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Morphometric analysis and genetic diversity in *Rindera* (Boraginaceae-Cynoglosseae) using sequence related amplified polymorphism

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Abstract. The genus *Rindera* comprises about 20–25 species distributed in central eastern Europe to central Asia. Ninety-five individuals related to six *Rindera* were collected in 9 provinces. A total of 147 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction amplifications (PCR) amplification of six *Rindera* species. These bands were produced with the combinations of 10 selective primers. The total number of amplified fragments ranged from 8 to 22.). The predicted unbiased heterozygosity (H) varied between 0.15 (*Rindera media*) and 0.30 (*Rindera regia*). High Shannon's information index was detected in *Rindera regia.* The genetic similarities between six species are estimated from 0.73 to 0.95. Clustering results showed two major clusters. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *Rindera regia* and *Rindera media* 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 *Rindera* species. Current results have implications in biodiversity and conservation programs.

Keywords: sequence-related amplified polymorphism, population structure, gene flow, network, genetic admixture, *Rindera.*

INTRODUCTION:

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; Guo, *et al.* 2021; Cheng, *et al.* 2021), and SRAP are potential markers to assess plant systematics and genetic diversity studies (Robarts and Wolfe 2014). These past studies showed that molecular markers, including SRAP markers, are efficient to investigate genetic diversity analyses and phylogenetic relationship among *Paracaryum* species in Boraginaceae family. The family Boraginaceae s.str consists of approximately 131 genera and 2,500 species, mainly distributed in dry, cliffy and sunny habitats of Eurasia, the Mediterranean region and the western North America (Binzet and Akcin 2009). They are mainly annual, bi-annual or perennial herbs and shrubs, some trees and a few lianes, distributed throughout the temperate and subtropical regions of the world (Retief and Vanwyk 1997), with a high distribution in Iran (Willis 1973). Given the negative impact of biodiversity threats and over exploitation of *Rindera* plant species in Iran, it is necessary to conduct genetic diversity studies on *Rindera* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017).

Subfamily Cynoglossoideae Weigend., is the largest subfamily having about 900 species and 50 genera. Recent molecular studies have shown that a wide range of the previously recognized tribes places into this subfamily (Chacón *et al.* 2016). The subtribe Cynogolossinae Dumort. (tribe Cynoglosseae W.D.J.Koch) is entirely restricted to the Old World, with a center of diversity in western Asia and the Mediterranean (Chacón *et al.* 2016).

The genus *Rindera* Pallas (1771: 486), comprises about 20–25 species distributed in central eastern Europe to central Asia (Bigazzi *et al.* 2006). This taxon is closely related to *Paracaryum* Boissier (1849: 128) and *Mattiastrum* Brand (1915: 150), nested in *Cynoglossum* Linnaeus (1753: 134) s.str. (Weigend *et al.* 2013, Weigend *et al.* 2016). All species of *Rindera* are perennial and linked to the dry and continental climate of the steppe and semidesertic belts (Bigazzi *et al.* 2006). *Rindera* is represented by 6 species in Iran, 4 of which *Rindera albida* (Wettst.) Kusn.; *Rindera bungei* (Boiss.) Gürke; *Rindera regia* Kusn., *rindera media* (Turrill) Riedl. are endemic (Khatamsaz 2001). *Rindera* is characterized by tubular corollas, stamens usually inserted at the throat of the corolla, with a style mostly exserted from the corolla, and usually eglochidiate large mericarpids with a broad, membranous wing (Bigazzi *et al.* 2006).

Rindera species are widely known as "Yünlü gelin" and used as an anti-inflammatory agent in Anatolian folk medicine (Altundag and Ozturk 2001). *R. lanata* is used to alleviate joint pains in Iranian folk medicine (Mosaddegh *et al.* 2012).

In order to develop conservation strategies and proper utilization of plant genetic resources, it is important to characterize plant species based on genetic studies (Kharazian *et al.* 2015), particularly this approach will serve better to understand genotypes of the geographically differentiated genus, such as *Echium L.* and *Onosma* (Boraginaceae) (Maria *et al.* 2007; Dana *et al.* 2007).

The present study investigated the molecular variation of six species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using WARD approaches. Current results have implications in breeding and conservation programs.

MATERIALS AND METHODS:

Plants collection

Ninety-five (95) individuals were sampled. Six *Rindera* species in west Azerbaijan, Mazandaran, Hamadan, Kurdistan, Esfahan, Semnan, Khorasan and Razavi Khorasan Provinces of Iran were selected and sampled during July-August 2018-2020 (Table 1). Morphometric and SRAP analyses on 95 plant accessions were carried out. Five to twelve samples from each population belonging to six different 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

Table 1. List of the investigated taxa including origin of voucher specimens. All material is collected by Majid Khayatneshad.

Taxa	Locality	Latitude	Longitude	Altitude(m)
Rindera albida (Wettst.) Kusn.	Kurdestan, Sanandaj Hamedan, 20km s of Nahavand	37°07'48"	49°54'04"	165
Rindera bungei (Boiss.) Gürke	Razavi Khorasan, Kashmar, Kuhsorkh District	37°07'08"	49°54'11"	159
Rindera lanata (Lam.) Bunge	Kurdestan, Sanandaj Esfahan, Ardestan on road to Taleghan	38°52'93"	47°25'92"	1133
Rindera cyclodonta Bunge	Bojnord, Ghorkhod protected area Semnan, 20km NW of Shahrud	38°52'93"	47°25'92"	1139
Rindera regia Kusn v	Mazandaran, 40 km Tonekabon to Janat abad Mazandaran, Nowshahr	35°50'36"	51°24'28"	2383
Rindera media (Turrill) Riedl n	West-Azarbaijan, Urumieh, Silvana	35°42'29"	52°20'51"	2421

Figure 1. Provinces and collection sites of *Rindera* species.

Table 2. SRAP primer information and results.

Primer name	NTL	NPL	P	PIC	RP
Em1-Me1	13	12	92.31%	0.44	43.77
Em2-Me2	12	12	100.00%	0.66	36.77
Em1-Me4	18	17	94.4%	0.43	40.46
Em2-Me4	15	15	100.00%	0.49	33.76
Em2-Me5	8	8	100.00%	0.44	50.99
Em3-Me4	10	10	100.00%	0.41	32.24
Em3-Me1	24	19	79.00%	0.30	26.55
Em4-Me1	11	11	100.00%	0.44	44.23
Em5-Me1	16	16	100.00%	0.47	38.55
Em5-Me2	22	22	100.00%	0.35	29.65
Mean	16	15	94.00%	0.48	37.55
Total	147	133			359.85

Abbreviations: NTL = Number of total loci; NPL = Number of polymorphic loci; P = Polymorphic ratio; PIC = Polymorphic information content; RP = Resolving power.

of species are mentioned (Table 1 and Fig 1).

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (3) and quantitative (4) 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:

Fresh leaves were used randomly from one to twelve plants. These were dried with silica gel powder. Genomic DNA was extracted while following previous protocol (Esfandani-Bozchaloyi *et al.* 2019). SRAP assay was performed as described previously (Li and Quiros 2001). Ten 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 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:

UPGMA (Unweighted paired group using average) ordination method was implemnented to assess morphological characters. ANOVA (Analysis of variance) was conducted to assess morphological differences among species. Principal component analysis (PCA) was implemented to identify variable morphological characters in *Rindera* species. Multivariate statistical analyses i.e., PC analysis, were performed in PAST software version 2.17 (Hammer *et al.* 2001).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were scored 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 calculated as previously suggested by Roldan-Ruiz *et al.* (2000). Resolving power for individual marker system was calculated as: Rp = ΣIb. 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). Pairwise genetic similarity between species was evaluated to reveal genetic affinity between species (Jaccard, 1908). Unbiased expected heterozygosity and Shannon information index were calculated 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 conducted in GenAlEx (Peakall and Smouse 2006). Mantet test was performed with 5000 permutations in PAST, version 2.17 (Hammer *et al.* 2001). The comparison of genetic divergence or genetic distances, estimated by pairwise FST and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test, as originally formulated in 1967,

$$Z_m = \sum_{i=1}^{n}\sum_{j=1}^{n} g_{ij} \times d_{ij}$$

where *gij* and *dij* are, respectively, the genetic and geographic distances between populations i and j, considering populations. Because Zmis given by the sum of products distances its value depends on how many populations are studied, as well as the magnitude of their distances. The Zm-value can be compared with a null distribution, and Mantel originally proposed to test it by the standard normal deviate (SND), given by SND =Zm/ var(Zm)1/2 (Mantel 1967). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software.

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 55% cumulative variation. The first PCA axis explained 40% 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 *Rindera* species are shown in WARD tree (Fig. 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Rindera species* and separated each groups. In *Rindera albida* and *R. bungei* nutlets are 8–14 mm, two-winged; outer wing 3 mm broad, margin undulate, inner 2 mm broad, incurved, margin cristate-dentate, glochids entirely absent, while in *R. lanata, R. cyclodonta* nutlets are 15.8–23 mm, smooth, wing with smooth or undulate often blue margin, without glochids.

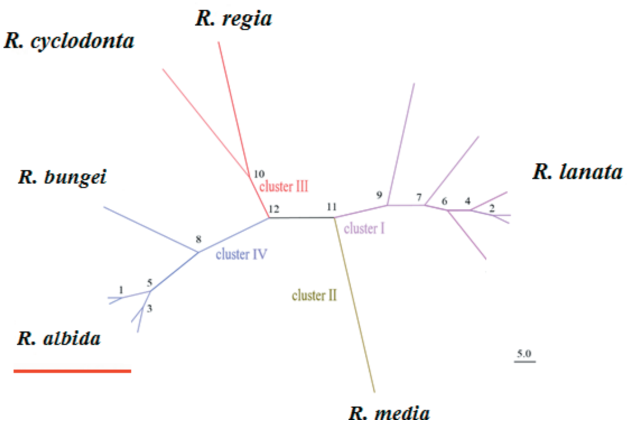

Figure 2. Morphological characters analysis of *Rindera* species by WARD.

Species identification and genetic diversity

Ten (10) suitable primer combinations (PCs), out of 25 PCs were screened in this research. Figure 3 illustrates the banding pattern of Em3-Me4, Em1-Me4, Em5-Me2 and Em1-Me1 primer by the SRAP marker profile. One hundered and thirty three (133) 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 22 and 8 for Em5-Me2 and 8 Em2-Me5, respectively. Each primer produced 15 polymorphic bands on average. The PIC ranged from 0.30 (Em3-Me1) to 0.66 (Em2-Me2) for the 10 SRAP primers, with an average of 0.48 per primer. RP of the primers ranged

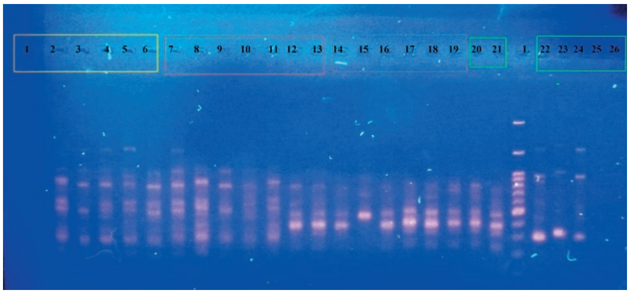

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,7,14,20: *Rindera albida* ; 2, 8,15,21: *Rindera bungei* ; 3,9, 16, 22: *Rindera lanata*; 4, 10, 17, 23: *R. cyclodonta;;* 5, 11, 18, 24: *Rindera regia* and 6, 12-13, 19, 25-26: *Rindera media;* L = Ladder 100 bp.

Table 3. Genetic diversity parameters.

SP	N	Na	Ne	I	He	UHe	P
Rindera lanata	8.000	0.333	1.016	0.192	0.17	0.22	48.23%
R. cyclodonta	12.000	1.155	1.190	0.271	0.184	0.192	55.91%
R. regia	5.000	0.358	1.440	0.374	0.30	0.29	66.50%
R. albida	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
R. bungei	5.000	0.462	1.095	0.288	0.25	0.22	62.05%
R. media	5.000	0.358	1.117	0.18	0.15	0.12	34.30%

Abbreviations: N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = genetic diversity, UHe = unbiased gene diversity, P = percentage of polymorphism, populations.

Table 4. Molecular variance analysis.

Source	df	SS	MS	Est. Var.	%	ΦPT
Among Pops	30	1501.364	92.789	16.154	82%	82%
Within Pops	100	334.443	3.88	2.888	18%	
Total	130	1955.807		20.060	100%	

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

from 26.55 (Em3-Me1) to 50.99 (Em2-Me5) with an average of 37.55 per primer (Fig. 3, Table 3). The calculated genetic parameters of *Rindera* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.15 (*Rindera media)* and 0.30 (*Rindera regia*) with a mean of 0.23. Shannon's information index (I) was maximum in *Rindera regia* (0.37), where as we recorded minimum Shannon's information index in *Rindera media* (0.18). The observed number of alleles (*N*a) ranged from 0.299 in *Rindera albida* to 1.155 in *Rindera cyclodonta*. The significant number of alleles (*N*e) ranged from 1.016 (*Rindera lanata*) to 1.440 (*Rindera regia*).

Analysis of Molecular Variance results in significant genetic difference ($p = 0.01$) among *Rindera* species. The majority of genetic variation occurred among species. AMOVA findings revealed that 82% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Rindera* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.66, $p = 0.01$) and D_est values (0.122, $p = 0.01$) .Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.77, P = 0.001). Therefore, the populations that are geographi-

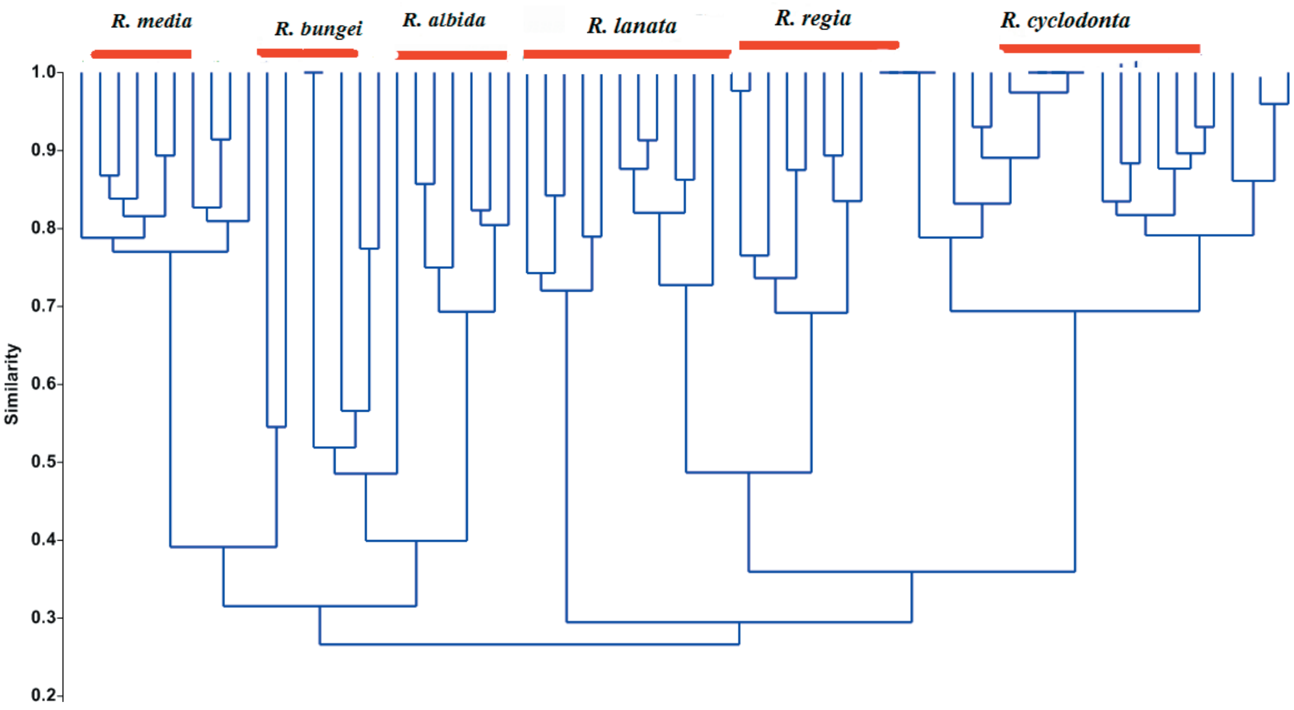

Figure 4. Dendrograms of *Rindera* species.

cally more distant have less amount of gene flow, and we have isolation by distance (IBD) in the *Rindera*.

The constructed dendrogram highlighted two major clusters (Fig. 4). Group A consisted of 3 species Rindera lanata; R. cyclodonta and Rindera regia .Two subclusters were in the B group: three species of Rindera bungei, Rindera albida and Rindera media.

We detected strong correlation between geographical *and gene*tic distances (r = 0.22, p=0.0002) and gene flow (N_m) score of 0.356 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 (0.95) between *Rindera lanata* and *R. cyclodonta*. On the contrary to this, *Rindera regia* and *Rindera media* (0.73) had lowest genetic resemblance.

DISCUSSION

In the present study, we used morphological and molecular (SRAP) data to evaluate species relationships in *Rindera* species. Morphological analyses of *Rindera* 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, nutlet shape, nutlet length, stamens position, nutlet margin, nutlet disc have the potentials to identify and delimitate *Rindera* species. Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Rindera* species. Morphological characters including nutlet shape, nutlet length, stamens position, nutlet margin 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 *Rindera* members.

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 0.30 to 0.66, with a mean value of 0.48. 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 *Rindera* species. In our study, SRAP markers detected average percentage of polymorphism (94%). Current research results also described average PIC values of SRAP makers (0.48) and average RP (resolving power) values i.e. 37.55 of SRAP markers. These current reported values are higher than other reported markers on *Rindera* species (Maria *et al.* 2007; Dana *et al.* 2007). In the recent study, low gene flow (N_m) was detected among *Rindera* species. Despite the presence of limited gene flow in *Rindera* species, two distinct ecotypes were reported previously. These ecotypes were formed due to reproductive isolation caused by altitude gradient and different niches (Moein *et al.,* 2019). The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Rindera* 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; Zhang *et al.* 2021; Zheng *et al.* 2021; Guo *et al.* 2021). The magnitude of variability among *N*a, *N*e, *H,* and *I* indices demonstrated a high level of genetic diversity among *Rindera* species. Dendrogram and principal component analysis results showed clear difference among *Rindera* species . This shows the high utilization of the SRAP technique to identify Salvia species. Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture.

CONCLUSIONS

The present study investigated the molecular variation of six species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Rindera* 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 six species was estimated from 0.73 to

0.95. SRAP (Sequence-related amplified polymorphism) markers analysis, showed that *Rindera regia* and *Rindera media* had the lowest similarity. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism envloved in the ecology of *Rindera* species. Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinitiy among *Rindera* 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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