC855, with an average of 1.41 alleles per primer. The Shannon information index (I) showed a minimum value (0.28) for UBC834 and a maximum value (0.58) for UBC855 with a mean of 0.38. The total gene diversity (Ht), ranged from 0.18 for UBC834 to 0.40 for UBC855, with an average of 0.25, while the gene diversity within accessions (Hs) varied from 0.14 for UBC834 to 0.33 for UBC855 (mean 0.20). Nei's coefficient of genetic differentiation (G_{ST}) was 0.164, meaning that 83.60% of total genetic variability is accounted among accessions and 16.40% within accessions.
The result of global AMOVA analysis (Table 4) revealed that the differentiation among accessions was very large (F_{ST}=0.286), indicating that 28.60% of total genetic variability was distributed among accessions and 71.40% resided within accessions. This result showed a great level of variability residing within accessions despite the highest F_{ST} value (0.28), which corresponds to the estimated low value for gene flow (Nm=0.64).
A hierarchical AMOVA analysis was performed to explore if there is any genetic differentiation between sampled four different regions. A low percentage of genetic variation, 13.24%, was revealed among regional group of accessions (Table 3). When assembling the accessions according to their bioclimatic type, the results of AMOVA showed only 1.31% of the ISSR variation between these bioclimatic groups. This result showed that bioclimatic type does not influence the genetic structuration of analyzed accessions.
The pairwise F_{ST} values and geographic distances between the 11 accessions are presented in Table 4. Among 55 pairwise F_{ST} values, 50 values are significant, indicating that the accessions are widely different from each other. The values varied from 0.006 (DEM/AGH; 153) to 0.564 (TAG/AMG; 120), which means that accessions DEM/AGH are the most genetically similar and TAG/AMG are the most divergent. The accessions specific F_{ST} indices were determined (data not shown). The finding revealed that the Imlil accession has the highest value of index (F_{ST}= 0.341), implying that this latter accession is the most divergent from other studies.

Table 1: Properties of 11 ISSR primers used in this study.

ISSR Loci	Sequence (5'-3')	Ta °C	Number of band amplified		PPB (%)	Pic	EMR	MI	RP
			Total	Plymorphic					
UBC 807	(AG) 8T	45.1	9	9	100	0.84	9	7.59	3.79
UBC 810	(GA)8T	44.1	10	7	70	0.88	4.9	4.3	2.0
UBC 811	GA(AG)7C	46.1	10	10	100	0.86	10	8.64	2.0
UBC 814	(CT) 8A	44.4	10	8	80	0.87	6.4	5.58	4.67
UBC 818	(CA) 8 G	43.5	7	6	85	0.80	5.14	4.12	7.09
UBC 834	(AG) 8YT	47.6	15	14	93	0.87	13.07	11.38	4.06
UBC 836	(AG) 8YA	48.3	17	17	100	0.91	17	15.51	8.27
UBC 840	(GA) 8YT	46.5	14	12	85	0.92	10.29	9.46	7.67
UBC 841	(GA)8YC	47.1	19	19	100	0.93	19	17.63	9.21
UBC 855	(AC) 8YT	45.5	9	6	66	0.86	4	3.45	0.70
UBC 889	(AC) 7	34.3	15	15	100	0.91	15	13.61	4.52
Average			12,27	11,18	91.00	0.88	10.35	9.21	4.91

Y = (C,T)

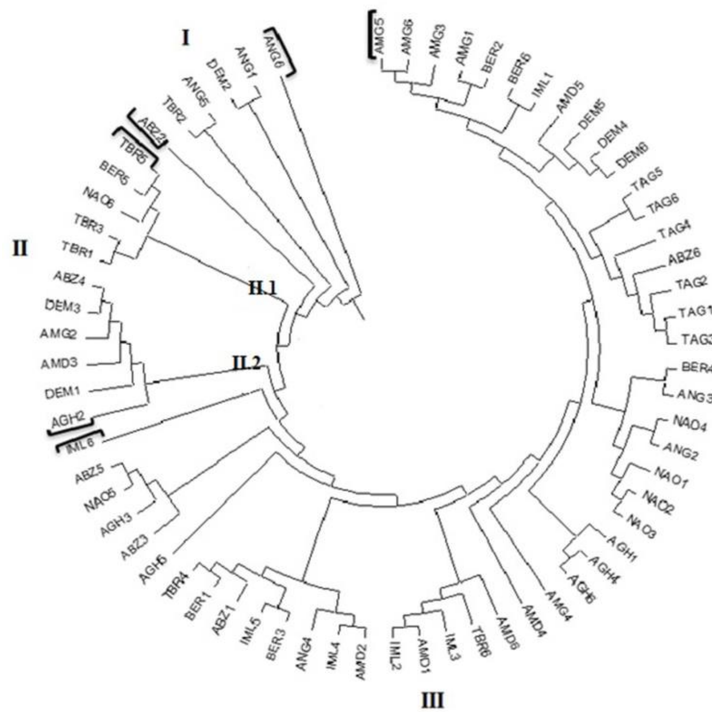

Fig 1. UPGMA dendrogram of 66 Moroccan walnut trees based on ISSR markers.

Table 2. Genetic diversity analysis of 11 accessions of Moroccan walnut based on ISSR markers.

ISSR Loci	Simple size	Na	Ne	I	Ht	Hs	Gst
UBC 807	66	2.00	1.46	0.45	0.29	0.25	0.13
UBC 810	66	1.7	1.25	0.28	0.32	0.16	0.13
UBC 811	66	2.1	1.36	0.37	0.24	0.22	0.07
UBC 814	66	1.8	1.46	0.34	0.26	0.22	0.13
UBC 818	66	1.86	1.56	0.43	0.3	0.17	0.31
UBC 834	66	1,93	1.25	0.28	0.18	0.14	0.11
UBC 836	66	2	1.48	0.44	0.28	0.22	0.18
UBC 840	66	2	1.48	0.44	0.29	0.25	0.10
UBC 841	66	1.79	1.31	0.31	0.2	0.17	0.11
UBC 855	66	2	1.68	0.58	0.4	0.33	0.19
UBC 889	66	1.8	1.39	0.35	0.22	0.21	0.07
Average		1.9	1.41	0.38	0.25	0.20	0.16

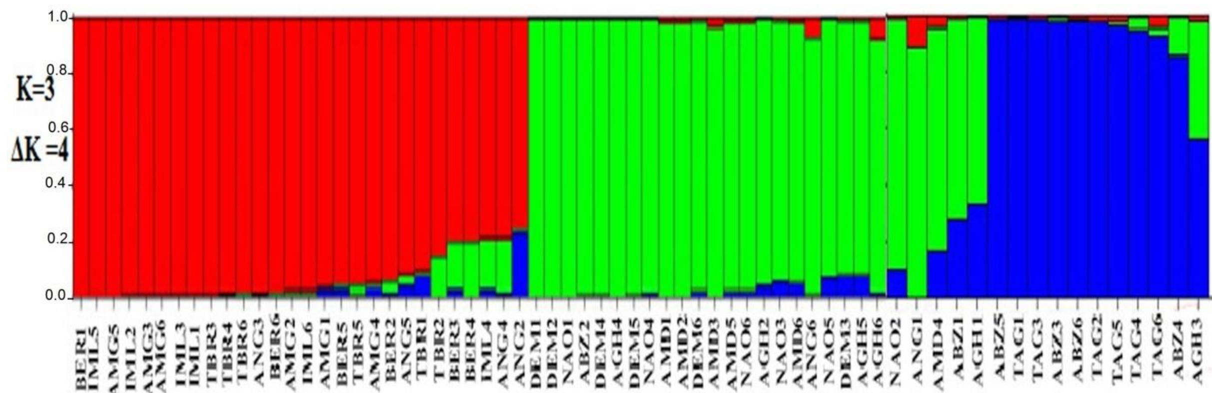

Fig 2. Genetic clustering obtained from the STRUCTURE analysis (N = 66). Each individual is represented by a single vertical column, divided into K colored segments that represent the individual's estimated proportion of membership to that genetic cluster. Thin lines separate individuals of different walnut accessions. Individuals are labeled below the Figure.

Table 3. Partition of the ISSR variation in its components for accessions of *juglans regia* L.

Source of variation	d.f	Sum of squares	Variance component	Percentage of variation	F-statistique
Global					
Among accessions	10	153.667	1.811 Va	28.70	FST=0.286***
Within accessions	55	247.500	4.500 Vb	71.30	
Hierarchical					
Among geographic group	3	75.528	0.856 Va	13.24	FCT=0.132
Among accessions within group	7	78.139	1.110 Vb	17.17	FSC=0.197***
Within accessions	55	247.500	4.500 Vc	69.59	FST=0.304***
Among bioclimatic group	2	33.492	0.083 Va	1.31	FCT=0.013
Among accessions within group	8	120.175	1.753 Vb	27.67	FSC=0.280***
Within accessions	55	247.500	4.500 Vc	71.01	FST=0.289***
Total	65	401.167	6.336		

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

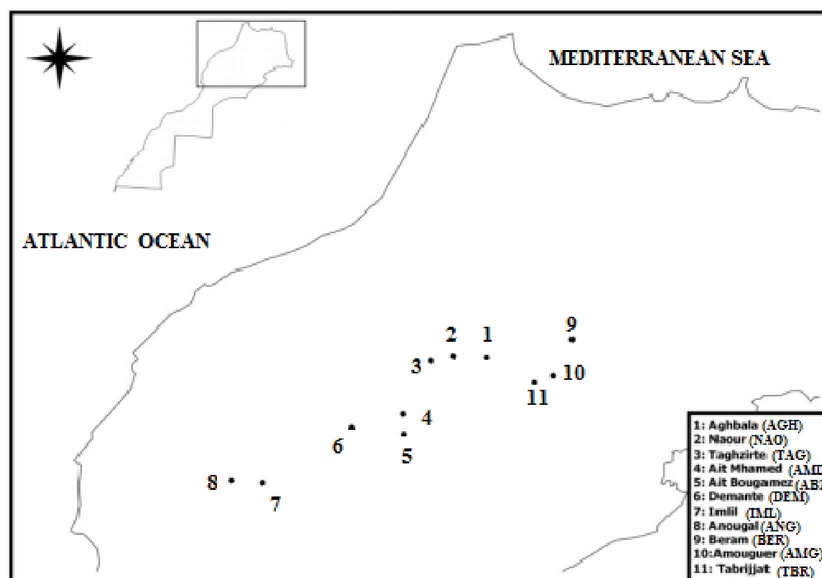

Fig 3. Map of Morocco showing locations of the walnut accessions analyzed.

Table 4. Pairwise F_{ST} values and corresponding geographic distances (in km, above diagonal) for 11 accessions of walnut analyzed by ISSRs.

	AMD	DEM	NAO	ABZ	TAG	AGH	AMG	BER	IML	TBR	ANG
AMD		51	84	26	63	101	152	184	158	133	186
DEM	0.009 NS		132	53	107	153	204	237	105	187	131
NAO	0.314**	0.310**		98	84	113	97	114	238	84	263
ABZ	0.103*	0.177**	0.376**		91	122	160	196	148	140	178
TAG	0.283***	0.365**	0.497**	0.195**		51	120	137	218	103	240
AGH	0.035 NS	0.006 NS	0.212**	0.054 NS	0.264**		71	86	265	56	286
AMG	0.293**	0.251**	0.513**	0.373**	0.564**	0.279***		49	309	20	337
BER	0.188**	0.229***	0.409**	0.286***	0.470**	0.214**	0.197**		345	62	369
IML	0.272***	0.340***	0.523***	0.368**	0.560**	0.313**	0.208***	0.110*		293	30
TBR	0.240***	0.245**	0.492**	0.373**	0.530**	0.272***	0.131**	0.040 NS	0.138*		316
ANG	0.159**	0.185*	0.438**	0.310***	0.497**	0.245**	0.168**	0.149*	0.203*	0.125NS	

Significant (p<0.05): *: Significant, ** highly significant, *** very highly significant, NS: no significant.

Table 5. Geographic and ecological characteristics of walnut accessions used in this study.

accession	Code	Number	Geographic origin	Altitude (m)	Latitude N	Longitude W	Zone	Rainfall average (mm)
Aghbala	AGH	1	32 Km North east of Aghbala	1673	32°32′	5°39′	Middle Atlas	450
Naour	NAO	2	Central Naour	1300	32°29′	5°58′	Middle Atlas	600
Taghzirte	TAG	3	12 Km East of Tagzirte	650	32 26	6° 12′	Middle Atlas	700
Ait Mhamed	AMD	4	20 Km South east of Azilal	1728	31° 25′	2° 28′	High Atlas	450
Ait Bougamez	ABZ	5	Ait Bougamez Centre	1996	31°38′	6° 28′	High Atlas	580
Demnate	DEM	6	3 km South east of Demnate	932	31° 43′	6° 58′	High Atlas	350
Imlil	IML	7	17 km South of Asni	1763	31° 8′	7° 55′	High Atlas	459
Anougal	ANG	8	40 km South of Amzmiz	1569	31° 9′	8° 15′	High Atlas	681
Beram	BER	9	5 km South of Midelt	1521	32° 40′	4° 44′	High Atlas	210
Amouguer	AMG	10	40 km West of Rich	1569	32° 12′	5° 8′	High Atlas	250
Tabrijjate	TBR	11	70 km East of Imilchil	1831	32° 16′	4° 56′	High Atlas	319

In contrast, the accession Ait Mhamed showed the lowest value (F_{ST}= 0.218), suggesting that it is the least different from the rest. The genetic distances between accessions were not related to their corresponding geographic distances after Mantel test execution (Mantel t-test= 0.8757, P = 0.81).

Cluster analyses

The UPGMA dendrogram, which was constructed based on the genetic distance matrix, showed that walnut trees were grouped into three major clusters (Fig. 1). A first minor cluster (I) was composed of six trees: ANG6, ANG1, DEM2, ANG5, TBR2 and ABZ2, all originating from High Atlas Mountain. The second cluster (II) bifurcates in two separate sub-clusters. The first sub-cluster consisted of five trees: TBR1, TBR3, TBR5 and BER5 coming from Hight Atlas Mountain, and NAO6 from Middle Atlas. In the second sub-cluster, six trees were grouped, namely ABZ4, DEM3, AMG2, AMD3 and DEM1 from High Atlas and AGH2 from Middle Atlas. The third cluster (III) contained the rest of the analyzed trees, in which many sub-clusters could be identified. The 66 trees of *juglans regia* analyzed were revealed to belong to 66 different ISSR haplotypes, reflecting high intra-accession diversity. The dendrogram showed that most individuals from a given accession tend to cluster together and are; therefore, more genetically similar than individuals from different accessions.

The genetic structure of Moroccan walnut accessions was further reconstructed by using the model-based Bayesian clustering approach (Pritchard et al., 2000) with three clusters (K = 3). The ad-hoc quantity based on the second order rate of change of the likelihood function (ΔK) (Evanno et al., 2005) showed an accurate representation of Moroccan walnut genetic structure of K =3 (ΔK = 4) (Fig. 2). Individuals with a membership coefficient less than 0.8 were considered as admixed. 61 individuals among the 66 studied (92.42%) were assigned to one of the model's defined groups. According to this model, walnut trees were assigned to three genetically different clusters. The first one (red) is composed of individuals from High Atlas Mountain accessions, Beram (BER), Imlil (IML), Amouguer (AMG), Tabrijjate (TBR) and Anougal (ANG) with a membership coefficient between 0.80 and 0.99, except individuals 2 and 4 from accession Anougal that could be considered as admixed (coefficients 0.75 and 0.78, respectively). The second cluster (green) contained individuals belonging to High Atlas Mountain accessions, Demnate (DEM), Ait Bougamez (ABZ), Ait Mhamed (AMD) and Anougal (ANG), and other arising from Middle Atlas accessions, Naour (NAO) and Aghbala (AGH). They all have membership coefficients greater than 0.80, except for individual 1 from accessions Ait Bougamez and Aghbala, which could be assumed to have an admixed ancestry (coefficients 0.71 and 0.67, respectively). The smallest cluster (blue) consisted of 11 individuals, with more than 80% of the assignation probability, originating from Middle Atlas Mountain accessions, Taghzirte (TAG) and Aghbala (AGH), and other rising in High Atlas accessions, Ait Bougamez (ABZ). The individual 3 from Aghbala showed an assigned probability of 56%, which could be considered as admixed. Accordingly, the genetic structure of investigated

walnut trees within three main gene pools was not correlated to the mountain range type.

Discussion

In general, the ISSRs markers were successfully used to evaluate the genetic diversity in many tree species (Reddy et al., 2002), including walnut (Potter et al., 2002; Pollegioni et al., 2003). In this study, the eleven tested ISSRs primers revealed a high percentage of polymorphism with an average of 91%. This finding is higher than that showed by Malvolti et al. (2010) for Italian walnut (73.8%) and Christopoulos et al. (2010) for Greek walnut (82.8). Nevertheless, it is lower than result reported by Aiqing et al. (2014) for Chinese walnut (92.31). In addition, PIC with an average of 0.88 was higher than several values reported in other studies with different markers like the work carried out for Greek walnut by Christopoulos et al. (2010) (0.28) using ISSRs markers and for Indian walnut by Shamasbi et al. (2018) (0.30) using ISSRs markers and Noor Shah et al. (2018) (0.16) using SSR markers. The MI values with an average of 9.21 was higher than that obtained in Greek walnut by Christopoulos et al. (2010) (3.19) using ISSRs and in Indian walnut by Noor Shah et al. (2018) (0.24) using SSRs, but lower than that showed by Shamasbi et al. (2018) using ISSR (28.9) for Indian walnut. In addition, The Rp used to determine the ability of primer to differentiate provenances, with an average of 4.91. This value was higher than that obtained in Indian walnut by Noor Shah et al. (2018) (2.37) using SSR, but comparable with that published by Christopoulos et al. (2010) (4.52) for Greek walnut. The primer UBC841 recorded a PPB value of 100%, which is higher than PPB reported by Malvolti et al. (2010) for the same primer in Italian walnut (71.4%). Moreover, this primer obtained higher values of PIC, MI and Rp, (0.93, 17.63 and 9.21 respectively). Consequently, the UBC841 was the most informative primer and the primer UBC855 with the low values of PPB, PIC, MI and Rp (66, 0.86, 3.45 and 0.7, respectively), were considered as the lowest informative primer. The high multi-locus value of Ht suggests the presence of a high level of polymorphism. This value was higher than that obtained by Aiqing et al. (2014) (Ht=0.13) in Chinese walnut using ISSR markers.

The high genetic diversity obtained in Moroccan walnut was in agreement with general trend for all plant species (Ht=0.30 from 584 entries), long-lived woody perennial species (Ht=0.28 from 195 entries) and for angiosperms species (Ht=0.28 from 73 entries) (Hamrik et al., 1992). This high diversity of Moroccan walnut genotypes may be attributed to life history traits of this species. The walnut has unisexual flowers and a monoecious plant, 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, which results in frequent gene flow among individuals and increases the chance of gene recombination (Aiqing et al., 2014). Depending on the G_{ST} value (G_{ST}=0.16), Moroccan walnut accessions were largely differentiated. This result is lower than G_{ST} value found by Aiqing et al. (2014) (G_{ST}=0.50). Likewise, the F_{ST} value (0.28) detected in this investigation showed a great level of differentiation, which is higher than that published by several walnut genetic studies (0.18, Wang and Pei, 2008; 0.12, Karimi et al., 2010; 0.021, Vischi et al., 2017), but lower than that published by Aiqing et al. (0.51, 2014). Together with large differentiation among accessions, there is also an important variation within accessions which can be explained by the traditional method of multiplication by seedling used by farmers. As pointed out by Telles et al. (2003), when gene flow is restricted, the population tends to have smaller effective size and greater inbreeding. As a result, a greater probability of inter-population differentiation may occur. Moreover, the adaptation of Moroccan walnut accessions to the local environment should increase this differentiation. Little amount of genetic variation among regional group of accessions (13.24%) and between bioclimatic groups (1.31%) indicates that there is no adaptation of Moroccan walnut accessions to the local environment and that bioclimatic type do not have effect on accessions genetic structuration. The genetic distances between Moroccan walnut accessions were not associated to their corresponding geographic distances (Mantel t-test= 0.87, P = 0.81). The same result was found by Vischi et al. (2017), which did not reveal any significant correlation between geographic and genetic distances among walnut populations from the Eastern Italian Alps. More, Mohsenipoor and Vahdati, (2010) reported that the geographical proximity of the populations was not correlated to their level of genetic relatedness. Nevertheless, Wang and Pei (2008) reported that the Mantel test has shown a significant correlation between pairwise genetic distance and geographic distance among nine walnut populations (r=0.35, P<0.005). Regarding the accessions genetic structure, three groups were observed independently from mountain range type. The genetic structure of natural populations of Maritime Pine (*Pinus pinaster* Aiton) was investigated by isozymes. This was revealed to be correlated to mountain range type in Morocco (Wahid et al., 2004). Furthermore, the accessions clustering was made independently of their geographic origin and bioclimatic zones, which was confirmed by the very low amount of genetic differentiation revealed by AMOVA for among geographic and bioclimatic groups. Similarly, Arzani et al. (2008) showed that clustering of walnut genotypes was not correlated to their geographical location in Iran. In this study, analysis of 66 trees showed 66 ISSR haplotypes, suggesting the existence of high intra-accessions variability.

Materials and methods

Plant material

Eleven accessions of *Juglans regia*, representing the main cropping area of this species in Morocco, were sampled on the basis of the difference in their geographical distances and altitude (Fig 3, Table 5). Six individual trees were sampled randomly from each of the 11 accessions. They belong to two range types of Mountain in Morocco: Middle Atlas (Aghbala, Naour and Taghzirte accessions) and High Atlas (Ait Mhamed, Ait Bougamez, Demnate, Imlil, Anougal, Beram, Amouguer and Tabrijjate accessions). The sampled young leaves were stored at -80°C pending DNA extraction.

DNA extraction and PCR reactions

Six individuals from each accession were taken at random for DNA extraction. Genomic DNA from young leaves was extracted with the method described by Doyle and Doyle

(1990) slightly modified. DNA concentration were determined spectrophotometerically and diluted to 10 ng/µl to carry out PCR amplification.

Eleven ISSR primers had previously displayed reliable banding patterns (Christopoulos et al., 2010; Aiqing et al., 2014; Shamasbi et al., 2018) and were used for 66 PCR amplification of the DNA templates (Table 2). The reaction mixture, in a final volume of 12.5µl, contained: 15ng of DNA template, 1x reaction buffer, 1 mM $Mgcl_2$, 0.8 mM dNTPs, 0.8 µM of each primer and 0.75 U of My Taq™ DNA polymerase. PCRs were run in a Multigene gradient (Labnet, NJ. USA) thermocycler through 45 cycles, each consisting of: 94°C for denaturation step (45s), determined temperature for each primer for annealing step (45s) (Table 1), 72°C for extension step (2 min) and 72°C for a final extension step (7 min). The optimum annealing temperature for each primer was determined by using the gradient PCR.

The ISSR products were separated by electrophoresis using 1.7% agarose gel submerged in 0.5x TBE buffer and then stained with 1 µg/µl of ethidium bromide. The DNAs were visualized under UV light using the Gel Doc system (Enduro^{TM} GDS, Labnet). A 1Kb DNA HyperLader™-Bioline was used for molecular weight estimation of PCR product.

Data analyses

Since ISSR primers are dominant markers, amplified bands were scored 1 for presence or 0 for absence. In order to determine the utility of these polymorphic markers, several parameters were calculated: the percentage of polymorphic band (PPB), polymorphic information content (PIC) value using formula: $PIC = 1 - \Sigma pi^2$ (where 'pi' is the frequency of each allele per primer) (Rohlf, 1998), effective multiplex ratio (EMR) was calculated (Powell et al, 1996), marker index (MI) measured as product of PIC and EMR and resolving power (Rp) of each primer was calculated according to Prevost and Wilkinson (1999). To evaluate the genetic diversity within and among accessions on the basis of Nei's formula, the following parameters are determined: numbers of alleles (Na), effective number of alleles (Ne), The diversity within the provenances (Hs), total gene diversity (Ht), coefficient of gene differentiation (G_{ST}) which calculated according to the following formula: $G_{ST} = Dst/Ht$, $Dst = Ht - Hs$ and Shannon's Information index (I), using POPGENE version 1.32 software (1999). The analysis of molecular variance (AMOVA) was conducted to investigate the genetic variation among accessions (F_{ST}) and within accessions; and also to research the amount of ISSR variation partitioned between four geographic groups of accessions: Beni Mellal (Naour, Aghbala and Taghzirte accessions), Azilal (Ait Mhamed, Ait Bougamez and Demnate accessions), Merrakech (Imlil and Anougal accessions) and Errachidia (Amouguer, Beram and Tabrijjate accessions); and between three bioclimatic zones of acessions: sub-humid (Taghzirte and Anougal), semi-arid (Aghbala, Naour, Ait Bougamez, Ait Mhamed and Imlil) and arid (Amouguer, Berram, Demnate and Tabrijjate) (Excoffier et al., 1992). The AMOVA was based on genetic distance calculated by the number of pairwise differences between haplotypes. The pairwise genetic differentiations (FST) among the 11 accessions were also generated by AMOVA. Accessions specific FST indices were also computed to explore which accessions is more differentiated from one another. 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 $Nm = 0.25 (1/FST - 1)$. The genetic distance matrix between 66 studied walnut 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. The Bayesian model-based clustering algorithms implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000) was used to infer the genetic structure of Moroccan walnuts. The STRUCTURE algorithm was run using putative population origin for each individual as prior information, a model with admixture and correlated allele frequencies. Each run involved a burning period length of 70,000 and a number of MCMC (Markov Chain Monte Carlo) reps after burnin of 1000 iterations for a number of clusters (K) ranging from 2 to 11 with 10 iterations per K. To identify the number of K clusters explaining the observed genetic structure, we used the STRUCTURE Harvester website (Earl and vonHoldt, 2012), which implements the Evanno method (Evanno et al., 2005).

Conclusion

Fingerprinting of the Moroccan walnut was carried out using ISSR markers to obtain molecular data of the national gene pool. The present study demonstrated that ISSR markers provide a practical and effective method to estimate the genetic diversity in this crop. The results led to reveal a great level of genetic diversity in Moroccan walnut genotypes which were structured in three gene pools independ of Mountain range type, geographic origin and bioclimatic type. The walnut accessions were largely differentiated in line with limited gene flow. These results could be exploited for future conservation, breeding program and to develop the walnut crop, which has an economic importance for mountain Moroccan farmers.

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References

Agostini G, Echeverrigaray S, Souza-Chies TT (2008) Genetic relationships among South American species of Cunila D. Royen ex L. based on ISSR. Plant Syst Evol. 274(3–4): 135–141.

Aiqing J, Yina W, Guoliang W, Wenjiang W, Hongyan Y, Qihai W (2014) Genetic diversity and population structure of North China Mountain walnut revealed by ISSR. Am J Plant Sci. 5: 3194-3202.

Ajal EA, Jbir R, Melgarejo P, Hernández F, Haddioui A, Salh Hannachi A (2014) Efficiency of Inter Simple Sequence Repeat (ISSR) markers for the assessment of genetic diversity of Moroccan pomegranate (*Punica granatum* L.) cultivars. Biochem Syst Ecol. 56: 24–31

Akbari V, Heidari R, Jamei R (2014) Fatty acid compositions and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Iran. Adv Herb Med. 1 (1): 36-41.

Amiri R, Vahdati K, Mohsenipoor S, Mozaffari MR, Leslie Ch (2010) Correlations between Some horticultural traits in walnut. Hort Sci. 45(11):1690–1694.

Angmo S, Acharyya P, Hasan MA (2013) Performance of indigenous walnut selections under climatic conditions of Ladakh. International Symposium on Agri-Foods for Health and Wealth. Bangkok, Thailand, p.167-176.

Arzani K, Mansouri-Ardakan H, Vezvaei A, Roozban MR (2008) Morphological variation among Persian walnut (*Juglans regia* L.) genotypes from central Iran. New Zeal J of Cr and Horti Sci. 36:159–168.

Bayazit S, Kazan K, Gulbitti S, Çevik V, Ayanoglu H, Ergul A (2007) AFLP analysis of genetic diversity in low chill requiring walnut (*Juglans regia* L.) genotypes from Hatay, Turkey. Sci Hort. 111:394–398

Ben Tamarzizt H, Ben Mustapha S, Baraket G, Abdallah D, Salhi Hannachi A (2015) Assessment of genetic diversity and relationships among wild and cultivated Tunisian plums (*Prunus* spp.) using random amplified microsatellite polymorphism markers. Genet Mol Res. 14: 1942–1956.

Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jeronimo C (2010) Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. Food Chem Toxico. 48:441–447.

Christopoulos V M, Rouskasb D, Tsantili E, Bebeli PJ (2010) Germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) cultivars and Greek local selections revealed by Inter-Simple Sequence Repeat (ISSR) markers. Sci Hortic. 125: 584–592.

Ciarmiello LF, Piccirillo P, Pontecorvo G, Luca AD, Kafantaris I, Woodrow P (2011) A PCR based SNPs marker for specific characterization of English walnut (*Juglans regia* L.) cultivars. Mol Biol Rep. 38:1237–1249

Doyle JJ and Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus12: 13–15.

Earl DA and von Holdt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour. 4: 359-361.

Ebrahimi A, Fatahi, Zamani Z (2011) Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSRs markers. Sci Horti. 130 :146–151.

Evanno G, Regnaut S and Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 14: 2611-2620.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver 30: An integrated software for population genetics data analysis. Evol Bioinform Onl. 1: 47-50.

Excoffier L, Smouse PE, Quatro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genet. 131: 479-491.

FAOSTAT (2018) Food and Agriculture Organization of the United Nations. FAO Statistics Division. http://faostat.fao.org/site/567/default.aspx#ancor..

Fatahi R, Ebrahimi A, Zamani Z (2010) Characterization of some Iranians and foreign walnut genotypes using morphological traits and RAPDs markers. Hort Environ Biotechnol. 51: 51–60.

Fjellstrom RG, Parfitt DE (1995) Phylogenetic analysis and evolution of the genus *Juglans* (*Juglandaceae*) as determined from nuclear genome RFLPs. Plant Syst Evol. 197:19–32

Foroni B, Malvoiti ME, Taurchini D, Finesch S (2001) Isozyme and organellar analysis of genetic diversity in natural European and Asiatic walnut (*Juglans regia* L.) populations. Acta Hortic. 544.

Gandev S (2007) Budding and grafting of the walnut (*Juglans regia* L.) and their effectiveness in Bulgaria (Review). Bulg J Agri Sci. 13: 683-689.

Germain E, (1992) Le noyer. In : Gallais A, Bannerot H (ed) Amélioration des espèces végétales cultivées, objectifs et critères de sélection, INRA Editions, Paris, pp. 620-632.

Hamrick JL, Godt MJW, Sherman-Broyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. New Forest. 6: 95-125.

Kafkas S, Ozkan H, Sutyemez M (2005) DNA polymorphism and assessment of genetic relationships in walnut genotypes based on AFLP and SAMPL markers. J Am Soc Hortic Sci. 130: 585–590.

Karimi R, Ershadi A, Vahdati KK, Woeste K (2010) Molecular characterization of Persian walnut populations in Iran with microsatellite markers. Hort Sci. 45: 1403–1406.

Karimi R, Ershadi A, Ehtesham Nia A, Sharifani M, Rasouli M, Ebrahimi A, Vahdati K (2014) Morphological and molecular evaluation of Persian walnut populations in Northern and Western regions of Iran. J Nutr. 5(2):21-31

Kodad O, Socias I, Company R, Sindic M (2014) Kernel quality in a local walnut (*juglans regia* L.) population grown under different ecological conditions in Morocco. FAO-CIHEAM Nucis-Newsl. 335 (16): 27-31.

Kumar M, Mishra GP, Singh R, Kumar J, Naik PK, Singh SB (2009) Correspondence of ISSR and RAPD markers for comparative analysis of genetic diversity among different apricot genotypes from cold arid deserts of trans-Himalayas. Physiol Mol Biol Plant. 15: 225–236.

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5:150-163.

Lansari A, Hassani El, Nabil D, Germain E (2001) Preliminary results on walnut germplasm evaluation in Morocco. Acta Hortic. 544 :27–35.

Li Z, Lanying Z, Qianwen X (2007) Identification of RAPD markers linked to thickness gene of shuck in walnut. Adv Biol Res. 1:137– 140

Mahmoodi R, Rahmani F, Rezaee R (2013) Genetic diversity among *Juglans regia* L. genotypes assessed by morphological traits and microsatellite markers. Spanish J of Agri Res. 11(2): 431-437

Malvolti ME, Pollegioni P, Bertani A, Mapelli S, Cannata F (210) *Juglans regia* provenance research by molecular, morphological and biochemical markers: A case study in Italy. Bioremed Biodiv Bioavailab. 4 (1):84-92.

Martins M, Tenreiro R, Oliveira MM (2003) Genetic relatedness of Portuguese almond cultivars assessed by RAPD and ISSR markers. Plant Cell Rep. 22(1):71-78.

Miltiadis VC, Dimos R, Eleni T, Penelope JB (2010) Germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) cultivars and Greek local selections revealed by Inter-Simple Sequence Repeat (ISSR) markers. Sc Hort.125 : 584–592

Mohsenipoor S, Vahdati K, Amiri R, Mozaffari MR (2010) Study of the genetic structure and gene flow in Persian walnut (*Juglans regia* L.) using SSR markers. In: McNeil DL (Ed): VIth Intl. Walnut Symposium Proc. Acta Hort. 861.

Nicese FP, Hormasa JI, Mc Granaham GH (1998) Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers. Euphy. 101:199–206.

Noor Shah U, Mir JI, Ahmed N, Fazili Kh M (2018) Assessment of germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) genotypes through microsatellite markers. J Saudi Soc Agri Sci. 17: 339-350.

Pollegioni P, Bartoli S, Cannata F, Malvolti ME (2003) Genetic differentiation of four Italian walnut (*Juglans regia* L.) varieties by inter simple sequence repeat (ISSR). J Genet Breeding. 57:231–240.

Pollegioni P, Woeste K, Mugnozza GS, Malvolti ME (2009) Retrospective identification of hybridogenic walnut plants by SSRs fingerprinting and parentage analysis. Mol Breeding 24: 321–335.

Potter D, Gao F, Aiello G, Leslie C, McGranahan G (2002) Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars. J Am Soc Horti Sci. 127:75–81.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The utility of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breeding. 2: 225-238.

Prevost A, Wilkinson MJ (1999) A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor Appl Genet. 98:107–112.

Pritchard JK, Stephens M and Donnelly P (2000) Inference of population structure using multilocus genotype data. Genet. 155: 945-959.

Reddy MP, Sarla N, Siddiq EA (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphy. 128:9–17.

Rholf FJ (1998) NTSYS-pc*:* Numerical taxonomy and multivariate analysis system Version 2.02. Applied Biostatistics, New York.

Ross-Davis A, Woeste K (2008) Microsatellite markers for *Juglans cinerea* L. and their utility in other *Juglandaceae* species. Conserv Genet. 9: 465–469.

Shamasbi FV, Nasiri N, Shokri E (2018) Genetic diversity of Persian ecotypes of Indian Walnut (*Aeluropus littoralis* (Gouan) Pari.) by AFLP and ISSR Markers. Cytol Genet. 52(3): 222–230.

Slatkin, M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. Evo. 43: 1349 –1368.

Tapia MI, Sanchez-Morgado JR, Garcıa-Parra J, Ramırez R, Hernandez T, Gonzalez-Gomez D (2013) Comparative study of the nutritional and bioactive compounds content of four walnut (*Juglans rejia* L.) cultivars. J of Food Compo and Analy. 31: 232-237.

Telles MPC, Coelho ASG, Chaves LJ, Diniz-Filho JAF, D'ayala VF, (2003) Genetic diversity and population structure of *Eugenia dysenterica* DC. (*Cagaiteira-Myrtaceae*) in Central Brazil: spatial analysis and implications for conservation and management. Conserv Genet. 4: 685 –695 (2003).

Vischi M, Chiabà C, Raranciuc S, Poggetti L, Messina R, Ermacora P, Cipriani G, Paffetti D, Vettori C, Testolin R, (2017) Genetic Diversity of Walnut (*Juglans Regia* L.) in the Eastern Italian Alps. Forests 8 (81): 2-14.

Vyas D, Sharma SK, Sharma DR (2003) Genetic structure of walnut genotypes using leaf isozymes as variability measure. Sci Hort. 97:141–152

Wahid N, Martínez CG, EL Hadrami I, Boulli A (2004) Genetic structure and variability of natural populations of Maritime Pine (Pinus pinaster Aiton) in Morocco. Silvae Genet. 53 (3): 93-99

Wang H, Pei D, Gu R, Wang B (2008) Genetic diversity and structure of walnut populations in central and southwestern China Revealed by microsatellite markers. J Am Soc Horti Sci. 133:197–203.

Yerlikaya C, Yucel S, Erturk Ü, Korukluoğlu M (2012) Proximate composition, minerals and fatty acid composition of *Juglans regia* L. genotypes and cultivars grown in Turkey. Braz Arch Bio Techn. 55 (5): 677-683.

Yu HH, Yang ZL, Sun B, Liu RN (2011) Genetic diversity and relationship of endangered plant *Magnolia officinalis* (*Magnoliaceae*) assessed with ISSR polymorphisms. Biochem Syst Ecol. 39 (2): 71–78.

Zietkiwewicz E, Rafalasky JA, Labua D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomi. 20: 176-183.

Zohary D, Hopf M (1993) Domestication of plants in the Old World. The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley. Clarendon Press, Oxford.