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Morphometric analysis and genetic diversity in *Hypericum* L. using sequence related amplified polymorphism

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Abstract. There are about 484 species of Hypericum in the Guttiferae family, which includes Hypericoideae. In Iran, species of this genus are mainly found in the north, northwest, and center of the country, and they are key contributors to the floral elements of the Hyrcanian mountains, Irano-Turanian, and Mediterranean regions (such as the Zagros). Medicinal, commercial, and horticultural values are associated with these plants.The genetic diversity was assessed through Sequence-related amplified polymorphism. To uncover genetic diversity and species characteristics in *Hypericum* species, were studied through a combination of morphological and molecular data. Eighty-five individuals related to 7 *Hypericum* were collected in 6 provinces. A total of 76 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction amplifications (PCR) amplification of seven *Hypericum* species. These bands were produced with the combinations of 5 selective primers. The total number of amplified fragments ranged from 10 to 20. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *H. perforatum* and *H. asperulum* had the lowest similarity. This study also detected a significant signature of isolation by distance (Mantel test results). Present results showed that sequence-related amplified polymorphism have the potential to identify and decipher genetic affinity in *Hypericum* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

Keywords: sequence-related amplified polymorphism, gene flow, genetic diversity, morphometric analysis, *Hypericum*.

INTRODUCTION:

Genetic diversity is a basic component of biodiversity and its conservation is essential for survival of any species in the changing environments (Si *et al.* 2020; Liu *et al.* 2021). Most authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Peng *et al.* 2021; Ma *et al.* 2021). This is very important in fragmented populations,

because they are more vulnerable due to the loss of allelic richness and increased population differentiation by genetic drift (decreased heterozygosity and eventual fixation of alleles) and inbreeding depression (increased homozygosity within populations; Chen *et al.* 2021; Bi *et al.* 2021). Therefore, understanding the genetic variability and diversity within and among different populations is crucial for their conservation and management (e.g., Esfandani-Bozchaloyi *et al.,* 2018a, 2018b, 2018c).

Sequence-related amplified polymorphism (SRAP) is PCR –based marker system. It is one of the efficient and simple marker systems to study gene mapping and gene tagging in plant species (Li and Quiros 2001), and SRAP are potential markers to assess plant systematics and genetic diversity studies (Wang *et al.* 2021; Yin *et al*. 2021; Zhao *et al*. 2021). Previously, Wu *et al*. (2010) assessed genetic diversity and population structure in *Pogostemon cablin* with the aid of SRAP markers. SRAP markers were successfully implemented in Lamiaceae family to study natural populations and variations within the family. These past studies showed that molecular markers, including SRAP markers, are efficient to investigate genetic diversity analyses and phylogenetic relationship among *Hypericum* species in Guttiferae, Hypericoideae family.

There are about 484 species of *Hypericum* in the Guttiferae family, which includes Hypericoideae. In Iran, species of this genus are mainly found in the north, northwest, and center of the country, and they are key contributors to the floral elements of the Hyrcanian mountains, Irano-Turanian, and Mediterranean regions (such as the Zagros). Medicinal, commercial, and horticultural values are associated with these plants. They prefer steep-sloped rocky and calcareous cliffs, as well as the edges of highland woods (Robson 1968; Azadi 1999). Robson (1968) expanded the Flora Iranica region by 21 species. H. fursei N. Robson and H. dogonbadanicum were described by Robson (1977) and Assadi (1980), respectively (1984). Assadi can only be found in Iran's north and southwestern regions. Azadi (1999) identified 19 species in the Flora of Egypt, four subspecies divided into five sections (Campylosporus (Spach) R. Keller, *Hypericum*, Hirtella Stef., Taeniocarpum Jaub. & Spach., and Drosanthe (Spach) Endl.) and two doubtful species (*H. heterophyllum* Vent. and H. Olivieri) and two doubtful species (H (Spach) The term "Hofariqun" was used by Bo Ebn Sina (or Bo Ali Sina) to denote *Hypericum* species in Iran (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 (Jia *et al.* 2020; Shi *et al.* 2021; Zheng *et al.* 2021; Zhu *et al.* 2021). The present study investigated the molecular variation of seven species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using NJ approaches. Current results have implications in breeding and conservation programs.

MATERIALS AND METHODS

Plants collection

Eighty-five (85) individuals were sampled. Seven *Hypericum* species in East Azerbaijan, Esfahan, Hamedan, Tehran, Mazandaran, Kermanshah and Kohgilouye-Boirahmad Provinces of Iran were selected and sampled during may-August 2014-2020 (Table 1). We employed 85 plant accessions (five to twelve samples from each community) from 7 distinct populations with various eco-geographic features for SRAP analysis, which were sampled and kept in -20 till further use. Table 1 provide further information on the geographical distribution of accessions.

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (10) and quantitative (11) morphological characters were studied. Data were transformed before calculation. Different morphological characters of flowers, leaves, and seeds were studied. Ordination analyses were conducted while using Euclidean distance (Podani 2000).

Sequence-related amplified polymorphism method

In each of the tested populations, fresh leaves were taken at random from one to twelve plants. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.,* 2019). SRAP assay was performed as described previously (Li and Quiros 2001). Five SRAP in different primer combinations were used (Table 2). A 25µl volume containing 10 mM of Tris-HCl buffer at pH 8; 50 mM of KCl; 1.5 mM of MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of

Table 1. Voucher details of *Hypericum* species in this study from Iran.

No	Section	Sp.	Locality
Sp1	*Hypericum*	*H. perforatum* L.	Esfahan:, Ghameshlou, Sanjab
Sp2	*Hirtella* Stef.	*H. lysimachioides* Boiss. & Noe in Boiss.	Kermanshah, Islamabad
Sp3		*H. asperulum* Jaub. & Spach.	Hamedan, Nahavand
Sp4		*H. helianthemoides* (Spach) Boiss.	Tehran, Damavand
Sp5		*H. vermiculare* Boiss. & Hausskn	Hamedan, Alvand
Sp6	*Taeniocarpium*	*H. hirsutum* L.	Mazandaran, Nowshahr
Sp7		*H. linarioides* Bosse.	Azarbaiejan, West of Tabriz

Table 2. SRAP primer information and results.

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	25	20	88.22%	0.29	34.71
Em2-Me2	14	14	100.00%	0.46	32.16
Em1-Me4	16	13	83.4%	0.35	40.16
Em2-Me4	13	13	100.00%	0.22	31.30
Em2-Me5	10	10	100.00%	0.40	49.94
Mean	17	16	93.50%	0.33	39.14
Total	76	70			198.33

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).

single primer; 20 ng of genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany) were subjected to PCR reactions. The overall reaction volume consisted of 25 µl. This PCR reaction was carried out in Techne thermocycler (Germany). The following cycles and programs were observed. The initial denaturation step was performed for 5 minutes at 94°C. The initial denaturation step was followed by 40 cycles for 1 minute at 94°C; 1 minute at 52-57°C, and 2 minutes at 72°C. The reaction was completed by a final extension step of 7-10 min at 72°C. Staining was performed with the aid of ethidium bromide. DNA bands/fragments were compared against a 100 bp molecular size ladder (Fermentas, Germany).

Data Analyses

Morphological characteristics were first normalized (Mean = 0, Variance = 1) before being utilized to calculate Euclidean distance between taxonomic pairs (Podani 2000). The UPGMA (Unweighted paired group using average) ordination techniques were utilized to group the plant specimens (Podani 2000).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were coded as binary characters (presence = 1, absence = 0). 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 calculated as previously suggested by Roldan-Ruiz *et al.* (2000). Resolving power for individual marker system was calculated as: $Rp = \Sigma Ib$. Ib (band informativeness) was estimated while following equation: proposed as: $Ib = 1 - [2 \times (0.5-p)]$. In the equation, p indicates the presence of bands (Prevost and Wilkinson, 1999). To quantify the capability of each primer to identify polymorphic loci among the genotypes, two measures, polymorphism information content (PIC) and marker index (MI) were utilized to assess its discriminatory ability (Powell *et al.* 1996). The Mantel test was used to see whether there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (2012) software were used to conduct these studies.

To reveal genetic differences across the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was implemented in GenAlex 6.4 (Peakall & Smouse 2006). Gene flow was calculated by I using PopGene ver. 1.32 (1997) to calculate Nm, an estimate of gene flow from Gst, as follows: Nm = 0.5(1 - Gst)/Gst. This method takes into account the same amount of gene flow in all populations. Gene flow was conducted in POPGENE software, version 1.32 (Yeh *et al*. 1999).

RESULTS

Mophometery

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 77% cumulative variation. The first PCA axis explained 43% of the total variation. The highest correlation (> 0.7) was shown by morphological characters such as calyx length, calyx width, corolla length, corolla color. The morphological characters of *Hypericum* species are shown in UPGMA tree (Figure 1). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Hypericum* species and separated each groups.

Species identification and genetic diversity

Five (5) suitable primer combinations (PCs), out of 10 PCs were screened in this research. Figure 2 illustrates the banding pattern of Em2-Me4, Em1-Me1 and Em2-Me2 primer by the SRAP marker profile. Seventy (70) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 150bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 20 for Em1-Me1 and 10 Em2-Me5, respectively. Each primer produced 16 polymorphic bands on average. The PIC ranged from 0.22 (Em2-Me4) to 0.46 (Em2-Me2) for the 5 SRAP primers, with an average of 0.33 per primer. RP of the primers ranged from 31.30 (Em2-Me4) to 49.94 (Em2-Me5) with an average of 39.14 per primer (Figure 2, Table 2). The calculated genetic parameters of *Hypericum* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.17 (*H. helianthemoides*) and 0.32 (*H. hirsutum*) with a mean of 0.32. Shannon's information index (I) was maximum in *H. hirsutum* (0.49), where as we recorded minimum Shannon's information index in *H. helianthemoides* (0.18). The observed number of alleles (*Na*) ranged from 0.113 in *H. perforatum* to 1.222 in *H. lysimachioides*. The significant number of alleles (*Ne*) ranged from 1.011 (*H. helianthemoides*) to 1.190 (*H. lysimachioides*).

Analysis of Molecular Variance results in significant genetic difference ($p = 0.01$) among *Hypericum* species. The majority of genetic variation occurred among species. AMOVA findings revealed that 75% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Hypericum* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.476, $p = 0.01$) and D_est values (0.843, $p = 0.01$) .

Different clustering and ordination methods produced similar results therefore, WARD clustering are presented here (Figure 3). In general, plant samples of each species belong to a distinct section, were grouped

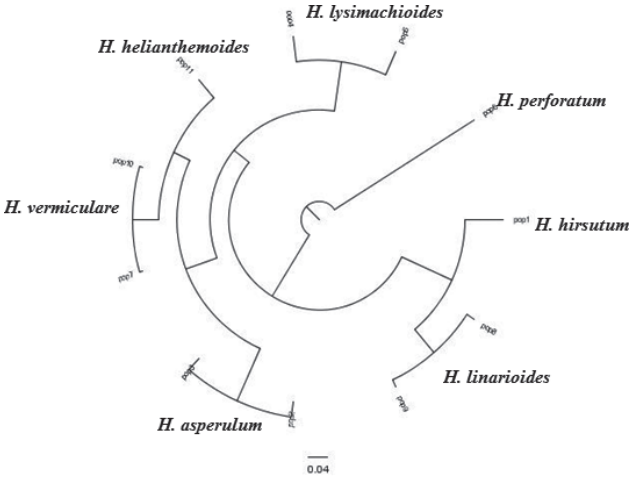

Figure 1. Morphological characters analysis of *Hypericum* species by UPGMA tree.

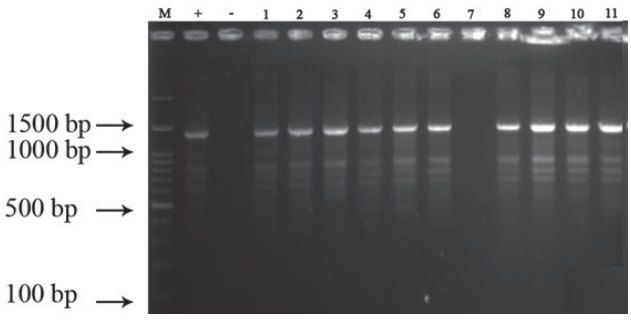

Figure 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,8: *H. perforatum*; 2, 9: *H. lysimachioides*; 3,10: *H. asperulum ;* 4, 11: *H. helianthemoides*; 5,12: *H. vermiculare*; 6,13: *H. hirsutum*; 7,14: *H. linarioides.*

Table 3. Genetic diversity parameters in the studied *Hypericum* species.

SP	N	Na	Ne	I	He	UHe	%P
H. perforatum L.	20.000	0.113	1.099	0.262	0.27	0.22	38.23%
H. lysimachioides Boiss. & Noe in Boiss.	17.000	1.222	1.190	0.211	0.284	0.292	25.91%
H. asperulum Jaub. & Spach.	12.000	0.228	1.180	0.414	0.22	0.25	46.50%
H. helianthemoides (Spach) Boiss.	15.000	0.288	1.011	0.181	0.19	0.17	16.11%
H. vermiculare Boiss. & Hausskn	9.000	0.352	1.083	0.27	0.29	0.24	45.05%
H. hirsutum L.	8.000	0.333	1.016	0.492	0.33	0.32	48.23%

Abbreviations: N = number of samples, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations.

Table 4. Molecular variance analysis.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	22	1116.114	77.111	24.100	75%	75%
Within Pops	112	55.455	18.27	10.133	25%	
Total	134	1656.127		34.022	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, ($P < 0.001$).

together and formed separate cluster. This result show that molecular characters studied can delimit *Hypericum* species in two different major clusters or groups. In the studied specimens we did not encounter intermediate forms. In general, two major clusters were formed in WARD tree (Figure 3), Populations of *H. perforatum* were placed in the first major cluster and were placed with great distance from the other species. The second major cluster included two sub-clusters. Plants of *H. hirsutum* and *H. linarioides* comprised the first sub-cluster, while plants of *H. lysimachioides*, *H. asperulum*, *H. helianthemoides* and *H. vermiculare* formed the second sub-cluster.

We detected strong correlation between geographical and genetic distances (r = 0.88, p=0.0002) and gene flow ($N_{m)}$ score of 0.265 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Supplementary Table). The findings suggested that there was the highest degree of genetic similarity (0.89) between *H. hirsutum* and *H. linarioides*. On the contrary to this, *H. perforatum* and *H. asperulum* (0.68) had lowest genetic resemblance.

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow or / and ancestrally shared alleles in the species studied. K-Means clustering showed k = 7 according to pseudo-F and k = 5 according to BIC. K = 7 is in agreement with WARD grouping and AMOVA. K = 7 reveal the presence of 7 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 7. The STRUCTURE plot (Figure not included) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and / or gene flow among *Hypericum* species. This plot revealed that genetic difference of species 1 and 2 (differently colored), as well as 3 and 4. This is in agreement with Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

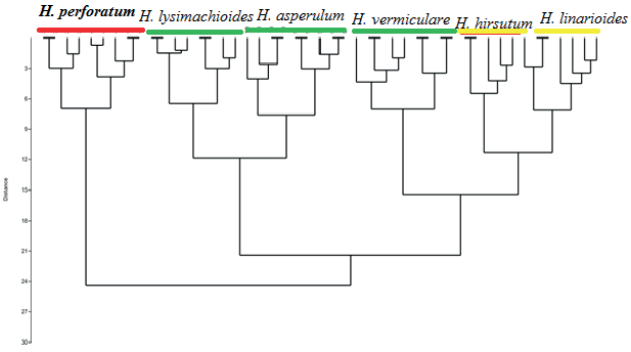

Figure 3. WARD tree of SRAP data revealing species delimitation in the *Hypericum* species.

The low Nm value (0.265) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species.

DISCUSSION

In the present study, we used morphological and molecular (SRAP) data to evaluate species relationships in *Hypericum* species. Morphological analyses of *Hypericum* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated from each other. PCA analysis suggests that morphological characters such as corolla color, pedicel hair, stem hair, leaf hair, petiole hair, width of petal have the potentials to identify and delimitate *Hypericum* species. Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Hypericum* species. Morphological characters including corolla color, pedicel hair, stem hair, leaf hair, petiole hair, width of petal 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 *Hypericum* members.

Given the negative impact of biodiversity threats and overexploitation of *Hypericum* plant species in Iran, it is necessary to conduct genetic diversity studies on *Hypericum* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017a,b,c,d). 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 this research, we reported PIC values of SRAP primers from 0.22 to 0.46, with a mean value of 0.33. PIC values indeed show low and high genetic diversity among genotypes. Values are ranging from zero to 0.25 show low genetic diversity; in contrast to this, 0.25 to 0.50 highlight mid-level of genetic diversity. In addition to this, values higher than 0.5 are associated with high genetic diversity (Tams *et al.* 2005). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Hypericum* species. In our study, SRAP markers detected average percentage of polymorphism (93.50%). Current research results also described average PIC values of SRAP makers (0.33) and average RP (resolving power) values i.e. 39.14 of SRAP markers. These current reported values are higher than other reported markers on *Hypericum* species (Maria et al. 2007; Dana et al. 2007). In the recent study, low gene flow (N_m) was detected among *Hypericum* species. The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Hypericum* species (Mantet test results). Several mechanisms, such as isolation, local adaptation, and genetic drift, shape the species or population differentiation (Frichot et al. 2013; De Kort et al. 2014). The magnitude of variability among *N*a, *N*e, *H,* and *I* indices demonstrated a high level of genetic diversity among *Hypericum* species. Dendrogram and principal component analysis results showed clear difference among *Hypericum* species. This shows the high utilization of the SRAP technique to identify *Hypericum* species . Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture.

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). 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).

Bi *et al* (2021) were conducted to study *Hypericum* genetic diversity by Random Amplified Polymorphic DNA (RAPD) from seventy plant specimens. They showed significant differences in quantitative morphological characters in plant species. *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 (*Na*) ranged from 0.22 to 0.53 in *H. dogonbadanicum* and *H. elongaturn*. Gene flow (Nm) was relatively low (0.87) in *Hypericum*.

Ma *et al.* (2021) conducted a study in Iran on identification of *Hypericum* population through morphological and ISSR Markers. They observed 10 primers produced 141 bands, of which 127 were polymorphic (95.78%). The obtained high average PIC and MI values revealed high capacity of ISSR primers to detect polymorphic loci among *Hypericum* species. The genetic similarities of 17 collections were estimated from 0.617 to 0.911. According to Inter-Simple sequence repeats (ISSR) markers analysis, *H. androsaemum* and *H. hirtellum* had the lowest similarity and the species of *H. perforatum* and *H. triquetrifolium* had the highest similarity.

Since widespread species may possess the higher levels of genetic diversity than narrowly distributed plants (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*. High among-population variation was previously reported in *Hypericum* species by Percifield *et al.* (2007), Pilepić *et al.* (2008), and Farooq *et al.* (2014). High differentiation among populations is mostly coupled with limited gene flow among them. The low gene flow and the high differentiation among populations has been explained mainly by founder events such as time since colonization (Jacquemyn et al., 2004).

CONCLUSIONS

The present study investigated the molecular variation of seven species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Hypericum* species. This was first attempt to assess genetic diversity through Sequence-related amplified polymorphism and morphometrics analysis in Iran. Current study reported two major clusters. These two major groups were separated on the basis of genetic and morphological characters. The genetic similarities between four species was estimated from 0.68 to 0.89. SRAP (Sequence-related amplified polymorphism) markers analysis, showed that *H. perforatum* and *H. asperulum* had the lowest similarity. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism envloved in the ecology of *Hypericum* species. Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinitiy among *Hypericum* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

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