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Original scientific article

SCOT MOLECULAR MARKERS AND POPULATION DIFFERENTIATION IN *Hedera helix* L.

Haiying LI[1,*], Yingxue WANG[1], Rabia IQBAL [2]

[1]North China University of Water Resources and Electric Power, Zhengzhou, Henan, P.R.China
[2]Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan

Li H., Y. Wang, R, Iqbal (2021). *SCoT molecular markers and population differentiation in Hedera helix L.*. - Genetika, Vol 53, No.2, 739-756.
Hedera helix L. is a specie that is used for its ornamental and medicinal properties widely. In spite of its very good biochemical characterization, the knowledge about the DNA variability is very limited and no DNA markers were used to analyses the genomic variability of the populations, up to date. In the present study, genetic diversity of 56 *Hedera helix*, individuals nine populations were studied using 10 Start Codon Targeted (SCoT) markers. High polymorphic bands (95.78%), polymorphic information content (0.25) and allele number (1.34) showed SCoT as a reliable marker system for genetic analysis in *Hedera helix*. At the species, the percentage of polymorphic loci [*P*] was 66.20%, Nei's gene diversity [*H*] was 0.159, Shannon index [*I*] was 0.148 and unbiased gene diversity [*UHe*] was 0.56. Genetic variation within populations (70%) was higher than among populations (30%) based on analysis of molecular variance (AMOVA). We used SCoT molecular marker for our genetic investigation with the following aims: 1— Investigate genetic diversity both among and with date *Hedera helix*, 2—Identify genetic groups within these nine populations ivy, and 3—produce data on the genetic structure of date ivy populations. The results obtained revealed a high within-population genetic variability.
Keyword: Genetic admixture, Gene flow, Genetic structure, SCoT, *Hedera helix* L.

INTRODUCTION

Genetic variability description specifies differencies among individuals or populations of the same species and serves as a very good tool for plant breeding and conservation programmes (MINN *et al*., 2015). Different types of DNA markers have been applied in evaluation of genetic diversity of different plants, considering also the effects of the plant growing environment and developmental stage (NADEEM *et al*., 2018; CHEN *et al.,* 2021; DONG *et al.,* 2021; HAN *et al*., 2021).

Corresponding author:
Haiying Li, North China University of Water Resources and Electric Power, No.136 Jinshui east road, Zhengzhou, Henan, P.R.China. E-mail: lhy9870654@126.com

The genus *Hedera* L. occupies forest understories and riparian vegetation in temperate latitudes throughout Europe, N Africa and Asia (MEUSEL *et al*., 1965; MABBERLEY, 1997; HOU *et al*., 2021; JI *et al*., 2021). The taxonomical treatments of *Hedera* published through the second half of the 20th century recognized between three and 19 species (SEEMANN, 1868; TOBLER, 1912; LAWRENCE and SCHULZE, 1942; POJARKOVA, 1951; JI *et al*., 2020a, 2020b). Previous molecular and cytogenetic studies identified two main centers of diversity for *Hedera* (Araliaceae), the eastern and western parts of the Mediterranean region.

Since McAllister and Rutherford's treatment, the identification and delimitation of *Hedera* species have mainly been based on a combination of trichome morphology (stellate-multiangulate, stellate-rotate, and scalelike hairs; SEEMANN, 1868; LUM and MAZE, 1989; MCALLISTER and RUTHERFORD, 1990; ACKERFIELD and WEN, 2002; VALCÁRCEL and VARGAS, 2010), juvenile leaf morphology (from entire to 3–7 lobate; RUTHERFORD *et al*., 1993; ACKERFIELD and WEN, 2002; VALCÁRCEL, 2008; VALCÁRCEL and VARGAS, 2010), and ploidy level (from 29 to 89; VARGAS *et al*., 1999; JIANG *et al*., 2021; SUN *et al.,* 2021).

Common ivy (*Hedera helix* L.) is one of the plant species documented with only a very limited information about its genomic variability assessed by DNA markers. Up to date, only a few data are available for markers based on the internal transcribed spacers (ITS), randomly amplified polymorphic DNA (RAPD) and polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP). Specific variable sites and polymorphism generated by ITS in ivy were determined by VARGAS *et al*. (1999) with regard to A/C substitution. Chloroplast, microsatellites and *trnK* PCR-RFLP analyses combined with haplotype sequencing define a total of 13 groups with natural occurence in ivy (GRIVET and PETITE, 2002; ZHANG *et al*., 2020b). Haplotype analysis for characterization of the *Hedera* genus was later used also by some other authors (ACKERFIELD and WEN, 2003; VALCÁRCEL *et al*., 2003; GREEN *et al*., 2011, 2013; SHEN *et al*., 2020). Invasive populations of *Hedera* were mapped in Pacific Northwest native forests with using RAPD markers (CLARKE *et al*., 2006; SHEN *et al*., 2021; WANG *et al.,* 2021). PBA markers were applied in analysis of European population of common ivy by BOŠEĽOVÁ and ŽIAROVSKÁ (2016). A poor knowledge of ivy's genome could be changed, having in mind the potential of DNA markers (KUMAR *et al*., 2014; ZHANG *et al*., 2016). *Hedera helix* is a multipurpose plant with possible many applications in future. Nowadays, about 500 different cultures of ivy are cultivated. Ivy is popular as a decorative plant and has many available cultivars including non-climbing cultivars used to cover the soil and to fix compact forms in plots. Owing to the evergreen and shade-loving qualities, the ivy is ideal for conservatories and can create attractive packaging for garden structures. Besides of the ornamental applications, ivy is a very promising plant for medicinal use (LUTSENKO *et al*., 2010; HOOSHYAR *et al*., 2014), for plant protection (PÂRVU *et al*., 2015; ZHANG *et al*., 2020a; ZHANG *et al*., 2021a, b, c) and technological use (nanoparticles) in the future (LENANGHAN *et al*., 2013). Starting a breeding and selection of ivy, a variability and genomic characterization of natural variability of its population is a strategic knowledge as this species has been recognised as an example of a genomic plasticity occurring during the typical developmental changes from the juvenile to the adult phase (OBERMAYER, 2000).

In recent years, a novel marker system termed start codon targeted (SCoT) markers was developed by COLLARD and MACKILL (2009) based on the short-conserved region flanking the

start codon (ATG) in plant genes. SCoT employs long primers (18-mers), and can generate polymorphisms that are reproducible. It is considered as a dominant marker system, requiring no prior sequence information, and the polymorphism is correlated to functional genes and their corresponding traits. Other excellent characteristics include their simplicity of use, high polymorphism, the use of universal primers, low cost and gene targeted markers. This technique has been successfully used to assess genetic diversity and structure (COLLARD and MACKILL, 2009; WU *et al*., 2013; LUO *et al*., 2011), construct DNA fingerprints, identify QTLs, and analyze differential gene expression and screen stress tolerance genes (ELSHIBLI and KORPELAINEN, 2008; RHOUMA *et al*., 2009).

The present study is the first attempt to use SCoT markers to assess the level of genetic diversity of *Hedera helix* which were collected from the wild populations. The main objectives of this study were to assess the genetic diversity and genetic relationship of *Hedera helix* in Iran. These results could benefit *Hedera helix* germplasm collection, conservation and future breeding.

MATERIALS AND METHODS

Plant materials

A total of 56 individuals were sampled representing nine natural populations of *Hedera helix* in Tehran; Mazandaran; Kermanshah and Ardabil Provinces of Iran during July-Agust 2018 (Table 1). For morphometric and ISSR analysis we used 56 plant accessions (four to eleven samples from each populations) belonging to nine different populations with different eco-geographic characteristics were sampled and stored in -20 till further use. More information about geographical distribution of accessions are in Table 1 and Fig. 1. Different references were used for the correct identification of species (*Hedera helix*) (SEEMANN, 1868; TOBLER, 1912; LAWRENCE and SCHULZE, 1942; POJARKOVA, 1951). Vouchers were deposited at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH).

Table 1. Populations studied, their locality and ecological features

Pop No.	Locality	No. of collected accessions	Voucher No.	Elevation (m)	Longitude	Latitude	Mean maximum temperature (C)	Mean minimum temperature (C)	Annual rainfall (mm)	Number of frost days
1	Tehran; Darband	11	IAUH-322	2398	354636.4	515869.6	40.12	-18.12	325	77
2	Mazandaran; Chalous	9	IAUH-546	2299	355514	521172.8	35.55	-20.34	378	75
3	Mazandaran, Kandovan	6	IAUH - 549	2597	368309	511855	41.34	-10.34	377	96
4	Tehran; Shemshak-Dizin road	6	IAUH - 876	3245	362229.8	512628.2	39.14	-17.55	390	73
5	Kermanshah; Islamabad	8	IAUH - 123	2308	355775.2	512954.5	36.88	-11.23	320	76
6	Kermanshah Paveh	4	IAUH - 980	2978	354349	521384.8	32.55	-22.45	334	88
7	Kermanshah; Bijar	5	IAUH - 765	2225	363107	5456	30.44	-18.66	229	120
8	Ardabil; Hur	6	IAUH-522	1493	381209.9	483909.2	32.88	-11.66	210	114
9	Ardabil; Sheikh Ahmad village	4	IAUH-526	1389	381235	481757.3	20.44	-25.66	478	220

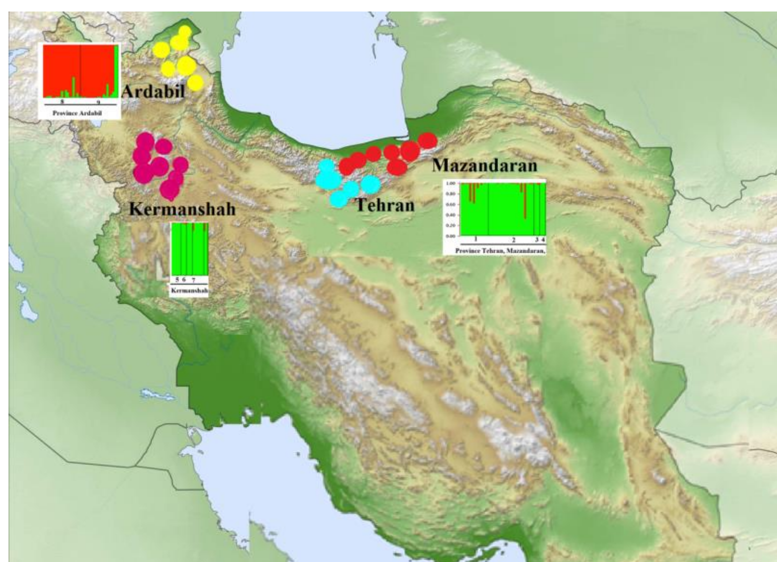

Fig. 1. Distribution map of the studied populations

Environmental variables

In this experiment, the data regarding climate variables included elevation, and geographic data (latitude and longitude), and this data was determined at each site using an electronic GPS. The climate variable data of mean annual temperature, mean maximum temperature (°C), mean minimum temperature (°C), annual rainfall (mm), number of frost days were downloaded from http://www.worldclim.org. (Table 1).

DNA extraction and SCoT-PCR amplification

Fresh leaves were used randomly from four to eleven plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (ESFANDANI-BOZCHALOYI *et al*., 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 SCoT primers developed by COLLARD and MACKILL (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen (Table 2). PCR reactions were carried in a 25μl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 μM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Morphological studies

In total 28 morphological (28 quantitative) characters were studied. Four to twelve samples from each population were randomly studied for morphological analyses (Appendix 1). Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (PODANI, 2000). For grouping of the plant specimens, The UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characters) as well as ordination methods of MDS (Multidimensional scaling) were used (PODANI, 2000). PAST version 2.17 (HAMMER *et al.*, 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses

Excel 2013 was used to calculate the total number of bands (TNB), the number of polymorphic bands (NPB), and the percentage of polymorphic bands (PPB). The polymorphism information content (PIC) of SCoT primers was determined using POWERMARKER v3.25 [53]. Binary characters (presence = 1, absence = 0) were used to encode SCoT bands and used for further analyses. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (WEISING *et al*, 2005; FREELAND *et al.* 2011).

Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (PEAKALL and SMOUSE, 2006)

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (FREELAND *et al*., 2011; HUSON and BRYANT, 2006). Mantel test checked the correlation between geographical and genetic distances of the studied populations (PODANI, 2000). These analyses were done by PAST ver. 2.17 (HAMMER *et al.*, 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006), and Nei˙s Gst analysis as implemented in GenoDive ver.2 (2013) (MEIRMANS and VAN TIENDEREN, 2004) were used to show genetic difference of the populations. Moreover, populations˙ genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (HEDRICK, 2005), and D_est = Jost measure of differentiation (JOST, 2008).

To assess the population structure of the *Hedera helix* accessions, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (PRITCHARD *et al*., 2000; FALUSH *et al*., 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every

individual (PRITCHARD *et al*., 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following EVANNO *et al.* (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (MEIRMANS, 2012).

Gene flow (Nm) which were calculated using POPGENE (version 1.31) program (YEH *et al*., 1999). Gene flow was estimated indirectly using the formula: Nm = 0.25(1 - FST)/FST. In order to test for a correlation between pair-wise genetic distances (FST) and geographical distances (in km) between populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; MILLER, 1997) (computing 999 permutations). This approach considers equal amount of gene flow among all populations.

RESULTS

SCoT polymorphisms

Twenty-five SCoT primers were tested with four *Hedera helix* accessions as DNA templates; all primers produced amplification products, and only primers showing clear and reproducible band patterns were selected for further analysis. Ten primers were then chosen for species identification and phylogenetic analysis. As shown in Table 2, all 10 primers used for SCoT analysis. A total of 135 fragments were obtained, and 130 of the fragments were polymorphic. The number of polymorphic fragments for each SCoT primer ranged from 8 (ST3) to 20 (ST14), with an average of 13. The percentage of polymorphic fragments was from 84.99% to 100.00%, with an average of 95.78% polymorphism. Polymorphism information content (PIC) values were 0.23 to 0.29, with an average of 0.25. The number of different alleles was 1.45 at the species (Table 3). These results indicated that a high level of polymorphism could be detected among *Hedera helix* accessions using SCoT markers.

Table 2. SCoT primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC
SCoT-1	CAACAATGGCTACCACCA	10	10	100.00%	0.25
SCoT-3	CAACAATGGCTACCACCG	9	8	84.99%	0.23
SCoT-6	CAACAATGGCTACCACGC	13	13	100.00%	0.24
SCoT-11	AAGCAATGGCTACCACCA	17	16	93.33%	0.27
SCoT-14	ACGACATGGCGACCACGC	20	20	100.00%	0.25
SCoT-15	ACGACATGGCGACCGCGA	18	17	94.74%	0.27
SCoT-16	CCATGGCTACCACCGGCC	13	12	92.31%	0.24
SCoT-17	CATGGCTACCACCGGCCC	11	11	100.00%	0.27
SCoT-18	ACCATGGCTACCACCGCG	9	9	88.89%	0.23
SCoT-19	GCAACAATGGCTACCACC	15	15	100.00%	0.29
Mean		14	13	95.78%	0.25
Total		135	130		

TNP: total number of bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content

Table 3. Genetic diversity parameters in the studied populations Hedera helix

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	11	1.320	1.191	0.310	0.210	0.139	82.02
Pop2	9	1.454	1.227	0.290	0.185	0.163	70.20
Pop3	6	1.599	1.193	0.298	0.190	0.145	75.39
Pop4	6	1.361	1.197	0.248	0.144	0.149	65.04
Pop5	8	0.917	1.192	0.195	0.128	0.130	42.36
Pop6	4	1.464	1.227	0.267	0.152	0.163	67.20
Pop7	5	1.588	1.193	0.298	0.191	0.145	75.38
Pop8	6	1.361	1.197	0.248	0.147	0.149	60.04
Pop9	4	0.919	1.162	0.188	0.111	0.129	40.36
Mean		1.387	1.183	0.255	0.159	0.148	66.20

N = number of samples, Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (p^2 + q^2)
I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q)), He = Expected Heterozygosity = 2 * p * q
UHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He, P%= percentage of polymorphism, populations
Where for Diploid Binary data and assuming Hardy-Weinberg Equilibrium, q = (1 - Band Freq.)^0.5 and p = 1 - q.

Populations, genetic diversity

Genetic diversity parameters determined in nine geographical populations of *Hedera helix* are presented in Table 3. The percentage of polymorphic loci (*P*) and Nei's gene diversity (*H*) were important parameters for measuring the level of genetic diversity. In Table 3, the genetic diversity parameters of the nine populations are shown. The highest value of percentage polymorphism (82.02%) was observed in Tehran, Darband (population No.1) which shows high value for gene diversity (0.210) and Shanon, information index (0.310). Population Ardabil; Sheikh Ahmad village (No.9) has the lowest value for percentage of polymorphism (40.36%) and the lowest value for Shanon, information index (0.188), and He (0.111).

Population genetic differentiation

AMOVA (PhiPT = 0.69, P = 0.010), revealed significant difference among the studied populations (Table 4). It also revealed that, 70% of total genetic variability was due to within population diversity and 30% was due to among population genetic differentiation.

Moreover, pair-wise AMOVA revealed significant genetic difference almost among all the studied populations. These results indicate that *Hedera helix* population is genetically differentiated and we can use such genetic difference in future breeding programs of this valuable plant species. The high degree of within population genetic variability is possibly due to flowers attract a range of insects seeking nectar and pollensuch as Aculeata (bees and wasps), Diptera (true flies), and Lepidoptera.

The pairwise comparisons of 'Nei genetic identity' among the studied populations *Hedera helix* (Table 5) have shown a higher a genetic similarity (0.933) between populations Tehran; Darband (pop. No 1) and Tehran; Shemshak-Dizin road (pop. No 4), while the lowest genetic similarity value (0.621) occurs between Tehran; Darband (pop. No.1) and Ardabil; Sheikh Ahmad village (pop. No. 9).

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	12	367.576	29.327	4.082	30%	30%
Within Pops	45	487.767	9.530	8.230	70%	
Total	57	888.342		12.513	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; *Φ*PT: proportion of the total genetic variance among individuals within an accession, ($P < 0.001$).

Stat	Value	P(rand >= data)
PhiPT	0.690	0.010

Probability, P(rand>=data), for PhiPT is based on permutation across the full data set.

PhiPT = AP / (WP + AP) = AP / TOT

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Table 5. Pairwise Population Matrix of Nei Unbiased Genetic Identity

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	
1.000									pop1
0.725	1.000								pop2
0.817	0.817	1.000							pop3
0.933	0.756	0.881	1.000						pop4
0.825	0.817	0.768	0.752	1.000					pop5
0.783	0.776	0.776	0.715	0.786	1.000				pop6
0.733	0.802	0.785	0.736	0.781	0.772	1.000			pop7
0.789	0.708	0.720	0.694	0.739	0.755	0.831	1.000		pop8
0.621	0.792	0.798	0.691	0.822	0.750	0.826	0.787	1.000	pop9

Populations' genetic affinity

NJ tree and Neighbor-Net network produced similar results therefore only NJ tree is presented and discussed (Figure. 2). The samples in populations 8&9 are to a great extent differentiated from the other populations. Most of the samples within each population also are placed close to each other, but some of them (pop. No 1-7) are dispersed among the other populations due to high within population genetic variability. This result show that molecular characters studied can delimit *Hedera helix* population in two different major clusters or groups. In general, two major clusters were formed in NJ tree (Fig. 2), ten accessions of populations Ardabil, Hur and Ardabil, Sheikh Ahmad village (pop. No 8-9) formed a single cluster, and these accessions were all from Ardabil province. The individuals of other populations were scattered among different clusters. All individuals were grouped into two main categories, as A and B. Most accessions from Tehran, Darband; Mazandaran, Chalous; Mazandaran, Kandovan; Tehran, Shemshak-Dizin road; Kermanshah, Islamabad; Kermanshah, Paveh and Kermanshah, Bijar (pop. No 1-7) merged together and formed cluster A. It contained 2 sub-clusters, including a total of 46 individuals. Cluster B contained two sub-clusters, and most of individuals were

primarily collected from Ardabil province. There were 10 individuals in this cluster. This result was similar to the result of the STRUCTURE analysis at $K = 2$.

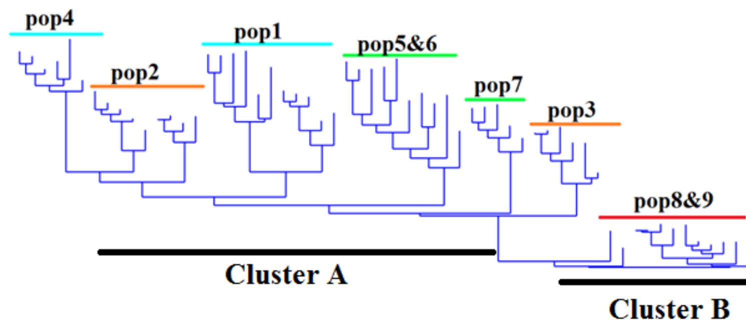

Fig. 2. NJ tree of populations in *Hedera helix* based on SCoT molecular markers, (Population numbers are according to Table 1.)

The principal coordinate analysis (PCoA) (Fig 3). for 9 populations of *Hedera helix* revealed that the populations 1 and 4, as well as populations 2 and 3, are separated from the other populations and also show closer genetic affinity. Similarly, the populations 8-9 are closer to each other. The results of PCoA were the same from the other cluster analyses as shown above.

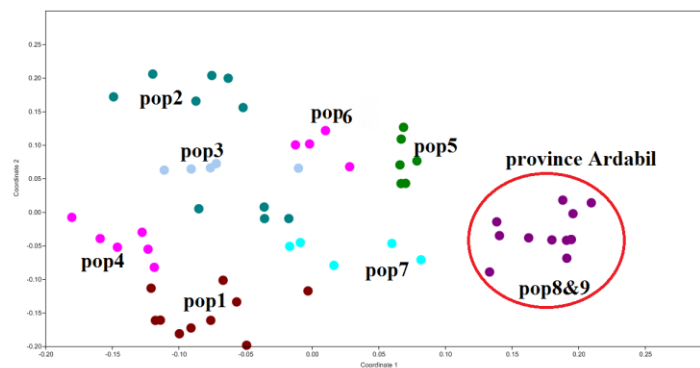

Fig. 3. PCoA plot of populations in *Hedera helix* based on SCoT molecular markers, (Population numbers are according to Table 1.)

This is in agreement with AMOVA and genetic diversity parameters presented before. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.22, P = 0.001). Therefore, the populations

that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Hedera helix*

Populations genetic structure

The number of genetic groups was determined by two methods of 1—K-Means clustering which is based on the maximum likelihood approach, and 2—Evanno test which is based on STRUCTURE analysis and is a Bayesian approach based method. K-Means clustering (Table 6), based on pseudo-F and BIC (Bayesian Information Criterion) recognized 2 and 5 genetic groups, respectively. This is in agreement with AMOVA result, showing significant genetic difference among date populations of ivy.

Table 6. K-Means clustering result. (Best clustering according to Calinski and Harabasz' pseudo-F: k = 2. Best clustering according to Bayesian Information Criterion: k = 5).*

k	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	AIC	BIC	Rho
1	1117.354	0	0	0	0	216.37	581.088	0
2*	1117.354	109.9	1013	0.114	10.676	209.45	577.617	0.210
3	1117.354	210.5	908.9	0.158	10.345	203.56	571.873	0.223
4	1117.354	292.3	827.1	0.261	9.189	198.98	578.493	0.335
5&	1117.354	367.5	751.9	0.328	9.409	192.49	544.084	0.388
6	1117.354	448.8	680.5	0.392	9.701	186.65	552.895	0.4
7	1117.354	498.3	621	0.445	9.634	181.54	550.22	0.426
8	1117.354	545.8	573.5	0.488	9.447	177.47	556.102	0.480
9	1117.354	571	538.4	0.519	9.846	174.81	555.325	0.492

* Best clustering according to Calinski & Harabasz' pseudo-F: k = 2
& Best clustering according to Bayesian Information Criterion: k = 5

Evan test based on delta k (Fig. 4) identified the optimum number of genetic groups 2. We performed STRUCTURE analysis based on k = 2, to identify the genetic groups (Fig. 5). In the plot of k = 2, the populations Tehran, Darband; Mazandaran, Chalous; Mazandaran, Kandovan; Tehran, Shemshak-Dizin road; Kermanshah, Islamabad and Kermanshah, Paveh (pop. No 1-7) (green colored) are placed in the first genetic group, while the other populations of ivy formed the second genetic group. These different genetic groups may be used in future breeding and hybridization programs of Iranian date ivy.

The mean Nm = 1.2 was obtained for all ISSR loci, which indicates high amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. This result is in accordance with grouping we obtained with PCoA plot, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Hedera helix* populations.

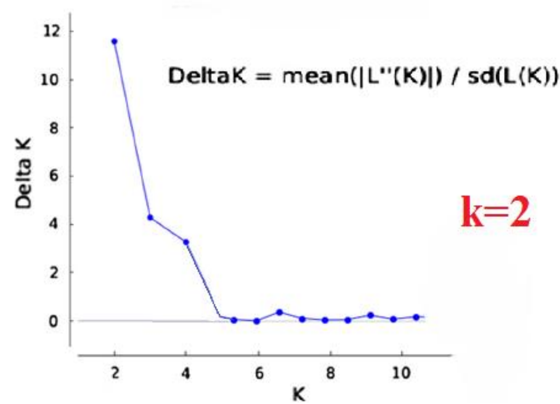

Fig. 4. Delta k plot of Evanno's test based on STRUCTURE analysis.

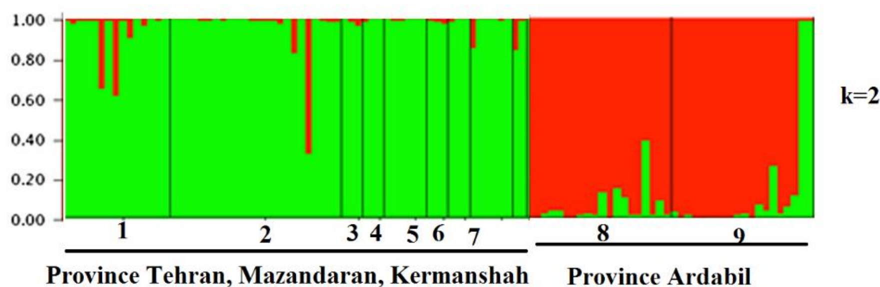

Fig. 5. STRUCTURE plot of *Hedera helix* populations based on k = 2, Numbers are according to Table 1.

Morphometric analyses

In present study we used 56 plant accessions (four to eleven samples from each populations) belonging to nine different populations. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 87% of the total variation. In the first PCA axis with 60% of total variation, such characters as shape of leaf, shape of sepals, peduncles and pedicels hair, stem hair, petioles hair, have shown the highest correlation (> 0.7), length of bract and pedicel, length and width of the petal, length and width of stem leaves, width of mericarp were characters influencing PCA axis 2 and 3, respectively.

Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Figs 6). The result showed morphological difference/ divergence among most of the studied populations. This morphological difference

was due to quantitative characters only. For example, character (Peduncle length), separated population No. 8-9, character (length of basal leaves) separated population No. 5-8, while character petal width, separated populations 1-4 from the other populations.

A consensus tree was obtained for both ISSR and morphological trees (no shown), to reveal the populations that are diverged based on both morphological and molecular features. Interesting enough, it showed divergence of almost all populations at molecular level as well as morphological characteristics.

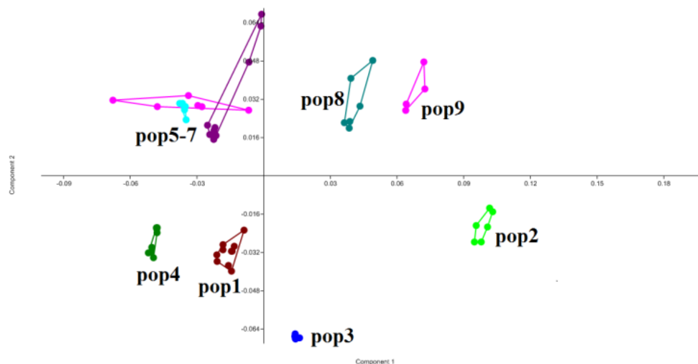

Fig. 6. PCA plot of *Hedera helix* populations based on morphological characters. Numbers are according to Table 1.

DISCUSSION

The existing genetic variability of the individual species within and among the populations is connected to this species ability to mirror the short- and long-term specific regimes of their living habitats (DAVIES *et al*., 2016). The analysis of the distribution of the genetic variability patterns specific for landscape and ecological parameters is valuable for identification of the taxa most vulnerable to the anthropogenic impacts (BRANDVAIN *et al*., 2014). The coupling of ecological and genetic data will provide the most suitable background for preserving the ability of the biota to respond the rapid environmental changes (ECKERT, 2011; GUGGER *et al*., 2011). The literature reports the following basic factors influencing the distribution of genetic variation: habitat specify, plant-insect interactions, connectivity and disturbance, dispersal ability, species lifespan, reproductive rates and existing genetic diversity (SCHIERENBECK, 2017; ESFANDANI –BOZCHALOYI *et al*., 2018a, 2018b, 2018c, 2018d). Genetic diversity when analysed by neutral markers does not correspond to the adaptive ability of plant populations, but these types of markers are very useful for the interpretation of the past landscapes, refugia and gene flow (HOLDEREGGER *et al*., 2006). That is, why the selected genes or markers of active parts of plant genomes are used to interprete the plant genome response to the changes to the local climate and environment (HOFFMAN and WILLI, 2008). Molecular-based population genetic data are very useful for determining the ecological and habitat events in the

past and for detection of patterns of the recent genetic divergence. This can be achieved using different types DNA markers (DAVEY and BLAXTER, 2010). SCoT markers are novel molecular markers that target the translation initiation site and preferentially bind to genes that are actively transcribed. These primers have been shown to exhibit relatively high levels of polymorphism (COLLARD and MACKILL, 2009). It was more informative than IRAP and ISSR for the assessment of diversity of plants (COLLARD and MACKILL, 2009).

In spite of an active pharmaceutical use of *Hedera helix* its molecular diversity based on the DNA markers is practically unknown. The only information about the applications of polymorphic markers are reported for ITS, cpDNA and RAPD (CLARKE *et al*., 2006; GREEN *et al*., 2011, 2013; VALCÁRCEL *et al*., 2003).

Grivet and Petit (2002) have reported that european ivies belong to eight different chloroplast haplotypes with the following characteristics: scotish ivies are of haplotype C1; slovak ivies are of A2, G, D and C1; croatian are of A1, A2 and H; german ivies are of haplotype A1, C1 and A2 and spanish are of E and G. This distribution is visible in the results of PBA based polymorphism, as the Spanish sample is the most distinct to the others and the common haplotype A2 is visible in the grouping of Slovak and Croatian samples and the haplotype C1 differ the samples from Scotland and Germany.

RAPD marker was not used directly to analyse the polymorphism of *Hedera helix* but an invading populations of *Hedera hibernica* (Kirchner) Bean and *Hedera helix* were identified based on the RAPD data (CLARKE *et al*., 2006). The first screening for the microsatellite, retrotransposon and miRNA based markers for the *Hedera helix* reported by ŽIAROVSKÁ *et al*. (2016). In this study, retrotransposon based types of markers – IRAP and iPBS were prooved to be an excellent for the ivy diversity analysis, microsatellite markers were prooved to be successfull based on the type of the repetition and miRNA as a novel types of DNA markers were prooved to be a very promising for ivy diversity analysis (ŽIAROVSKÁ *et al*., 2016).

BOŠELOVÁ and ŽIAROVSKÁ (2016) evaluate a cytochrome P450 sequences based polymorphism by PBA technique. Using a set of three PBA primers and their combinations, the number of amplified fragment levels obtained by individual primer combinations was for both of them 11 and the obtained polymorphism was 91% or 100% respectivelly. The PCoA analysis was performed and the genotypes belonging to a different geographic localities were grouped together.

ŽIAROVSKÁ *et al*., (2019) analyzed the genetic diversity within ivy (*Hedera helix*, L.) using iPBS retrotransposons. Their results shown that natural and planted European populations of ivy were analysed using an iPBS marker 5´ACCTGGCGTGCCA3´ with a total number of 238 fragments generated. Of these, 86% were polymorphic. There were determined certain attributes of this marker such as the diversity index (DI) and polymorphism information content (PIC). The value of the diversity index was 0.79 and the polymorphic information index was 0.78. The proportion of polymorphisms of the individual amplified loci ranged from 0.32% to 6.98%. Cluster analysis was performed to determine the relationships among the European ivy populations where the distribution in the dendrogram under the habitat specifity was found for the used iPBS marker.

The present study revealed interesting data about its genetic variability, genetic stratification and morphological divergence in north and west part of Iran. The studied

populations have a high level of genetic diversity (He = 0.111- 0.210). The Genetic diversity is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to the cope with changes in the environment (WARBURG, 1938; GUITTONNEAU, 1972). Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/ cross breeding brings about higher level of genetic variability in the studied taxon (KNUTH, 1908). Flowers of common ivy provide late season pollen and nectar for several insect groups, and its fruits are a winter and spring food source for frugivorous birds. Ivy benefits from insect pollination in order to set fruit, but it is unknown which flower-visiting insects are the most effective pollinators (VEZZA *et al*., 2006).

Low genetic variability may also occur due to small size of the populations and genetic drift (DAHLGREN, 1980). These species tend to perform inbreeding as also evidenced by very low Nm value and IBD obtained for the studied species. However, limited gene flow was not solely due to geographical distance among the species, but some of the species, which grew in adjacent areas with overlapping zones, did not form any hybrids or intermediate forms as evidenced by morphological and ISSR clusters obtained (WEBB and CHATER, 1968)

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SCOT MOLEKULARNI MARKERI I DIFERENCIJACIJA POPULACIJE KOD *Hedera helix* L.

Haiying LI[1,*], Yingxue WANG[1], Rabia IQBAL [2]

[1]Severnokineski Univerzitet za vodne resurse i električnu energiju, Zhengzhou, Henan, Kina
[2]Departman za biljne nauke, Quaid-i-Azam Univerzitet Islamabad, Pakistan

Izvod

Hedera helix L. je vrsta koja se široko koristi zbog svojih ukrasnih i lekovitih svojstava. Uprkos vrlo dobroj biohemijskoj karakterizaciji, znanje o promenljivosti DNK je vrlo ograničeno i do danas nisu korišćeni DNK markeri za analizu genomske varijabilnosti populacija. U ovoj studiji, proučavana je genetska varijabilnost 56 *Hedera helix*, individua unutar devet populacija koristeći 10 markera Start Codon Tarnged (SCoT). Visoko polimorfne trake (95,78%), polimorfni sadržaj informacija (0,25) i broj alela (1,34) pokazali su da su SCoT pouzdan sistem markera za genetsku analizu u *Hedera helix*. Kod svih vrsta, procenat polimorfnih lokusa [P] iznosio je 66,20%, raznolikost gena Nei [H] bila je 0,159, Šenonov indeks [I] bio je 0,148, a nepristrasna raznolikost gena [UHe] bila je 0,56. Genetske varijacije unutar populacija (70%) bile su veće nego među populacijama (30%) na osnovu analize molekularne varijanse (AMOVA). Koristili smo SCoT molekularni marker za naše genetsko istraživanje sa sledećim ciljevima: 1 - ispitati genetsku raznolikost *Hedera helix*, 2 - identifikovati genetske grupe unutar ovih devet populacija bršljana i 3 - proizvesti podatke o genetskoj strukturi populacije bršljana. Dobijeni rezultati otkrili su visoku genetsku varijabilnost unutar populacije.

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