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ASSESSMENT OF GENETIC DIVERSITY OF WILD STRAWBERRY TREE (*ARBUTUS UNEDO* L.) GENOTYPES FROM MOROCCO USING ISSR MARKERS

SUMMARY

The aim of this study was to evaluate the genetic diversity among 36 wild strawberry tree (*Arbutus unedo* L.) genotypes belonging to different altitude and geographical origins, using 16 ISSR (Inter Simple Sequence Repeats) markers. Results revealed that ISSR primers produced a total of 344 bands, of which 94.1% were polymorphic, indicating a high level of genetic variation among the studied populations. The mean values of polymorphism information content (PIC), efective multiplex ratio (EMR), resolving power (Rp) and marker index (MI) were 0.34, 19.13, 23.46 and 6.45, respectively. The total gene diversity (Ht), gene diversity within populations (Hs) and total genetic differentiation coefficient among the populations (Gst) values were 0.33, 0.15 and 0.50, respectively. The genetic distance between all pairwise combinations of the genotypes ranged from 0.113 to 0.512. The cluster analysis revealed three main groups and which are subdivided into eight subgroups and a two independant branches. Therefore, the grouping of strawberry tree genotypes was independent of their geographical origins and altitude with exception for the genotypes belonging to Bin El-ouidane and Laanoucer populations. The results showed that ISSR markers are suitable tools for the evaluation of genetic diversity among strawberry tree genotypes. The data of this work will be useful for strawberry tree breeding programs and conservation strategies.

Keywords: *Arbutus unedo* L., strawberry tree, genetic diversity, ISSR markers, polymorphism, Morocco

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INTRODUCTION

Strawberry tree (*Arbutus unedo* L.), is an evergreen shrub or small tree belonging to the *Ericaceae* family and the genus *Arbutus*. It is native to Mediterranean countries such as Algeria, Morocco, Tunisia, Turkey, Syria, Greece, Croatia, France, Portugal and Spain (Serçe *et al*. 2010, Ruiz-Rodriguez *et al.* 2011). Furthermore, the plant 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, Ruiz-Rodriguez *et al.* 2011, Abbas, 2015). Strawberry tree prefers siliceous or decarbonated substrata and can grow on alkaline and relatively acidic soils (pH 5-7.2) (Torres *et al*. 2002, Godinho-Ferreira *et al.* 2005, Celikel *et al.* 2008, Gomes, 2011).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). The plant has a huge ecological importance since it prevents erosion of the soils and has also the capacity to regenerate itself rapidly after fires, surviving quite well in poor soils (Gomes and Canhoto, 2009, Takrouni *et al.* 2012). It is widely used in traditional medicine, such as antiseptics, diuretics, and laxatives as well as for its uses in the treatment of hypertension, atherosclerosis, thrombosis (Ziyyat *et al.* 2002, Mekhfi *et al.* 2006) and diabetes (Bnouham *et al.* 2007). The strawberry tree fruit is suitable for the production of alcoholic beverages, jams, jellies, and marmalades (Pallauf *et al.* 2008).

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). Morphological and biochemical markers have been widely exploited in breeding studies and in the investigations into diversity of species and the relationship between genotypes, and their wild parent (Vidrih *et al.* 2013). Conventional plant breeding depends on phenotypic selection in the first step (Ercisli *et al.* 2012). However, the morphological and biochemical traits have some limitation, they are strongly influenced by environmental factors and they varied with plant developmental stage (Kercher and Sytsma, 2000, Ouinsavi and Sokpon, 2010). In recent years, various types of molecular markers that are based on DNA have been developed to study genetic diversity and genotype identification of plant (Sorkheh *et al.* 2009, Zaefizadeh and Goliev, 2009). Among these genetic markers, RAPD (Randomly Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), SNPs (Single Nucleotide Polymorphic) and ISSR (Inter Simple Sequence Repeats) techniques, are commonly used for genetic studies of strawberry tree in Tunisia and Portugal (Takrouni and

Boussaid, 2010; Rodriga de Sà *et al*. 2011; Gomes *et al.* 2012; Lopes *et al*. 2012; Fazenda and Miguel, 2013; Ribeiro *et al*. 2017; Fazenda *et al*. 2019). The low reproducibility of RAPD, and the necessity to know the flanking sequences to develop species specific primers for SSR polymorphism are some limitations of these primers (Reddy *et al.* 2000, Gupta *et al.* 2010). ISSR's are best to overcome these limitations (Reddy *et al.* 2002). The ISSR markers are simple, fast and highly reproducible. They also require small amounts of DNA and do not require any prior sequence information of amplified locus (Zietkiewics *et al*. 1994, Pradeep *et al.* 2002).

Although there are works published about strawberry tree fruits in Morocco regarding biochemical composition (Zitouni *et al*. 2020, Zitouni *et al.* 2022), as well as morphological variability (Zitouni *et al.* 2021), the genetic diversity within the species is still largely unknown. Thus, the objective of this study was to evaluate a genetic diversity of strawberry tree genotypes belonging to different bioclimates and geographical origins, using ISSR molecular markers. Herein, we intend to present a complete database regarding genetic diversity of Moroccan strawberry tree in order to provide useful information for future conservation of these genetic resources and breeding programs.

MATERIAL AND METHODS

Plant material

Thirty-six genotypes were selected from natural populations of Strawberry tree (*Arbutus unedo* L.) representing the natural range of the species in Morocco.

Table 1. The geographical characteristics of the studied strawberry tree genotypes

Localities	Sample Code	Geographic Zone	Longitude (W)	Latitude (N)	Altitude (m)
Bin El Ouidane	BNO	Middle Atlas	6°30'	32°05'	1313
Tamscart	TAM	Middle Atlas	6°16'	32°16'	1520
Ksiba	KSB	Middle Atlas	6°11'	32°31'	1338
Ouaouizerth	OUA	Middle Atlas	6°21'	32°09'	1050
Tahnaout	TAH	High Atlas	7°55'	31°18'	1200
Khénifra	KHN	Middle Atlas	5'27'	32°53'	1613
Laanoucer	LAN	Middle Atlas	4°54'	33°42'	1700
Oulmès	OUL	Central Plateau	6°02'	33°33'	935
Chefchaoun	CHF	Western Rif	5°17'	35°07'	534
Ouazzane	OUZ	North West	5°32'	34°55'	202
Moulay Driss Zerhoune	MDZ	Middle Atlas	5°30'	34°02'	820
Bab Marzouka	BMR	Rif- Middle Atlas	4°08'	34°10'	801

The main geographical characteristics of the populations are reported in Table 1 and Figure 1.

Young leaves were collected and stored at -80°C, then the samples were freeze-dried in a WPA Bio-wave S2100 freeze-dryer to be used for DNA extraction.

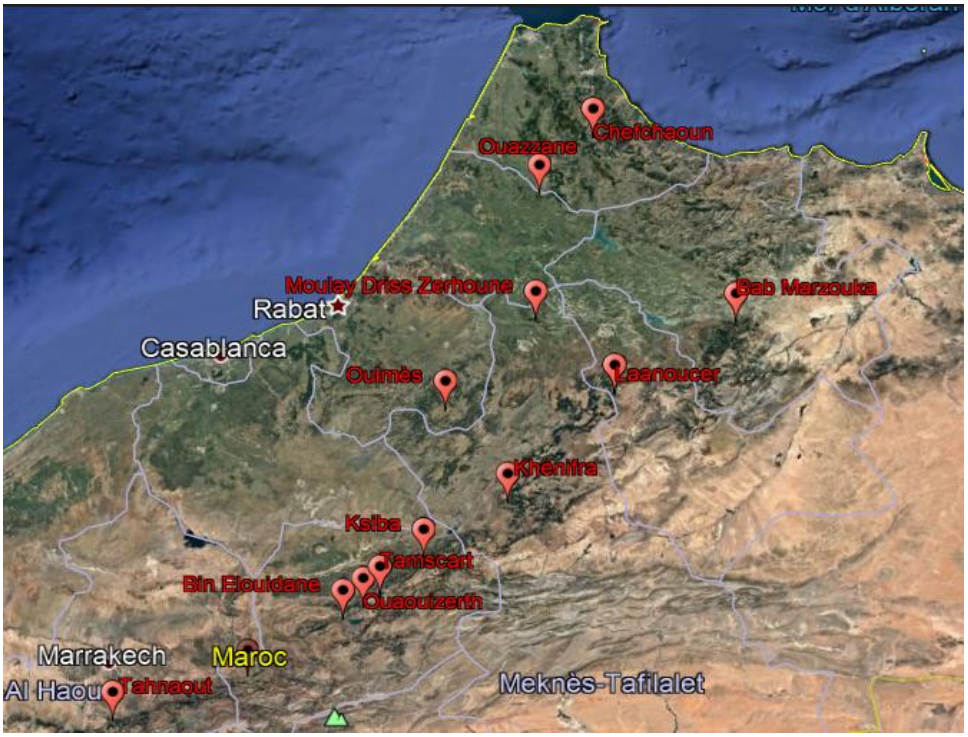

Figure 1. Geographical Origin of the studied strawberry tree (*Arbutus unedo* L.) populations

DNA extraction and quantification

Genomic DNA was extracted from young leaves using the Cetyl Trimethyl Ammonium Bromide (CTAB) method described by Saghai-Maroof *et al.* (1984). The quantity and quality of the DNA obtained was assessed spectrophotometrically by a Nanodrop (BioDrop µLite+, 2019) at 230, 260 and 280 nm.

ISSR primer screening

A total of 20 ISSR primers was tested and 16 primers were selected (Table 2). Gradient PCR was used to adjust the annealing temperature of each primer.

Table 2. The 16 ISSR primers used in this study and their annealing temperature

Primers	Sequence (5'-3')	H.T (°C)	BS (bp)
F2	[CA] 6GC	54.8	141 - 4392
F8	[AG] 8CC	50.7	188 - 3587
F10	[CA] 8AG	42	208 - 4676
F11	[CA] 8AC	54.8	162 - 5319
F12	[GA] 8CC	45.4	594 - 14000
IMA 834-1	[AG] 8YT	53.5	134 - 2441
IMA 834-2	[AG] 8YT	45.4	362 - 13362
IMA 834-Z	[AG] 8YT	50.7	281 - 8985
IMA 9-Z	[GA] 8CG	54.8	156 - 3412
IMA 12-1	[CA] 8TC	50.7	177 - 3632
UBC 808-2	[AG] 8C	54.8	145 - 3371
UBC 807	[AG] 8T	50	402 - 7143
UBC 817	[CA] 8A	50	470 - 11244
UBC 818	[CA] 8G	42	216 - 3153
ISSR 1	[AG] 8CA	45.4	457 - 11267
ISSF 1	[AG] 8TA	45.4	654 - 8094

H.T: Hybridization temperature, BS: band size

Statistical analysis

Band size was measured with Mesurim Pro V3.4 software. A binary data matrix was created based on the presence of bands (1) or their absence (0) in each genotype. The polymorphism information content (PIC), linked to the genetic diversity for each primer, was evaluated according to the formula of De Riek *et al.* (2001), PIC=2i (1-fi), where f is the band frequency in the data set. The Effective multiple ratio (EMR) was obtained according to Powell *et al.* (1996) as follow: EMR=np (np/n), where np is the number of polymorphic loci and n is the total number of loci. The Marker index (MI) was estimated according to Chesnokov and Artemyeva, (2015) as the product of PIC value and EMR value (MI=PIC×EMR). The Marker index (MI) is a statistical parameter used to estimate the total utility of the maker system. The Resolving power (Rp) of each ISSR primer was calculated according to Prevost and Wilkinson, (1999), as follow: Rp=∑lb, where lb=1−[2×(0.5−p)], where p is the proportion of genotypes containing the i th band. Parameters of genetic diversity were calculated: numbers of alleles (Na), effective number of alleles (Ne), the diversity within the

populations (Hs), total gene diversity (Ht), coefficient of gene differentiation (GST) using POPGENE version 1.32 software (1999). Genetic distances were calculated using Simple Coefficient Matching (SCM) by Clustering Calculator software program established by Brzustowski, (2002). A histogram of pairwise comparisons between all genotypes according to the number of markers that differentiate them was established. Then, a dendrogram was constructed on the basis of the similarity matrix data using an Unweighted Pair Group Mean with Arithmetic Average (UPGMA) method to better understand the patterns of variability among the genotypes.

RESULTS AND DISCUSSION

ISSR polymorphism

The 16 ISSR primers amplifed produced a total of 344 bands, which 324 were polymorphic. The band size ranged from 134 to 14000 base pairs (Table 2). The number of amplified fragments ranging from 14 bands (IMA 834-1) to 28 bands (F2) with an average of 21.5. The number of polymorphic bands per primer varied from 14 (ISSF1, IMA 834-1) to 27 (F2), with a mean of 20.25. Moreover, the percentage of polymorphism ranged from 86.9% for ISSR1 and F8 primers to 100% for F12, IMA 834-2, IMA 834-1, F11, F10 and UBC 818, with an average of 94.1% (Table 3). The high percentage of polymorphic bands per primer revealed the high level of polymorphism showed by the studied primers among the strawberry tree genotypes.

The Polymorphism Information Content (PIC) measures the ability of the markers to detect polymorphisms, the PIC values ranging from 0.28 for primer ISSF1 to 0.38 for primers F2, F11 and UBC 817 with a mean value of 0.34 (Table 3).

Regarding the effective multiplex ratio (EMR), the highest value of EMR (26.04) and the lowest (12.25) were observed for primers IMA 12-1 and F8, respectively, with an average of 19.13. The marker index (MI) used to estimate the overall utility of each marker system (Sorkheh *et al.* 2007). The values of MI ranged from 3.80 in the primer F8 to 9.56 in the primer F2 with a mean of 6.45. The F2 primer seems to be the most appropriate primer it had the highest value of PIC and MI and consequently is the most informative. Furthermore, the resolving power (Rp) which indicates to the efficiency of the ISSR primers used in discriminating strawberry tree genotypes, the values varied from 14.61 for the IMA 834-1 primer to 34 for F12 with an average value of 23.46 (Table 3). In general, the use of ISSR markers was found to be very efficient and yielded a high polymorphism rate.

The number of bands obtained in the current study with an average value of 21.5 bands were higher than those recorded by Lopes *et al*. (2012) in 46 Portuguese strawberry tree genotypes. They found a total of 45 and 56 bands using 5 ISSR and 6 RAPD primers with an average value of 9 and 9.3 bands, respectively. Gomes *et al*. (2012) tested 20 RAPD primers across 27 Portuguese

strawberry tree genotypes and they found 124 bands, that ranged from 1 to 13 with a mean value of 6.2 bands. In another study, Takrouni and Boussaid, (2010) assessed the genetic diversity of Tunisian strawberry tree using 9 RAPD markers. They reported a total of 88 bands, out of which 65 were polymorphic, that varied between 8 and 13 bands per primer.

Table 3. Characteristics and amplification results of 16 ISSR primers in the studied genotypes

Primer	TNB	NPB	NMB	PPB (%)	PIC	EMR	MI	Rp
F2	28	27	0	96.43	0.38	25	9.56	23.22
F8	23	20	2	86.96	0.31	12.25	3.80	24
F10	23	23	0	100	0.35	23	8.00	23.05
F11	26	26	1	100	0.38	20.05	7.62	22.55
F12	25	25	3	100	0.35	17.39	6.02	34
IMA 834-1	14	14	2	100	0.37	13.24	4.88	14.61
IMA 834-2	23	23	2	100	0.37	16.2	5.99	24
IMA 834-Z	22	21	0	95.45	0.30	14	4.15	22.16
IMA 9-Z	21	19	1	90.48	0.33	21.04	7.02	22
IMA 12-1	23	22	1	95.65	0.31	26.04	7.99	23.11
UBC 808-2	25	22	0	88	0.31	26	8.18	28.22
UBC 807	17	15	3	88.23	0.36	17.39	6.18	21.94
UBC 817	20	18	0	90	0.38	23	8.66	22
UBC 818	15	15	2	100	0.33	17.19	5.67	18.22
ISSR 1	23	20	3	86.96	0.30	19.36	5.82	29.61
ISSF 1	16	14	0	87.5	0.28	15	4.19	22.66
Average	21.5	20.25	1.25	94.10	0.34	19.13	6.45	23.46

TNB: Total number of bands, NPB: Number of polymorphic bands, NMB: Number of monomorphic bands, PPB: Percentage of polymorphic bands, EMR: Effective multiple ratio, MI: Marker index, PIC: polymorphic information content, Rp: Resolving power.

Comparing our results to previous studies carried out in other species belonging to *Ericaceae* family, the value of total markers found by Carvalho *et al.* (2018) was lower than the one we obtained in the current study. The 10 ISSR primers used by these authors to analyzed 16 blueberry (*Vaccinium corymbosum*) cultivars and three wild populations of *Vaccinium myrtillus* produced a total of 122 markers among the genotypes. Generally, the high total number of bands found in our research could be explained by the relatively larger number of primers used and by the types of genetic material used.

The results concerning the percentage of polymorphism detected in our study (94.10%) were higher than those reported in Portuguese strawberry tree by Lopes *et al.* (2012) using ISSR (86.7%) and RAPD (83.9%) markers and by Gomes *et al.* (2012) based on RAPD (57.3%) and SSR markers (80%) and by Rodriga de Sà (2010) using RAPD (83.9%) and ISSR (86.7%) markers, and in Tunisian strawberry tree by Takrouni and Boussaid (2010) using RAPD (65%) and Takrouni *et al.* (2012) using isozymes (63.33%). In other reports, the mean value of polymorphism detected by Carvalho *et al*. (2018) in blueberry (*Vaccinium corymbosum*) cultivars was lower (83.20%) than the value obtained in strawberry tree genotypes.

According to Roldan-Ruiz *et al.* (2000), the closer the PIC value to 0.5, the higher the polymorphism index of the primer in question. In our study, the primers F2, F11 and UBC 817 had the highest values of PIC, indicating that these primers are informative and presented a high performance in the genetic exploration of this species. Gomes *et al.* (2012) obtained much higher average PIC value of 0.71 in Portuguese strawberry tree using 11 SSR markers. Comparing our results to previous studies carried out in *Vaccinium* species, the PIC average value (0.30) reported by Carvalho *et al.* (2018) in *Vaccinium corymbosum* cultivars was lower than the value obtained in our study. For *Vaccinium myrtillus* populations, the mean value of PIC obtained by the same researchers was much lower (0.05) than our results. In plum (*Prunus salicina* L) genotypes, Hamdani *et al*. (2022) reported similar average value of PIC (0.34) using 20 ISSR primers.

Primers with high Rp values were generally more effective in distinguishing between genotypes and showed higher number of polymorphic bands (Prevost and Wilkinson, 1999, Debnath, 2007). In our study, the primer F12 showed to be the most effective (Rp=34). Comparing the results obtained in this work to previous studies carried out in *Vaccinium species*, the value of Rp found in strawberry tree was higher than those reported by Debnath, (2007), Debnath, (2009) and Carvalho *et al*. (2018). The Rp values recorded by Debnath, (2007) varied between 2.1 and 9.4 in 43 lingonberry (*Vaccinium vitis-idaea* L.) clones collected from four Canadian provinces using 15 ISSR primers. In another study, Debnath, (2009) reported values of Rp ranged from 1.9 to 8.1 in 43 wild lowbush blueberry (*Vaccinium angustifolium*) clones by using ISSR markers. In Portugal, Carvalho *et al.* (2018) analyzed *Vaccinium corymbosum* and *Vaccinium myrtillus* cultivars using 10 ISSR primers and the Rp values were 11.40 and

14.50, respectively. The results concerning MI (6.45) were lower than those found by Carvalho *et al.* (2018). They found an average value of 24.60 in *Vaccinium corymbosum* cultivars, whereas they reported a mean value much lower (0.50) in *Vaccinium myrtillus* using ISSR markers. The results obtained clearly demonstrate that ISSR markers are a high efficient marker to characterize strawberry tree genotypes, as has been referred in other horticultural species.

Genetic diversity analysis

The results of genetic diversity analysis were presented in Table 4. The number of alleles observed (Na) varied from 1.87 (ISSF1, UBC 807 and UBC 817) to 2 (F12, IMA 834-2, IMA 834-Z, IMA 834-1, F11 and UBC 818) with an average of 1.94. The mean effective number of alleles (Ne) was 1.55, the values ranged from 1.45 for F8 to 1.68 for F12. Moreover, the Shannon information index (I) values ranged from 0.43 for F8 to 0.56 for F12 and F11 with a mean of 0.49. The total gene diversity (Ht) and the gene diversity within populations (Hs) ranged respectively, from 0.28 (F8) to 0.38 (F12, F11) and from 0.11 (F8) to 0.20 (F11), with an average of 0.33 and 0.15. The coefficient of genetic differentiation (Gst) among the populations was 0.50 and the mean value of gene flow (Nm) was 0.75 (Table 4).

Table 4. Genetic diversity analysis of the studied strawberry tree genotypes

ISSR Loci	Simple size	Na	Ne	I	Ht	Hs	Gst	Nm
F12	36	2	1.68	0.56	0.38	0.19	0.51	0.62
ISSF1	36	1.87	1.48	0.46	0.30	0.13	0.54	0.54
IMA 834-2	36	2	1.58	0.52	0.35	0.17	0.53	0.65
IMA 834-Z	36	2	1.51	0.46	0.30	0.17	0.39	1.5
ISSR1	36	1.92	1.52	0.48	0.32	0.15	0.50	0.68
UBC 807	36	1.87	1.55	0.48	0.32	0.13	0.56	0.55
UBC 817	36	1.87	1.48	0.44	0.29	0.14	0.50	0.63
IMA 834-1	36	2	1.56	0.51	0.34	0.18	0.43	0.96
IMA 12-1	36	1.96	1.56	0.49	0.33	0.13	0.56	0.55
F2	36	1.96	1.51	0.47	0.31	0.16	0.42	1.11
F11	36	2	1.66	0.56	0.38	0.20	0.43	1.01
F8	36	1.88	1.45	0.43	0.28	0.11	0.52	0.83
F10	36	1.96	1.53	0.49	0.32	0.15	0.51	0.75
IMA 9Z	36	1.91	1.57	0.49	0.33	0.15	0.51	0.65
UBC 808-2	36	1.88	1.56	0.48	0.32	0.12	0.60	0.49
UBC 818	36	2	1.59	0.53	0.35	0.16	0.53	0.51
Mean		1.94	1.55	0.49	0.33	0.15	0.50	0.75

Na: Observed number of alleles; Ne: Effective number of alleles; I: Shannon's Information index; Ht: Total genetic diversity; Hs: Genetic diversity within group; Gst: Genetic differentiation among group; Nm: Gene flow

The number of alleles observed (Na) with an average of (1.94) was higher than that obtained in Portuguese strawberry tree by Lopes *et al.* (2012) (Na=1.66) using ISSR markers, but lower than that showed by Gomes *et al.* (2012) using SSR markers (Na=11.6) for Portuguese strawberry tree. Our results regarding the Shannon's information index (I=0.49) given by the primers tested was higher than that found in Portuguese strawberry tree by Lopes *et al.* (2012) using ISSR markers. They found an average value of I=0.39 that vary between 0.29 and 0.52.

The high value of (Ht=0.33) suggests the presence of a high level of polymorphism. This value was similar to that reported by Rodriga de Sà, (2010) (Ht=0.33) in the Portuguese strawberry tree using RAPD and ISSR markers and by Kabiri *et al.* (2022) (Ht=0.33) in Moroccan *Juglans regia* using ISSR markers. However, it was higher than that obtained by Lopes *et al*. (2012) (Ht=0.30) in Portuguese strawberry tree using ISSR markers. Indeed, the recorded polymorphism is confirmed by the Shannon index value (0.49). The high genetic diversity obtained in Moroccan strawberry tree was generally 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) (Hamrik *et al*. 1992). The high diversity of Moroccan strawberry tree genotypes could be explained by seed and pollen migration between populations (Mesléard and Lepart, 1991). It could be attributed also to life history traits of this species.

Nevertheless, our results showed low levels of variation within strawberry tree populations (Hs=0.15). The Hs value found in this study was relatively lower than the results recorded by other authors. In Portuguese strawberry tree, Lopes *et al.* (2012) recorded low to moderate levels of variation within populations (Hs=0.23) using ISSR markers. In Tunisia, Takrouni and Boussaid, (2010) reported also, a low level of genetic variation within strawberry tree populations (Hs=0.21) using RAPD markers.

This low level of variation could be the resulted of inbreeding, dictated by the selfing mating system and by the small population size. According to Barrett and Kohn, (1991), small population sizes can cause both genetic drift and inbreeding leading to loss of genetic variation within populations. Moreover, the failure of strawberry tree seeds to germinate, due to specific requirements for seed germination (Mereti *et al*. 2003) would result in the population decline and in the decrease of genetic diversity. In other reports, has been documented that selfers plant populations have, in general, low level of genetic variation (Rossetto *et al.* 1995, Hamrick and Godt, 1996, Godt *et al*. 2001).

The mean value of Gst among the populations was large (Gst=0.505), indicating that 50.5% of total genetic variability was distributed among the populations, while 49.5 % of the total genetic diversity was within populations. Depending on the obtained Gst value, Moroccan strawberry tree genotypes were largely differentiated. Our results were higher than that reported by Lopes *et al.* (2012) (Gst=0.22) and by Rodriga de Sà, (2010) (Gst=0.26) in Portuguese strawberry tree using ISSR markers, and by Ribeiro *et al*. (2017) (Gst=0.29)

using SSR markers for Portuguese strawberry tree. In addition, our results were higher than that found by Takrouni and Boussaid, (2010) (Gst=0.31) in Tunisian strawberry tree using RAPD markers. According to Slatin, (1987), a value of Gst>0.25 is generally regarded as the threshold quantity beyond which significant population differentiation occurs.

The high level of differentiation between populations was in agreement with the restricted gene flow (0.75) that could be due to presence of geographical barriers. According to Wright, (1978), the Nm was divided into three grades: high (≥1), medium (0.25-0.99) and low (0-0.249) (Govindaraju, 1988), and when Nm>1, there was certain gene flow between populations. 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).

Genetic distance

The genetic distance between the different strawberry tree genotypes was calculated based on the 344 bands obtained. The genetic distance varied from 0.113 to 0.520 (Table 5 and 6), with an average of 0.343.

Table 5. Genetic distances between the studied strawberry tree genotypes

	CHF1	CHF2	CHF3	OUZ1	OUZ2	OUZ3	MDZ1	MDZ2	MDZ3	LAN1	LAN2	LAN3	BMR1	BMR2	BMR3	OUL1	OUL2	OUL3
CHF2	0,238																	
CHF3	0,172	0,172																
OUZ1	0,358	0,363	0,326															
OUZ2	0,413	0,465	0,427	0,311														
OUZ3	0,491	0,497	0,488	0,244	0,259													
MDZ1	0,262	0,250	0,235	0,299	0,424	0,387												
MDZ2	0,326	0,372	0,363	0,317	0,331	0,317	0,331											
MDZ3	0,291	0,349	0,340	0,311	0,366	0,346	0,314	0,320										
LAN1	0,381	0,422	0,401	0,390	0,299	0,326	0,358	0,375	0,288									
LAN2	0,410	0,445	0,465	0,390	0,276	0,291	0,410	0,340	0,323	0,297								
LAN3	0,480	0,445	0,424	0,401	0,340	0,326	0,392	0,311	0,294	0,273	0,262							
BMR1	0,456	0,456	0,453	0,378	0,259	0,279	0,398	0,317	0,334	0,279	0,326	0,267						
BMR2	0,480	0,485	0,483	0,384	0,305	0,297	0,410	0,328	0,352	0,308	0,291	0,291	0,233					
BMR3	0,395	0,413	0,410	0,346	0,273	0,305	0,395	0,314	0,355	0,311	0,305	0,299	0,235	0,224				
OUL1	0,439	0,456	0,448	0,378	0,328	0,302	0,416	0,311	0,346	0,314	0,291	0,297	0,291	0,308	0,288			
OUL2	0,305	0,363	0,302	0,302	0,358	0,331	0,323	0,340	0,270	0,302	0,343	0,326	0,326	0,390	0,340	0,291		
OUL3	0,282	0,334	0,273	0,302	0,311	0,355	0,282	0,311	0,276	0,326	0,302	0,297	0,326	0,331	0,305	0,331	0,203	
KHN1	0,291	0,349	0,328	0,311	0,326	0,358	0,314	0,256	0,355	0,352	0,363	0,340	0,369	0,352	0,343	0,346	0,334	0,288
KHN2	0,363	0,416	0,390	0,302	0,311	0,279	0,352	0,265	0,305	0,308	0,326	0,308	0,250	0,291	0,241	0,285	0,291	0,291
KHN3	0,285	0,291	0,265	0,311	0,355	0,416	0,256	0,395	0,326	0,375	0,387	0,404	0,398	0,439	0,366	0,433	0,334	0,282
TAH1	0,288	0,363	0,326	0,308	0,299	0,349	0,317	0,323	0,282	0,314	0,302	0,291	0,314	0,349	0,270	0,297	0,291	0,262
TAH2	0,366	0,395	0,404	0,328	0,302	0,340	0,372	0,326	0,384	0,346	0,305	0,328	0,323	0,311	0,331	0,294	0,328	0,305
TAH3	0,302	0,314	0,328	0,346	0,343	0,387	0,337	0,343	0,349	0,363	0,375	0,387	0,358	0,416	0,343	0,363	0,299	0,294
OUA1	0,323	0,363	0,355	0,343	0,358	0,384	0,340	0,334	0,323	0,331	0,343	0,349	0,355	0,401	0,346	0,337	0,302	0,308
OUA2	0,241	0,270	0,244	0,326	0,427	0,500	0,294	0,410	0,346	0,395	0,436	0,442	0,471	0,512	0,398	0,465	0,331	0,308
OUA3	0,253	0,317	0,256	0,273	0,404	0,436	0,270	0,375	0,317	0,395	0,407	0,401	0,442	0,471	0,369	0,424	0,308	0,291
BNO1	0,334	0,398	0,384	0,291	0,334	0,360	0,381	0,288	0,282	0,372	0,349	0,343	0,355	0,349	0,340	0,360	0,320	0,279
BNO2	0,355	0,401	0,358	0,311	0,331	0,363	0,372	0,302	0,308	0,369	0,334	0,317	0,346	0,340	0,343	0,352	0,323	0,282
BNO3	0,384	0,372	0,369	0,363	0,378	0,410	0,366	0,366	0,349	0,404	0,398	0,433	0,410	0,416	0,355	0,427	0,358	0,334
KSB1	0,267	0,331	0,294	0,340	0,384	0,427	0,302	0,326	0,256	0,369	0,358	0,346	0,346	0,369	0,314	0,346	0,270	0,247
KSB2	0,244	0,314	0,265	0,363	0,430	0,509	0,326	0,436	0,326	0,387	0,445	0,474	0,485	0,520	0,448	0,485	0,334	0,328
KSB3	0,279	0,337	0,288	0,363	0,372	0,433	0,302	0,349	0,326	0,381	0,363	0,369	0,381	0,392	0,337	0,410	0,323	0,282
TAM1	0,390	0,349	0,358	0,381	0,366	0,392	0,401	0,384	0,390	0,392	0,369	0,398	0,369	0,410	0,297	0,381	0,340	0,340
TAM2	0,430	0,413	0,416	0,427	0,413	0,427	0,442	0,360	0,459	0,468	0,387	0,445	0,410	0,416	0,366	0,422	0,375	0,369
TAM3	0,459	0,477	0,468	0,381	0,273	0,299	0,430	0,320	0,384	0,334	0,311	0,299	0,253	0,276	0,227	0,276	0,358	0,334

The genotypes were dissimilar with a number of markers ranged from 39 to 176 using a pairwise comparisons (Figure 2). The lowest genetic distance (0.113) was found between the genotypes BNO2 and BNO1 with only 39 dissimilar markers differentiated both genotypes. The genetic similarity between the genotypes can be explained by the geographic proximity as well as the common

ancestor. However, the highest genetic distance (0.520) was recorded between the genotypes KSB2 and BMR2, indicating that these genotypes are genetically distinct with 176 dissimilar markers.

The variation of genetic distances (0.113-0.520) was similar to the results of Hamdani *et al.* (2022), who reported values of genetic distances ranged between 0.136 and 0.619 with a mean of 0.365 in plum (*Prunus salicina* L) genotypes using 20 ISSR primers.

Table 6. Genetic distances between the studied strawberry tree genotypes (suite)

	KHN1	KHN2	KHN3	TAH1	TAH2	TAH3	OUA1	OUA2	OUA3	BNO1	BNO2	BNO3	KSB1	KSB2	KSB3	TAM1	TAM2
KHN2	0,299																
KHN3	0,291	0,328															
TAH1	0,247	0,250	0,235														
TAH2	0,233	0,259	0,337	0,265													
TAH3	0,291	0,328	0,331	0,288	0,273												
OUA1	0,294	0,273	0,305	0,279	0,323	0,294											
OUA2	0,328	0,401	0,235	0,302	0,422	0,299	0,302										
OUA3	0,334	0,366	0,259	0,320	0,369	0,305	0,302	0,192									
BNO1	0,270	0,291	0,392	0,285	0,276	0,299	0,320	0,366	0,285								
BNO2	0,291	0,282	0,390	0,270	0,291	0,320	0,311	0,392	0,334	0,113							
BNO3	0,372	0,346	0,355	0,369	0,355	0,366	0,381	0,381	0,311	0,212	0,233						
KSB1	0,314	0,311	0,285	0,259	0,331	0,279	0,259	0,294	0,253	0,288	0,302	0,337					
KSB2	0,390	0,427	0,308	0,328	0,442	0,314	0,317	0,230	0,282	0,375	0,366	0,424	0,273				
KSB3	0,326	0,358	0,279	0,270	0,360	0,326	0,299	0,288	0,276	0,334	0,308	0,349	0,180	0,244			
TAM1	0,401	0,352	0,326	0,358	0,407	0,372	0,375	0,352	0,317	0,410	0,395	0,279	0,355	0,442	0,285		
TAM2	0,436	0,392	0,413	0,392	0,407	0,395	0,456	0,433	0,392	0,439	0,430	0,320	0,430	0,488	0,360	0,157	
TAM3	0,360	0,270	0,436	0,305	0,326	0,390	0,346	0,480	0,462	0,358	0,320	0,424	0,390	0,506	0,378	0,326	0,349

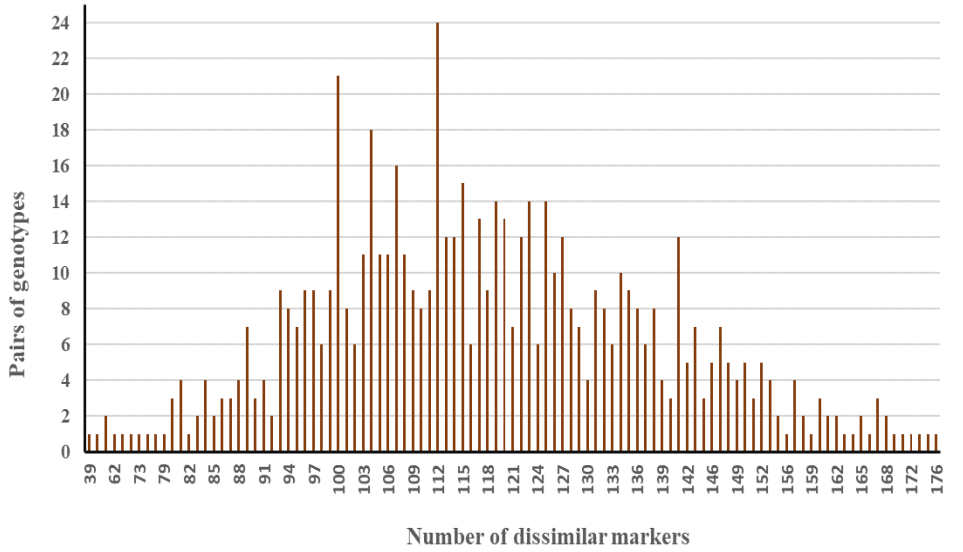

Figure 2. Frequency distribution of genetic dissimilarity for all pairwise combinations among the studied Moroccan strawberry tree genotypes

Mantel test performed in the Portuguese strawberry tree genotypes by Ribeiro et al. (2017) showed no correlation between genetic and geographic distances. Similarly, no correlation between geographic and genetic distances was

found by Gomes *et al*. (2012) on Portuguese strawberry tree (r=0.01, p<0.57). However, Rodriga de Sà, (2010), showed that there was a positive correlation between geographical and genetic distances (r=0.53, p<0.001) in the Portuguese strawberry tree genotypes.

Cluster Analysis

A dendrogram, based on the genetic distance matrix, was created using the UPGMA (Unweighted Pair Group Method using Arithmetic Averages) cluster analysis (Figure 3). Cluster analysis data revealed three main groups, with two independent branches (MDZ2, OUA1). The first group divided into twenty genotypes (the largest group) subdivided into four main subgroups. The first subgroup was constituted by eleven genotypes belonging to the different altitudes and geographical zones. This subgroup contained the genotypes (CHF1, CHF3 and CHF2) from the Western Rif (very low altitude), the genotypes (MDZ1, KHN3, OUA2, OUA3, KSB2, KSB1 and KSB3) from the Middle Atlas (low and moderate altitudes) and the genotype (TAH1) from the High Atlas (moderate altitude). The second subgroup was mainly formed by the genotype (MDZ3) from the Middle Atlas and the genotypes (OUL2 and OUL3) from the Central Plateau. This group characterized by low altitudes. The third minor subgroup contained the genotype (KHN1) from the Middle Atlas and the genotypes (TAH2 and TAH3) from the High Atlas. This group presented moderate altitudes. The last subgroup included only the genotypes (BNO1, BNO2 and BNO3) from the Middle Atlas with moderate altitude. The second group included twelve genotypes subdivided into three main subgroups. The first subgroup contained only two genotypes (OUZ1 and OUZ3) from the North West (very low altitude). The second subgroup included the genotype (OUZ2) from the North West (very low altitude), the genotypes (BMR1, BMR2 and BMR3) from the Rif- Middle Atlas (low altitude), the genotype (OUL1) from the Central Plateau (low altitude) and the genotypes (TAM3 and KHN2) from the Middle Atlas (moderate altitudes). The last subgroup contained only the genotypes (LAN1, LAN2 and LAN3) from the Middle Atlas (moderate altitude). The third group included only two genotypes (TAM1 and TAM2) from the Middle Atlas (moderate altitude) (Table 7).

Both morphological and molecular dendrogram clustered the genotypes into three main groups but the genotypes did not grouped in the same cluster. In molecular dendrogram, Bin El Ouidane and Laanoucer genotypes formed distinct subgroups. However, in morphological dendrogram, these genotypes did not formed separate groups. Generally, the molecular data (ISSR loci) dit not showed positive correlation with of the morphological traits (data not shown). There are several studies on different fruit trees such as plums (Shimada *et al*., 1999) and grapevine (Vidal *et al*., 1999) reported correlation between molecular data and morphological traits. However, there is no need to be a necessarily positive correlation between morphological and molecular markers (Zhang *et al.,* 2010).

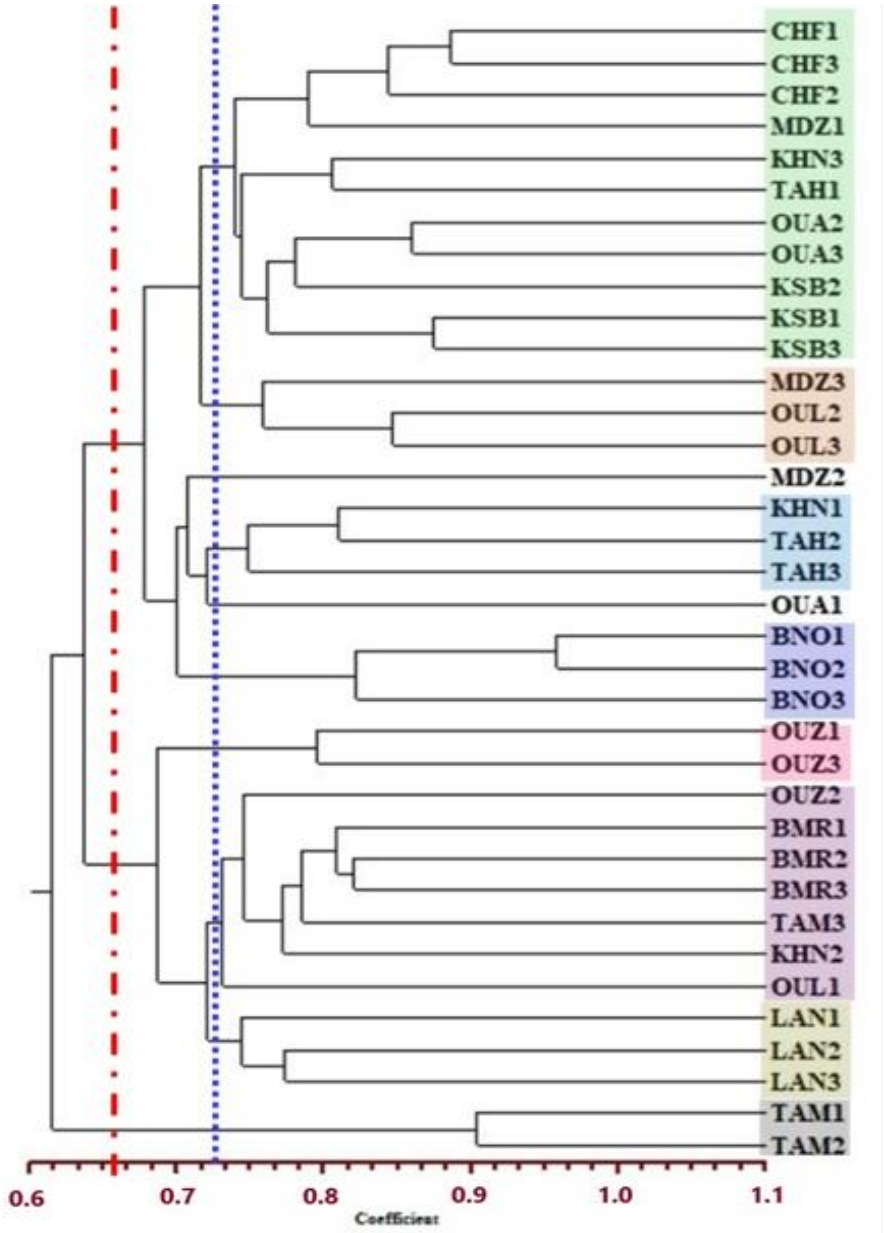

Figure 3. Dendrogram of the studied Moroccan strawberry tree genotypes generated by ISSR using UPGMA cluster analysis

Table 7. Genotypes clustering obtained from ISSR markers using UPGMA method

Groups	Subgroups	Genotypes	Total number of genotypes
Group I	Subgroup 1	CHF1, CHF3, CHF2, MDZ1, KHN3, TAH1, OUA2, OUA3, KSB2, KSB1 and KSB3.	11
	Subgroup 2	MDZ3, OUL2 and OUL3.	3
	Subgroup 3	KHN1, TAH2 and TAH3.	3
	Subgroup 4	BNO1, BNO2 and BNO3.	3
Group II	Subgroup 1	OUZ1 and OUZ3.	2
	Subgroup 2	OUZ2, BMR1, BMR2, BMR3, TAM3, KHN2 and OUL1.	7
	Subgroup 3	LAN1, LAN2 and LAN3.	3
Group III		TAM1 and TAM2	2
Branches		MDZ2 and OUA1.	2

Nevertheless, the genetic relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflect what may be observed with respect to morphological traits (Métais *et al*., 2000).

The dendrogram obtained based on UPGMA analysis with ISSR data, did not cluster together the genotypes according to their population affinity. The finding revealed that the grouping of strawberry tree genotypes was independent of their geographic origin with exception for the genotypes belonging to Bin El Ouidane and Laanoucer populations. These genotypes formed distinct subgroups. The results showed also that the genotypes KHN1, KHN2 and KHN3 appeared dispersed throughout the groups I and II.

Lopes *et al*. (2012) clustered forty-six Portuguese strawberry tree genotypes in two main groups, based on ISSR markers. They found that the genotypes from the same population did not cluster together, with the exception for some genotypes. In another study, Ribeiro *et al.* (2017) identifed four groups, based on SSR markers, in Portuguese strawberry tree genotypes belonging to different ecological conditions. Twenty seven strawberry tree genotypes were screened by Gomes *et al.* (2012) with 20 RAPD and 11 SSR markers. They reported that clustering of Portuguese strawberry tree was not correlated to their geographical origin. Also, Takrouni and Boussaid, (2010) assessed the genetic diversity of nine Tunisian strawberry tree populations using RAPD markers. They showed the lack of correlation between population groupings and geographic origin. However, Rodriga de Sà, (2010), showed that the clustering of Portuguese strawberry tree populations resulting from the UPGMA dendrogram is in agreement with their distant geographical positions. The grouping of genotypes

belonging to several geographic origin could be attributed to environmental similarities (temperature and rainfall) (Lopes *et al*. 2012).

In general, the differentiation among strawberry tree 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 (Debussche and Isenmann, 1989, Aparicio *et al*. 2008). 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 Moroccan strawberry tree populations was not related to geographic distance (Hamrick and Godt, 1990).

CONCLUSIONS

The finding of this study revealed that ISSR markers are highly informative and effective in the detection of polymorphism in strawberry tree genotypes. These markers are suitable tools for the evaluation of genetic diversity among strawberry tree genotypes. The cluster analysis revealed three main groups and two independent branches. The grouping of genotypes was not related with their altitude and geographical origins with exception for the genotypes belonging to Bin El-ouidane and Laanoucer populations. The present work provides important data that could be exploited for future conservation of strawberry tree and various breeding programs. The results obtained with ISSR markers will be confronted with the morphological and biochemical evaluation of the genotypes investigated in the present work.

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