UDC 575. 630
https://doi.org/10.2298/GENSR2202553H
Original scientific article

MORPHOMETRIC ANALYSIS AND GENETIC DIVERSITY IN *Hedera* L. (Araliaceae) SPECIES POPULATIONS USING SEQUENCE RELATED AMPLIFIED POLYMORPHISM

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Hai-Yan Y., Z. Li, L. Jian-Cheng (2022). *Morphometric analysis and genetic diversity in Hedera L. (Araliaceae) species populations using sequence related amplified polymorphism*. - Genetika, Vol 54, No.2, 553-564.

In temperate latitudes across Europe, North Africa, and Asia, the genus *Hedera* L. colonizes forest understory and riparian vegetation. The goal of this research was to investigate SRAP (Sequence-related enhanced polymorphism**)** markers in overall 70 successions of *Hedera* types, that is comprised of three species *Hedera helix* L.; *Hedera colchica* (K.Koch) K.Koch and *Hedera pastuchovii* Woronow. Overall, 76 (Number of total loci) (NTL) DNA bands were created via polymerase chain reaction amplifications (PCR) amplification of three *Hedera* types. These bands were obtained by combining five distinct selective primers. The overall amount of amplified varied from 10 to 18. The projected impartial gene diversity (UHe) ranged from 0.013 (*Hedera colchica*) to 0.34 (*Hedera colchica*) (*Hedera helix*). The genetic similarity of three varieties is considered to be between 0.63 to 0.90. The clustering findings revealed two main groups. *Hedera helix* and *Hedera colchica* have the least affinity in the SRAP (Sequence-related amplified polymorphism) indicators study. Our findings revealed excellent molecular recognition of every genotypes tested, indicating that the *Hedera* accessions exhibit a lot of genetic variation. This discovery might be useful in breeding control techniques for genetic preservation and cultivar growth.

Key words: Genetic diversification, gene transfer; *Hedera,* Sequence-related enhanced polymorphism

INTRODUCTION

In temperate latitudes across Europe, North Africa, and Asia, the genus *Hedera* L. colonizes forest understory and riparian vegetation (MEUSEL *et al*., 1965). Between three and 19

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species were identified in taxonomic studies of *Hedera* released in the second half of the twentieth century (LAWRENCE and SCHULZE, 1942). Prior molecular and cytogenetic investigations highlighted the eastern and western Mediterranean regions as two key sources of diversification for *Hedera* (Araliaceae). Ever since the work of MCALLISTER and RUTHERFORD (1990), the recognition and delimitation of *Hedera* species has mostly relied on a mixture of trichome shape and other factors. (stellate-multiangulate, stellate-rotate, and scalelike hairs; MCALLISTER and RUTHERFORD, 1990; ACKERFIELD and WEN, 2002; VALCA´RCEL and VARGAS, 2010), juvenile leaf morphology (from entire to 3–7 lobate; RUTHERFORD *et al*., 1993; ACKERFIELD and WEN, 2002; VALCA´RCEL and VARGAS, 2010), and ploidy level (from 29 to 89; VARGAS *et al*., 1999).

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 tests, in combination with haplotype sequencing, identify 13 distinct units of ivy (GRIVET and PETITE, 2002). Several additional writers then employed haplotype analysis to characterize the *Hedera* genus (ACKERFIELD and WEN, 2002). The RAPD indicators were used to map invading Hedera populations in native Pacific Northwest forests (CLARKE *et al*., 2006). BOEOVÁ and IAROVSKÁ (2016) used PBA identifiers in their study of the European common ivy population. Having a limited understanding of ivy's genome could be modified, with the potential of DNA identifiers in mind (KUMAR *et al*., 2014). *Hedera helix* is a versatile plant that might have a range of uses in the future. Ivy is grown in around 500 distinct cultures worldwide. Ivy is a renowned aesthetic plant with a multitude of cultivars, including non-climbing types which are intended to cover the soil and keep plots compact. Ivy is good for conservatories and could make appealing wrapping for garden constructions due to its evergreen and shade-loving properties. Ivy is a potential plant for medical use (LUTSENKO *et al.*, 2010), plant protection (PÂRVU *et al*., 2015), and technical usage (nanoparticles) in the future, in addition to its decorative uses (LENANGHAN *et al*., 2013). Starting from reproduction and selection, a genomic assessment of natural diversity in its population is crucial information, as this type has been identified as an instance of genomic plasticity happening during the usual developmental transitions from juvenile to adult phase (OBERMAYER, 2000). The study's goals were to: a) assess genetic diversity; and b) use NJ methods to analyze population linkages. The current findings have ramifications for reproduction and conservation efforts. The current work is the first to use SRAP indicators to investigate genetic diversity as well as phylogenetic connections across and within *Hedera* populations in Iran.

MATERIALS AND METHODS

Plants collection

Three *Hedera* species (*Hedera helix* L.; *Hedera colchica* (K.Koch) K.Koch and *Hedera pastuchovii* Woronow) in Kermanshah, Gorgan, Tehran, Mazandaran and Guilan Provinces of

Iran were selected and sampled throughout July-August 2018-2020 (Table 1). Morphometric and SRAP analyses on 70 plant accessions were carried out. Five to twelve samples from each population fitting the three various species were selected based on other eco-geographic characteristics. Samples were stored at - 20 °C till further use. Detailed information about locations of samples and geographical distribution of species are stated (Table 1, Fig 1).

Table 1. List of the investigated the populations of Hedera species including origin of voucher specimens.

No	Sp.	Locality
Sp1	*Hedera helix* L.	Kermanshah; Paveh
Sp1	*Hedera helix* L.	Gorgan; Ziyarat Village
Sp1	*Hedera helix* L.	Tehran, Darakeh
Sp2	*Hedera colchica* (K.Koch) K.Koch	Giulan; Talesh
Sp2	*Hedera colchica* (K.Koch) K.Koch	Giulan; Astara; Lisa Rood
Sp2	*Hedera colchica* (K.Koch) K.Koch	Mazandaran; Ramsar
Sp3	*Hedera pastuchovii* Woronow	Gorgan; Nahar Khvoran
Sp3	*Hedera pastuchovii* Woronow	Mazandaran; Savadkuh

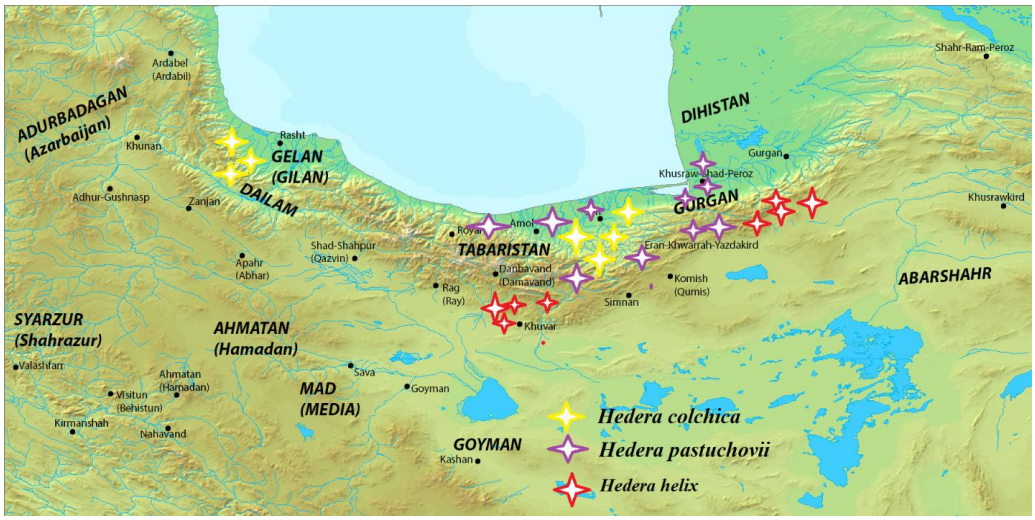

Fig. 1. Map of distribution of populations *Hedera*

Morphological analyses

Morphological investigations were conducted on a total of 23 morphological (23 quantitative) features. For morphological studies, four to twelve specimens from each population

were selected at random. Morphological characteristics were initially normalized (Mean = 0, Variance = 1) before being utilized to calculate Euclidean distance between taxonomic pairs (PODANI, 2000). The UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characteristics) as well as MDS (Multidimensional scaling) ordination methods were employed to group the plant specimens (Podani, 2000). For multivariate statistical analysis of morphological data, HAMMER *et al*. (2012) employed PAST version 2.17.

Sequence-related amplified polymorphism method

Fresh leaves were randomly employed from one to twelve plants. These were dried with silica gel powder. Genomic DNA was removed while following previous procedure (ESFANDANI-BOZCHALOYI *et al*., 2019). SRAP assay was implemented as portrayed previously (LI and QUIROS, 2001). 5 SRAP in different primer combinations were used (Table 2).

Table 2. SRAP primer information and results

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me4	22	18	75.55%	0.29	10.22
Em2-Me5	14	14	100.00%	0.44	30.92
Em3-Me4	11	11	100.00%	0.41	42.21
Em4-Me1	17	13	79.00%	0.32	24.23
Em5-Me1	12	10	90.00%	0.34	28.11
Mean	16	15	85.20%	0.38	29.55
Total	76	65			

a: Number of total loci (NTL); b: Number of polymorphic loci (NPL); c: Polymorphic ratio(P %); d: Polymorphic information content (PIC); e: Resolving power (Rp)

Data Analyses

UPGMA (Unweighted paired group using average) ordination process was implemnented to assess morphological characters. ANOVA (Analysis of variance) was conducted to evaluate morphological differences amongst classes. Principal component analysis (PCA) was implemented to recognize adjustable morphological characters in *Hedera* species. Multivariate statistical analyses i.e., PC analysis, were performed in PAST software version 2.17 (HAMMER *et al.*, 2001).

MOLECULAR STUDIES

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were recorded as present (1) and absent (0), respectively. Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Furthermore, the polymorphic ratio was assessed based on NPL/NTL values. Polymorphism information content was evaluated as previously suggested by ROLDAN-RUIZ *et al.* (2000). Resolving power for individual marker system was evaluated as: $Rp = \Sigma Ib$. Ib (band informativeness) was estimated while following equation: proposed as: Ib= 1 - [2 x (0.5-p)]. In the equation, p indicates the

presence of bands (PREVOST and WILKINSON, 1999). The amount of efficient alleles, Nei's gene variation (H), Shannon information index (I), and proportion of polymorphism (P percent = number of polymorphic loci/number of total loci) were all calculated (WEISING *et al*., 2005; FREELAND *et al*., 2011). Neighbor Joining (NJ) clustering and Neighbor-Net networking were based on Nei's genetic distance between populations (FREELAND *et al*., 2011; HUSON and BRYANT, 2006). The Mantel test was used to see if there was a link between the analyzed populations' geographical and genetic distances (PODANI, 2000). PAST 2.17 (HAMMER *et al*., 2012), DARwin 5 (2012), and SplitsTree4 V4.13.1 (2013) tools were used to conduct these studies. A heuristic technique based on Bayesian clustering algorithms was used to examine the population structure of the pistachio genotypes. To better discover population substructures, the clustering approach based on the Bayesian-model performed in the software program STRUCTURE (PRITCHARD *et al*., 2000; FALUSH *et al*., 2007) was applied to the same information set. The program predicts allele frequencies in each cluster and population memberships for each person, relying on an algorithm that allocates genotypes to homogeneous units, given a number of clusters (K) and presuming Hardy-Weinberg and linkage equilibrium among clusters (PRITCHARD *et al*., 2000). The number of possible subpopulations ranged from two to ten, and their influence to accessions' genotypes was determined using 50,000 iteration burn-ins and 100,000 iteration sampling intervals. Following EVANNO *et al*. (2005) the most likely number (K) of subpopulations was determined. Two summary statistics, pseudo-F and Bayesian Information Criterion (BIC), give the best fit for k in K-Means clustering (MEIRMANS, 2012). Pairwise genetic similarity between species was evaluated to reveal genetic affinity between species (JACCARD, 1908). Unbiased expected heterozygosity and Shannon information index were evaluated in GenAlEx 6.4 software (PEAKALL and SMOUSE, 2006). Gene flow was conducted in POPGENE software, version 1.32 (Yeh et al. 1999). Analysis of molecular variance test was performed in GenAlEx (PEAKALL and SMOUSE, 2006). Mantet test was performed with 5000 permutations in PAST, version 2.17 (HAMMER *et al*., 2001).

OUTCOMES

Mophometery

ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 62% cumulative variation. The initial PCA axis explained 44% of the overall deviation. The highest correlation (> 0.7) was shown by morphological characters such as leaf shape, sepal form, peduncles as well as pedicels hair, stem hair, petioles hair, bract and pedicel length, petal length and width, stem leave's length and width The morphological characters of three *Hedera* species are shown in PCoA plot (Figure 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Hedera* species and separated each groups.

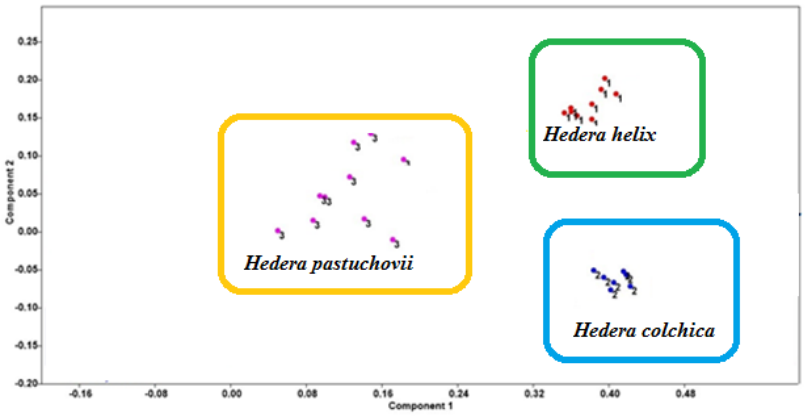

Fig 2. Morphological characters analysis of the *Hedera* species by PCoA plot.

Species identification and genetic diversity

Five (5) suitable primer combinations (PCs), out of 15 PCs were screened in this research. Sixty five (65) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 100bp to 3000 bp. Highest and lowest quantity of polymorphic bands were 18 and 10 for Em1-Me4 and Em5-Me1, respectively. Each primer produced 15 polymorphic bands on average. The PIC ranged from 0.29 (Em1-Me4) to 0.44 (Em2-Me5) for the 5 SRAP primers, with a mean of 0.38 for each primer. RP of the primers varied between 10.22 (Em1-Me4) and 42.21 (Em3-Me4) with a mean of 29.55 for each primer (Table 2). The calculated genetic parameters of *Hedera* species are shown. The impartial heterozygosity (H) ranged from 0.013 (*Hedera colchica*) to 0.34 (*Hedera helix*) with a mean of 0.22. Shannon's information index (I) was maximum in *Hedera helix* (0.45), where as we recorded minimum Shannon's information index in *Hedera colchica* (0.035). The observed number of alleles (*N*a) varied between 1.200 in *Hedera colchica and* 1.727 in *Hedera pastuchovii.* The significant amount of alleles (*N*e) varied from 1.137 (*Hedera helix*) to 1.449 (*Hedera pastuchovii*).

Analysis of Molecular Variance results in major genetic distinction (p = 0.01) amongst *Hedera* classifications. The majority of genetic variation occurred among species. AMOVA findings implied that 80% of the overall distinction was between types as well as comparatively less genetic variation was recorded at the species level (Table 3). Genetic difference between *Hedera* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.399, p = 0.01) and D_est amount (0.381, p = 0.01).

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	76	2245.364	22.789	66.154	80%	
Within Pops	120	115.443	133.99	10.888	20%	80%
Total	196	2345.807		76.060	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, ($P < 0.001$).

WARD tree and UPGMA clustering created parallel outcomes hence only WARD tree is given and debated (Figure. 3). This outcome implies that molecular characters investigated can delimit *Hedera* classes in two distinct chief clusters or units, In overall, two key clusters were produced in WARD tree (Fig. 3), 10 individual of *Hedera colchica* formed a single cluster. Cluster II contained two sub-clusters, and most of individual *Hedera helix* and *Hedera pastuchovii* formed cluster II. There were 60 individuals in this cluster.

We detected strong association amid geographical plus genetic distances (r = 0.71, p=0.0002) and gene flow ($N_{m)}$ score of 0.75 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Table not included). The findings suggested that there was the utmost degree of genetic resemblance (0.90) among *Hedera helix* and *Hedera pastuchovii.* On the contrary to this, *Hedera helix* and *Hedera colchica* (0.63) had lowest genetic resemblance.

The Evanno test ΔK =3 (Figure Table not included), showed the genetic details of the *Hedera* species. According to STRUCTURE analysis, the *Hedera* species are genetically differentiated due to different allelic structures (Figure not included). Limited gene flow results were supported by K-Means and STRUCTURE studies too. We could not detect substantial gene transfer amongst the *Hedera* species. This outcome is aligned with grouping we acquired with WARD tree (Figure 3), since these populations were clustered together. These common alleles form a relatively small percentage of the genomes in these populations, as indicated by the STRUCTURE diagram based on the admixture model, and all of these data concur in indicating a high level of genetic stratification among *Hedera* populations.

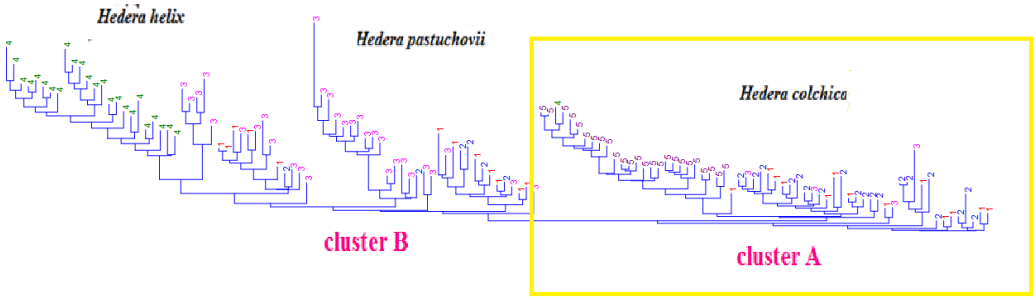

Fig. 3. WARD tree of populations in *Hedera* species based on SRAP molecular markers.

DISCUSSION

In the present study, we used morphological and molecular (SRAP) data to evaluate species relationships in *Hedera.* Morphological analyses of *Hedera* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated from each other. PCA analysis suggests that morphological characters including leaf form, sepal form, peduncles and pedicels hair, stem hair, petioles hair, bract as well as pedicel length, petal length and breadth, stem leaf length plus width have the potentials to identify and delimitate *Hedera* species. Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Hedera* species. Morphological characters including leaf form, sepal's shape, peduncles and pedicels hair, stem hair, petioles hair, length of bract and pedicel, length and width of the petal, length and width of stem leaves play key role in plant systematics and taxonomy. Our work also highlighted the significance of morphological characters and molecular data to identify and study species genetic diversity. In general, genetic relationships obtained from SRAP data coincides with morphometric results. This is in accordance with the parameters of AMOVA and genetic diversity results. SRAP molecular markers detected clear genetic difference among species. These results indicate that SRAP have potentials to study plant systematics and taxonomy in *Hedera* members.

Given the negative impact of biodiversity threats and overexploitation of *Hedera* plant species in Iran, it is necessary to conduct genetic diversity studies on *Hedera* species. Genetic diversity based studies pave our understanding to develop conservation strategies (ESFANDANI-BOZCHALOYI *et al.*, 2017; ZHU, ET AL., 2021; Zhao *et al.* 2021; YIN, *et al.* 2021; MA, *et al.* 2021; PENG, *et al.* 2021; SI, *et al*., 2020; JIA, *et al.* 2020; BI, *et al*. 2021). Genetic diversity studies are conducted through appropriate selection of primers and indexes including Polymorphic information content (PIC) and marker index (MI) are important indexes to fathom genetic variation in species (SIVAPRAKASH *et al.*, 2004). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (SIVAPRAKASH *et al*., 2004). In the present work, 3 *Hedera* species were categorized with 5 SRAP indicators. The outcomes verified the effectiveness of microsatellite indicators for fingerprinting resolutions. Our results implied that The PIC ranged from 0.29 (Em1-Me4) to 0.44 (Em2-Me5) for the 5 SRAP primers, with a mean of 0.38 for each primer. RP of the primers varied from 24.23 (Em4-Me1) to 42.21 (Em3-Me4) with an average of 21.77 per primer.

According to GRIVET and PETIT (2002), European ivies belong to eight distinct chloroplast haplotypes, each of which has the following features: Scottish ivies have the haplotype C1; slovak ivies have the haplotypes A2, G, D, and C1; croatian ivies have the haplotypes A1, A2, and H; german ivies have the haplotypes A1, C1, and A2; and spanish ivies have the haplotypes E and G. The findings of PBA-based polymorphism show that the Spanish sample is by far the most dissimilar from the others, and that the common haplotype A2 is apparent in the cluster of Slovak and Croatian specimens, while the haplotype C1 distinguishes the specimens from Scotland and Germany. Although the RAPD identifier was not employed directly to analyze the polymorphism of *Hedera helix*, the RAPD information was exploited to identify invasive populations of *Hedera hibernica* (Kirchner) Bean and *Hedera helix* (CLARKE *et al*., 2006). iarovská et al. published the very first screening for microsatellite, retrotransposon,

and miRNA-based indicators for the *Hedera helix* (2016). Retrotransposon-based indicators such as IRAP and iPBS were found to be outstanding for ivy diversity analysis, microsatellite identifiers were found to be effective based on the type of repetition, and miRNA as a novel class of DNA indicator was found to be very appealing for ivy variety analysis (IAROVSKÁ *et al.*, 2016). BOELOVÁ and IAROVSKÁ (2016) use the PBA approach to analyze a cytochrome P450 sequence-based polymorphism. The amount of enhanced segment levels achieved by individual primer combinations was 11 for both of them, and the acquired polymorphism was 91 percent or 100 percent, accordingly, using a set of three PBA primers and their combinations. Employing iPBS retrotransposons, IAROVSKÁ *et al*. (2019) investigated the genetic diversity of ivy (*Hedera helix*). Their findings revealed that an iPBS identifier 5'ACCTGGCGTGCCA3' was used to analyze natural and planted European populations of ivy, yielding a total of 238 pieces. 86 percent of them were polymorphic. Various characteristics of this indicator, such as the diversity index (DI) and polymorphism information content, were evaluated (PIC). The polymorphic information index was 0.78, while the diversity index was 0.79. The percentage of polymorphisms in each amplified locus varied from 0.32 percent to 6.98 percent. Cluster research was utilized to evaluate the linkages across European ivy populations, and the distribution of the employed iPBS indicator in the dendrogram under habitat specifity was determined.

Received, December 10th, 2020
Accepted November 28th, 2021

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MORFOMETRISKA ANALIZA I GENETIČKI DIVERZITET POPULACIJA VRSTE *Hedera* L. (Araliaceae) PRIMENOM *SRAP* MARKERA

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Izvod

U umerenim geografskim širinama širom Evrope, severne Afrike i Azije, rod *Hedera* L. kolonizuje šumsko podlogu i obalsku vegetaciju. Cilj ovog istraživanja je bio da se ispitaju markeri SRAP (*Sequence-related enhanced polymorphism*) u ukupno 70 sukcesija tipova *Hedera*, koje se sastoje od tri vrste *Hedera helix* L.; *Hedera colchica* (K.Koch) K.Koch i *Hedera pastuchovii* Woronow. Sveukupno, 76 (broj ukupnih lokusa) (NTL) DNK traka je stvoreno putem amplifikacije lančane reakcije polimerazom (PCR) tri tipa *Hedera*. Ove trake su dobijene kombinovanjem pet različitih selektivnih prajmera. Ukupna količina amplifikacija varirala je od 10 do 18. Projektovana nepristrasna genska raznolikost (UHe) kretala se od 0,013 (*Hedera colchica*) do 0,34 (*Hedera colchica*) (*Hedera helix*). Smatra se da je genetska sličnost tri varijeteta između 0,63 i 0,90. Rezultati grupisanja dali su dve glavne grupe. *Hedera helix* i *Hedera colchica* imaju najmanji afinitet u studiji indikatora SRAP (*Sequence-related amplified polimorphism*). Naši rezultati su otkrili odlično molekularno prepoznavanje svih testiranih genotipova, što ukazuje da *Hedera* uzorci pokazuju mnogo genetskih varijacija. Ovo otkriće može biti korisno u tehnikama kontrole kod genetičke prezervacije i za samo gajenjevrsta iz roda *Hedera*.

Primljeno 10.XII.2020.
Odobreno 28. XI. 2021.