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Original scientific article

MOLECULAR IDENTIFICATION AND GENETIC DIVERSITY IN *Hypericum* L.: A HIGH VALUE MEDICINAL PLANT USING RAPD MARKERS MARKERS

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Genus *Hypericum* (Guttiferae, Hypericoideae) is perennial, belonging to the Hypericaceae family, having 484 species in forms of trees, shrubs, and herbs, distributed in 36 taxonomic sections. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Hypericum* genetic diversity. Therefore, we collected and analyzed six species from five provinces of Iran regions. Overall, seventy plant specimens were collected. Our aims were 1) to assess genetic diversity among *Hypericum* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa. We showed significant differences in quantitative morphological characters in plant species. *H. dogonbadanicum* depicted unbiased

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expected heterozygosity (UHe) in the range of 0.10. Shannon information was high (0.32) in *H. perforaturm*. *H. dogonbadanicum* showed the lowest value, 0.17. The observed number of alleles (*N*a) ranged from 0.22 to 0.53 in *H. dogonbadanicum* and *H. elongaturn*. Gene flow (Nm) was relatively low (0.87) in *Hypericum*. The Mantel test showed correlation (r = 0.45, p=0.0001) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Hypericum* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Hypericum* species.

Keywords: Gene flow, *Hypericum*, Random Amplified Polymorphic DNA (RAPD), morphometry

INTRODUCTION

Genetic diversity is a vital feature that helps plant species survive in an ever-changing environment, and it sheds light on understanding the phylogenetic affinity among the species (ERBANO *et al.* 2015). Quite a significant number of genetic resources and materials programs of plant species have been carried out to preserve the plant species worldwide. Scientific data indicate that genetic diversity plays a pivotal role in conservation programs (GOMEZ *et al.* 2005).

Genus *Hypericum* (Guttiferae, Hypericoideae) is perennial, belonging to the Hypericaceae family, having 484 species in forms of trees, shrubs, and herbs, distributed in 36 taxonomic sections (CROCKETT and ROBSON 2011). The species of the family are distributed worldwide in the temperate zones but are absent in extreme environmental conditions such as deserts and poles. Iranian species of this genus grow mainly in north, northwest and center of Iran and form floristic elements of Hyrcanian mountainous areas, Irano-Turanian, Mediterranean and Zagros elements. They generally prefer steep slopes of rocky and calcareous cliffs and margin of mountainous forests (ROBSON 1968; AZADI 1999). ROBSON (1968) introduced 21 species in the area covered by Flora Iranica. ROBSON (1977) and ASSADI (1984) reported *H. fursei* N. Robson and *H. dogonbadanicum* Assadi as two endemics of North and South West of Iran. In Flora of Iran, AZADI (1999) identified 19 species, 4 subspecies arranged in 5 sections (comprising *Campylosporus* (Spach) R. Keller, *Hypericum*, *Hirtella* Stef., *Taeniocarpum* Jaub. & Spach. and *Drosanthe* (Spach) Endl.), and two doubtful species including *H. heterophyllum* Vent. and *H. olivieri* (Spach) Boiss. *Hypericum* species are generally known locally in Iran with the names "Hofariqun" as Ebn Sina called it (RECHINGER, 1986). St. John's wort (*Hypericum perforatum* L.) is the most important medicinal species of the genus and its main uses in medicine includes treatment of mild and moderate depression, skin wounds and burns (BARNES *et al.* 2001). The plant contains a vast array of secondary metabolites, among which naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin and adhyperforin) and essential oil can be mentioned (MORSHEDLOO *et al.* 2012; RADUSIENE *et al.* 2005).

To better understand genetic diversity, the biologist study population size. Population size is considered one of the central factors to understand the variability in a gene (TURCHETTO *et al.* 2016). Genetic variation and diversity are essential for species to survive because individuals are separated due to genetic or geographical barriers, often resulting in scattered populations. Since these individuals have limited gene flow, there is a greater chance of a decline in

population size (FRANKHAM 2005). Given the significance of genetic diversity in conservation strategies, it is of utmost importance to disentangle genetic diversity in plant species, particularly threatened and rare species (ESFANDANI-BOZCHALOYI *et al.* 2018a, 2018b, 2018c, 2018d).

Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (ESFANDANI-BOZCHALOYI *et al.* 2017). Taxonomical Systematics studies were conducted in the past to identify the *Hypericum* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied seventy samples. Our aims were 1) to assess genetic diversity among *Hypericum* species 2) is there a correlation between species and geographical distance? 3) Genetic structure of populations and taxa?

MATERIALS AND METHODS

Plant materials

Six *Hypericum* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. Seventy plant samples (5-20 per plant species) were examined for morphometry purposes. The random amplified polymorphic DNA analysis method was limited to 70 samples. According to previous references, all the species were identified (RECHINGER, 1986).

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxa	Locality	Latitude	Longitude	Altitude(m)
H. dogonbadanicum Assadi	Hamedan, 20km s of Nahavand	37° 07' 48 "	49° 54' 04"	165
H. androsaemum L.	Razavi Khorasan, Kashmar, Kuhsorkh District	37° 07' 08"	49°54' 11"	159
H. tetrapterum Fries.	Esfahan, ardestan on road to taleghan	38 ˚ 52' 93"	47 ˚25' 92"	1133
H. perforaturn L.	Hamedan, Alvand	38˚52' 93"	47 ˚25' 92"	1139
H. triquetrifolium Turra	Mazandaran, 40 km Tonekabon to janat abad	35 ˚50' 36"	51˚ 24' 28"	2383
H. elongaturn Ledeb	West-Azarbaijan, Urumieh, Silvana	35 ˚42'29"	52 ˚20'51"	2421

Morphometry

In total, 11 quantitative and 15 qualitative characters were studied**.** Data were transformed (Mean= 0, variance = 1) prior to ordination . Euclidean distance was implemented to cluster and ordinate plant species (PODANI 2000). Morphological characters studied are: corolla shape, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length, corolla length, corolla width, corolla apex, leaf length and leaf width, leaf apex, leaf margins, leaf shape, leaf gland and bract margins.

2.3. Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (ESFANDANI-BOZCHALOYI *et al.* 2019). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD sets. We selected those primers (5) which could show clear bands and polymorphism (Table 2). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after these forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

We used an Unweighted pair group method with arithmetic mean (UPGMA) and Ward methods. Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (PODANI 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (PODANI 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (HAMMER *et al.* 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism information content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes. Marker index was calculated according to the previous protocol. Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diversity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) . Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (HUSON and BRYANT 2006; FREELAND *et al.* 2011). The Mantel test was carried out to find the correlation between genetic and geographical distances (PODANI 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (PEAKALL and SMOUSE 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in PopGene ver. 1.32 (YEH *et al.* 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (EVANNO *et al*. 2005).

RESULTS

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Principal component results explained 66% variation. In the first PCA axis with 42% of total variation, such characters as corolla shape, calyx shape, calyx length, bract length and leaf shape have shown the highest correlation (>0.7), leaf apex, corolla length, leaf length, leaf width were characters influencing PCA axis 2 and 3 respectively.Unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA) plots showed symmetrical results (Figure 1, Figure 2).

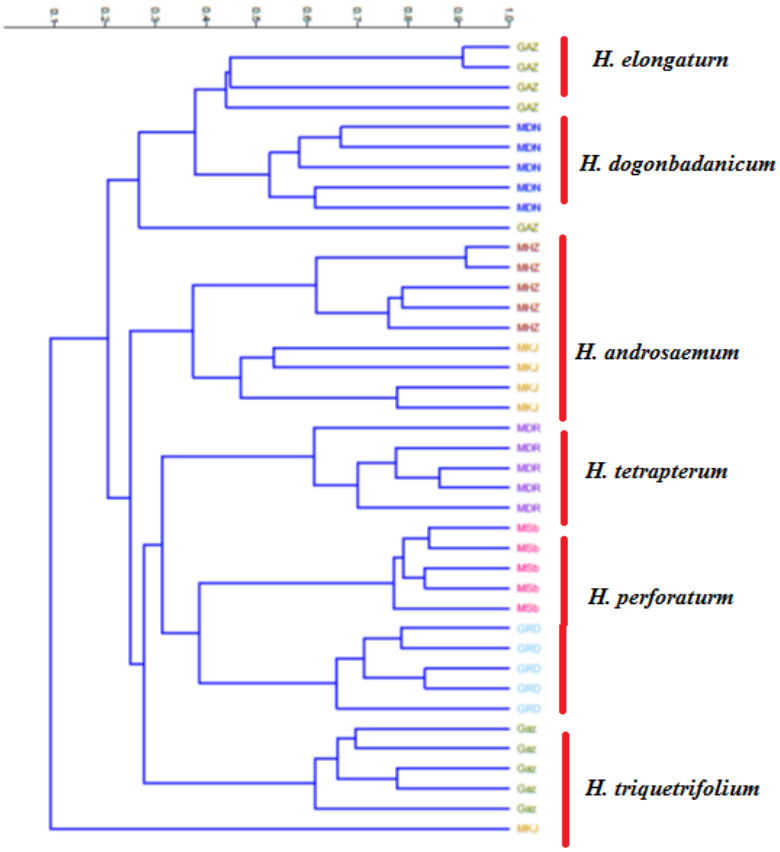

Figure 1. UPGMA tree produced using morphological characters revealing species delimitation in *Hypericum* species.

Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Morphological characters divided *Hypericum* species

into two groups, as evident in the UPGMA tree (Figure 1). Populations belonging to *Hypericum dogonbadanicum* and *Hypericum elongaturn* were in the first group. On the other hand, the second group consisted of two sub-groups. *H. androsaemum* formed the first sub-group. *H. tetrapterum*, *H. perforaturm* and *H. triquetrifolium* formed the second sub-group. These groups and sub-groups were formed due to morphological differences among the individuals of *Hypericum*. Our PCA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 2). Identical results were also reported in the UPGMA tree (Figure 1).

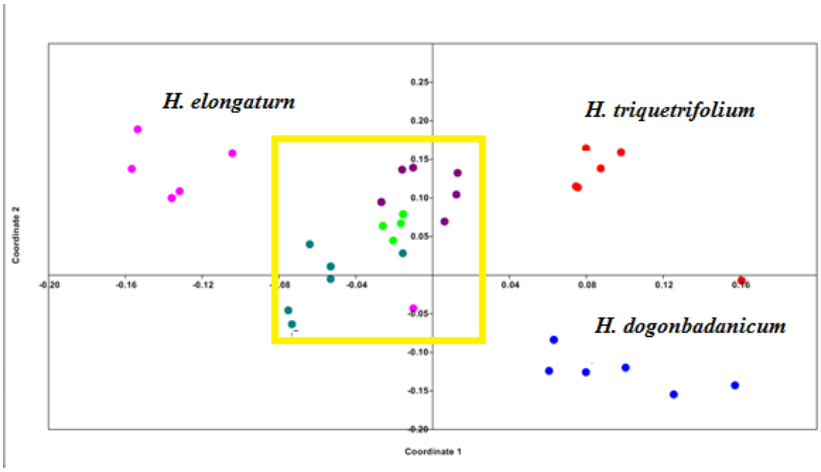

Figure 2. PCA plot morphological characters revealing species delimitation in *Hypericum* species.

Species Identification and Genetic Diversity

The primers, i.e., OPD-02 and OPB-02, could amplify plant (*Hypericum* species) DNA (Figure not included). 59 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPD-02. OPB-02 had the lowest polymorphic bands. The average polymorphic bands ranged to 12.45 for each primer. The polymorphic information content (PIC) had values in the range of 0.46 (OPD-02) to 0.56 (OPB- 02). Primers had 0.51 average polymorphic information content values.

Marker index (MI) values were 3.33 (OPD-03) to 6.87 (OPC-04), with an average of 5.2 per primer. Effective multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 7.34 (OPB-02) to 12.88 (OPD-02) EMR values. EMR values averaged 9.44 per primer (Table 2). All the necessary genetic features calculated of six *Hypericum* species are shown (Table 3). *H. dogonbadanicum* depicted unbiased expected heterozygosity (UHe) in the range of 0.10. Shannon information was high (0.32) in *H. perforaturm*. *H. dogonbadanicum* showed the lowest value, 0.17. The observed number of alleles (*N*a) ranged from 0.22 to 0.53 in *H. dogonbadanicum* and *H. elongaturn*.

Table 2. RAPD primers and other parameters.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPB-01	5′-GTTTCGCTCC-3′	15	15	100.00%	0.55	4.32	8.55	3.98
OPB-02	5′-TGATCCCTGG-3′	11	8	82.89%	0.56	6.56	7.34	5.18
OPC-04	5'-CCGCATCTAC-3'	11	11	100.00%	0.49	4.25	11.11	6.87
OPD-02	5′-GGACCCAACC-3′	17	17	100.00%	0.46	4.86	12.88	4.45
OPD-03	5′-GTCGCCGTCA-3′	12	9	84.99%	0.53	3.51	9.43	3.33
Mean		13.78	12.45	90.88%	0.51	3.5	9.44	5.2
Total		66	59					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers.

TABLE 3. Genetic diversity variables of Hypericum species

taxon	N	Na	Ne	I	He	UHe	%P
H. androsaemum	5.000	0.336	1.034	0.25	0.25	0.29	51.83%
H. tetrapterum	6.000	0.458	1.039	0.22	0.19	0.18	40.38%
H. perforaturm	6.000	0.448	1.049	0.32	0.38	0.33	69.38%
H. elongaturn	8.000	0.539	1.067	0.24	0.22	0.24	59.26%

(N = NUMBER OF SAMPLES, NE = NUMBER OF EFFECTIVE ALLELES, I= SHANNON'S INFORMATION INDEX, HE = GENE DIVERSITY, UHE = UNBIASED GENE DIVERSITY, P%= PERCENTAGE OF POLYMORPHISM IN POPULATIONS).

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Hypericum* species (P = 0.001). AMOVA showed that 55% of genetic variation was among the species. Relative less variation (45%) was reported within the species. Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.643, P = 0.001) and D_est values (0.872, *p* = 0.001). The MDS plot also revealed some major groups (Figure 3). The MDS plot also repeated the same pattern as indicated in figures 1 and 2. In current work, molecular findings also coincided with the traditional taxonomical (morphology) approaches for *Hypericum* species.

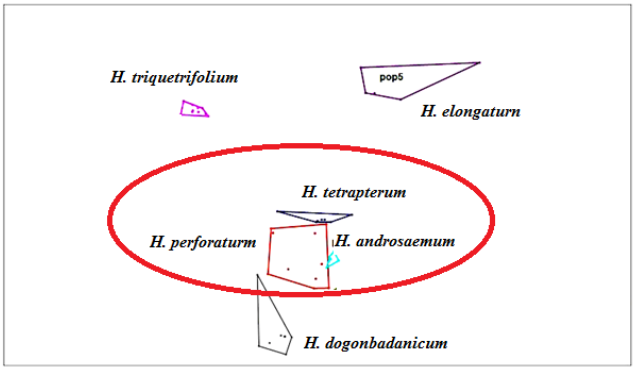

Figure 3. MDS plot of RAPD data revealing species delimitation in *Hypericum* species.

Gene flow (Nm) was relatively low (0.87) in *Hypericum* species. Genetic identity and phylogenetic distance in the *Hypericum* members are mentioned (Table 4). *H. tetrapterum* and *H. perforaturm* were genetically closely related (0.89) to each other. *H. androsaemum* and *Hypericum dogonbadanicum* were dissimilar due to low (0.64) genetic similarity. The mantel test showed correlation (r = 0.67, p=0.0001) between genetic and geographical distances.

The Evanno test showed ΔK =6 (Figure 4). Figure 4, showed the genetic details of the *Hypericum* species. According to STRUCTURE analysis, the *Hypericum* species are genetically differentiated due to different allelic structures (Figure 4). Limited gene flow results were supported by K-Means and STRUCTURE analyses too. We could not identify substantial gene flow among the *Hypericum* species. This result is in agreement with grouping we obtained with UPGMA tree (Figure 1), 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 *Hypericum* populations.

Table 4. The Nei genetic similarity (Gs) estimates using RAPD markers.

H. dogonbadanicum	*H. androsaemum*	*H. tetrapterum*	*H. perforaturn*	*H. triquetrifolium*	*H. elongaturn*	
1.000						*H. dogonbadanicum*
0.640	1.000					*H. androsaemum*
0.707	0.828	1.000				*H. tetrapterum*
0.825	0.753	0.896	1.000			*H. perforaturn*
0.837	0.834	0.874	0.832	1.000		*H. triquetrifolium*
0.719	0.726	0.705	0.792	0.816	1.000	*H. elongaturn*

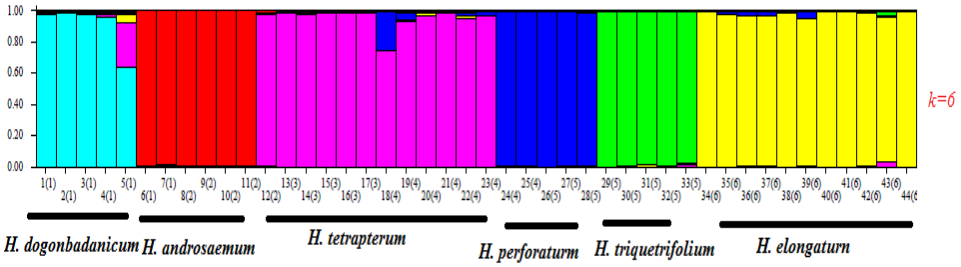

Figure 4. STRUCTURE in *Hypericum* species based on RAPD data.

DISCUSSION

The *Hypericum* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Hypericum* species (ROBSON 1968; AZADI 1999). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (ERBANO *et al.* 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (ERBANO *et al.* 2015). We examined genetic diversity in *Hypericum* by morphological and molecular methods. We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Hypericum* species. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Hypericum* species. PCA plot results also confirmed the application of morphological characters to separate *Hypericum* species. The present study also highlighted that morphological characters such as corolla shape, calyx shape, calyx length, bract length and leaf shape could delimit the *Hypericum* species group. The *Hypericum* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits.

In our study, morphology in six taxa of *Hypericum* species are given in detail for the first time. The aim of the present study was to find diagnostic features to separate species of *Hypericum* species in Iran. Morphological characters are considered as an useful tool for the identification of the species, as indicated previously (AZADI 1999).

Polymorphic information content (PIC) values are useful to detect genetic diversity. The current study recorded average PIC values of 0.51. This value is sufficient to study genetic diversity in the population (KEMPF *et al.* 2016). High genetic diversity among the *Hypericum* population was reported in the present study. Genetic analysis conducted via analysis of molecular variance and STRUCTURE showed genetic differences among the species. Interestingly, STRUCTURE results showed the presence of shared alleles in *Hypericum* species. Present findings revealed limited gene flow, and it is quite logical to report low gene flow. The Mantel test indicated a positive correlation between genetic and geographical distances. Therefore, it is concluded that isolation by distance and limited gene determines the *Hypericum* population genetic structure.

A high level of variation among *H. perforatum* populations was also reported by PERCIFIELD *et al.* (2007) which confirms results of the present study. Similar results have been reported on this species using the RAPD markers by HAZLER PILEPIC *et al.* (2008). The high genetic diversity of *H. perforatum* populations is as a result of its mating systems. In fact, propagation method(s) of plant species is considered as one of the most important factors determining their levels of genetic diversity (HAMRICK 1982; HAMRICK and GODT 1989). Self-incompatibility is a wide spread phenomenon in the genus *Hypericum* (ROBSON 1981), resulting in the high levels of genetic variability (BORBA *et al*. 2001). Furthermore, this perennial plant produces a great number of seeds every year in favor of the high amounts of diversity in this species (ZHAO *et al*. 2007).

Since widespread species may possess the higher levels of genetic diversity than narrowly distributed plants (HAMRICK and GODT 1996; SINGH *et al.* 1998), the wide range of *H. perforatum* distribution is an important factor in this respect. Considering the low level of gene

flow rate among studied wild populations of *H. perforatum*, therefore, genetic drift might be inevitable. In *H. perforatum*, the low rate of gene flow may be due to factors such as prevailing apomixes and short distance of seed dispersal as stated by HAZLER PILEPIC *et al.* (2008). Molecular markers have been used to investigate the genetic diversity, population structure, and reproductive biology of *H. perforatum* (ARNHOLDT-SCHMITT, 2000; HALUŠKOVÁ and KOŠUTH, 2003; BARCACCIA *et al.*, 2006; PERCIFIELD *et al.*, 2007).

Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Hypericum* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Hypericum* population. We also reported high genetic diversity, which clearly shows the *Hypericum* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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MOLEKULARNA IDENTIFIKACIJA I GENETIČKI DIVERZITET *Hypericum* L.: VISOKO VREDNA MEDICINSKA BILJKA PRIMENOM RAPD MARKERA

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Izvod

Hypericum (Guttiferae, Hypericoideae) pripada familiji Hypericaceae, koja sadrži 484 vrsta u formi drveća, žbunja i lekovitog bilja, raspoređenih u 36 taksonomskih sekcija. Detaljne RAPD studije nisu rađene za ispitivanje genetičkog diverziteta *Hypericum*. Zato smo skupili i analizirali šest vrsta iz pet provincijau Iranu. Ukupno 70 genotipova je skupljeno, Cilj rada je 1) da se ispita genetički diverzitet *Hypericum* vrsta 2) da li postoji korelacija između genetičke i geografske ddistance 3) genetička struktura populacija. Utvrđena je značajna razlika u kvantitativnim momorfološkim osobinama. *H. dogonbadanicum* očekivana heterozigotnost je 0.10. Shannon indeks je visok (0.32) kod *H. perforaturm*. *H. dogonbadanicum* je pokazala najnižu vrednost 0.17. Broj alela (*N*a) je bio u opsegu od 0.22 do 0.53 kod *H. dogonbadanicum* i *H. elongaturn*. Protok gena (Nm) je bio relativno nizak (0.87) kod *Hypericum*. Mantel test je pokazao korelaciju (r = 0.45, p=0.0001) uzmeđu genetičke i geografske distance. Visok genetički diverzitet jasno pokazuje da *Hypericum* vrste mogu da se adaptiraju na promene spoljašnje sredine pošto je visoki genetički diverzitet povezan sa adaptabilnošću vrsta. Dobijeni rezultati ističu primenu RAPD markera i morfometriske metode za ispitivanje genetičkog diverziteta *Hypericum* vrsta.

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