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MOLECULAR AND MORPHOLOGICAL STUDY OF THE GENUS *Senecio* L. (ASTERACEAE: SENECIONEAE) IN IRAN

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The genus *Senecio* (Asteraceae, Senecioneae) with about 1250 species is one of the largest genera in the family. Due to historical and present time inter-specific hybridization and reticulate evolution in the genus, the morphological and molecular phylogenetic evolution are disjunct. The genus contains 17 species belonging to four sections, sect. *Crociseris*, sect. *Quadridentati*, sect. *Jacobaea* and sect. *Senecio*, in Iran out of which, six are endemic. Therefore, the present study was performed with the aim to provide data on the above issues. In general, ISSR molecular markers could delimit the studied *Senecio* species and revealed the species relationships, but did not support any of the sections. The ITS and cp-DNA sequencing of six species of *S. iranicus, S. vulcanicus, S. kotschyanus, S. paulsenii subsp. khorasanicus* and *S. joharchii* were obtained for the first time. S-DIVA suggests three possible ancestral ranges, of Kordestan (A), Mazandaran (G), and West-Azarbayejan (D), for *Sececio* species in Iran. These areas are located in the western parts of Iran. Mazandaran province played important role in the speciation process that led to the formation of endemic *Sececio*

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species in the country, while, Kordestan and West-Azarbayejan are the main road for the entrance of *Sececio* species from Europe via neighboring countries. The morphological characters used could also delimit the four sections of the genus *Senecio*. However, ISSR, cp-DNA and nuclear genes sequences-based phylogenetic trees portrayed the species relationship much different from that of morphological dendrogram.

Keywords: cpDNA, divergence time, ISSR, ITS, *Senecio* sect. *Crociseris*, *Senecio* sect. *Senecio*, *Senecio* sect. *Jacobaea*, *Senecio* sect. *Quadridentati*

INTRODUCTION

The genus *Senecio* L. (Compositae, Senecioneae) comprises ca. 1250 species and is one of the largest genera of flowering plants (BREMER, 1994; CALVO *et al.*, 2015). It is almost cosmopolitan, but occurs mainly in America, Asia and Africa. Fewer species occur in Australasia and Mesoamerica (NORDENSTAM *et al.*, 2009). Many of the *Senecio* species are poisonous due to the presence of pyrrolizidine alkaloids (PAs) (JEFFREY, 1978), while some species are ornamental, and some have antimicrobial properties and have been used as folk medicine (BELAUNDE *et al.*, 2007).

Previous study on species delimitation and species relationship performed in this genus revealed that *Senecio* s.str. is either para- or poly-phyletic (for example see BAIN and GOLDEN, 2000; COMES and ABBOTT, 2001; PELSER *et al.*, 2002; COLEMAN *et al.*, 2003; SWENSON and MANNS, 2003). The large size of the genus, the remarkable amount of morphological variation and widespread inter-specific hybridization are the main obstacles in the way of reaching to a proper infra-generic classification in *Senecio* (BREMER, 1994; PELSER *et al.*, 2007).

Species delimitation is a difficult task in species complexes (MEDRANO *et al.*, 2014). Hybrid speciation and reticulate evolution can be a component in the evolution of a species complex as also evidenced in many *Senecio* species (COMES and ABBOTT, 1999; ABBOTT *et al.*, 2000).

Attempts to reconstruct the phylogeny of speciose genera such as *Senecio* always faces difficulties in terms of sampling taxa to achieve the stable and effective results.

The genus *Senecio* in Iran is represented by four sections (NORDENSTAM, 1989): 1- *Senecio* sect. *Crociseris* (Rchb.) Boiss. 2- *Senecio* sect. *Senecio*, 3- *Senecio* sect. *Jacobaea* (Mill.) Dumort., and 4- *Senecio* sect. *Quadridentati* Boiss.

The genus *Senecio* after NORDENSTAM (1989) draft of Flora Iranica has been subject of several studies, partly circumscription of the genus have been changed. JEFFREY (1992) based on anatomy character transferred species that belonged to *Senecio* Sect. *Quadridentati* Boiss. to the genus *Iranecio*. Also PELSER *et al*. (2006, 2007) and NORDENSTAM (2006) based on molecular systematic studies regarded the section *Jacobaea* (Mill.) Dumort. as a distinct genus, in this paper only the genus *Senecio* sensu NORDENSTAM (1989) has been subject of studies and the genus *Jacobaea* Mill. is included in the genus *Senecio*. There are no reports available on the species delimitation, population structure, molecular features and interspecies relationships. In addition, we have no idea about divergence time of these species and their ancestral distribution area within the country. In the present study, we used 17 species of the genus *Senecio* with the following aims: 1- to study the species relationship and 2- to provide insight on divergence time of the species diversification in Iran.

MATERIALS AND METHODS

Plant material

Eighty accessions of 17 *Senecio* species were collected from natural habitats in Iran in 2014 (Table 1). *Senecio* species collected were from four sections: Sect. *Crociseris* (Reichenb.) Hall & Wohlf. (*Senecio pseudo-orientalis* Schischk., *S. doriiformis* subsp. *orientalis* (Fenzl) Matthews., *S. paulsenii* subsp. *khorasanicus* (Rech. f. & Aell.) B. Nord. and *S. joharchii* F. Ghahremaninjad, Ezazi, Rahchamani & Attar), Sect. *Quadridentati* Boiss. (*S. taraxacifolius* (M. B.) DC., *S. davisii* Matthews., and *S. lipskyi* Lomak.), Sect. *Jacobaea* (Mill.) Dumort. (*S. mollis* Willd., *S. erucifolius* subsp. *grandidentatus* (Ledeb) B.Nord.), and Sect. *Senecio* (*S.vulgaris* L., *S. iranicus* B. Nord., *S. breviflorus* (Kadereit) Greuter., *S. kotschyanus* Boiss., *S. krascheninnikovii* Schischk., *S. glaucus* L., *S. vulcanicus* Boiss., and *S. leucanthemifolius* subsp. *vernalis* (Waldst. & Kit.) Greuter). The voucher specimens are deposited in Herbarium of Tehran University (TUH), Herbarium of Shahid Beheshti University (HSBU) and Iranian Research Institute of Plant Protection (TARI) (Table 1).

Table 1. Senecio species studied, their locality information and voucher number.

Species	Locality	Lat	Long	Elevation (m)	Voucher number
1- *S. breviflorus* (Kadereit) Greuter	Karaj, Azimieh	51.00	35.50	1469	HSBU- 4369
2- *S. paulsenii* subsp. *khorasanicus* (Rech.f. & Aellen) B.Nord	Mazandaran, Balade to Noor	51.55	36.24	919	TUH- 38605
3- *S. pseudo-orientalis* Schischk.	West Azarbaijan, Shahindej	46.41	36.32	1799	TARI- 69861
4- *S. mollis* Willd	Kordestan, Sanandaj	47.05	35.39	2056	TUH- 40393
5- *S. lipskyi* Lomak.	East Azarbaijan, Mishodagh	45.47	38.13	1911	TUH-11951
6- *S. krascheninnikovii* Schischk.	Kohgiloye and	51.05	30.40	2586	TUH- 38628
7- *S. kotschyanus* Boiss.	Boyrahmad, Yasuj	57.14	29.26	3059	TUH- 23455
8- *S. iranicus* B.Nord.	Kerman, Gughar village Mazandaran, Polur	52.02	35.51	4300	IRAN-53397
9- *S. davisii* Matthews.	West Azarbaijan-Silvana	73.57	40.48	35	TARI- 69903
10- *S. vulgaris* L.	Tehran,Velenjak	51.23	35.48	1758	HSBU- 4372
11- *S. doriiformis* subsp. *orientalis* (Fenzl) V. A. Matthews.	Kordestan, Paveh	46.21	35.03	2600	TUH-7980
12- *S. erucifolius* subsp. *grandidentatus* (Ledeb.) V.E.Avet.	Golestan, Gorgan	55.39	37.21	531	IRAN-35506
13- *S. glaucus* L.	Kashan, Abyaneh	51.35	33.35	2216	HSBU- 4373
14- *S. joharchii* Ghahremaninejad et al.	Khorasan,Esfarayen	57.30	37.04	1253	FAR- 4692
15- *S. vulcanicus* Boiss.	Mazandaran,Kelardasht	51.08	36.29	4000	HSBU- 4374
16- *S. taraxacifolius* (M. B.) DC.	East Azarbaijan,	46.41	37.54	3700	
17- *S. leucanthemifolius* subsp. *vernalis*(Waldst. & Kit.) Greuter	Bostan abad Mazandaran, Alasht	52.50	36.04	1684	

Abbreviations: Lat = Latitude, Long = Longitude.

Morphological studies

Eighty plant accessions were collected from 25 geographical populations belong to 17 species of the genus *Senecio* and used for the present study. Morphological characters studied were: number of involucre bracts, number of calyculus bracts, number of lobes in disc flowers, length of corolla, length of anther in disc flowers, length of involucre bracts, length of style in disc flowers, ratio between number of ray florets and number of involucre bracts, type of inflorescence, length of petiole in basal leaf, limb of ray florets, nut indumentum, life forms or life-history strategies, indumentums of involucre bracts, base of anther and status of caulis leaf lobes.

DNA extraction and molecular assays

Fresh leaves were collected from randomly selected plants and dried in silica gel powder. Genomic DNA was extracted using a CTAB activated charcoal protocol (SHEIDAI *et al.*, 2013). The quality of extracted DNA was examined by electrophoresis on 0.8% agarose gels. Ten ISSR primers custom synthesized by UBC (the University of British Columbia) were used: $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9C$, UBC807, UBC811, UBC834 and UBC823. PCR reactions were performed in a 25µl volume containing at final concentrations 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany). The DNA amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 94°C, 30s at 94°C; 35s at 54.7°C and 1 min at 72°C. The reaction was completed with a 7 min extension step at 72°C. The amplification products were visualized by electrophoresis on 2% agarose gels, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). The quality and quantity of extracted DNA were assessed by running on 0.8% agarose gel and NanoDrop® spectrometer respectively.

The ITS1 region was amplified with 0.2 µM primer ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´; Bioron, Germany), and primer ITS2 (5'- GCT GCG TTC TTC ATC GAT GC-3') (White et al. 1990). PCR reactions were performed in a 25µl volume containing at final concentration 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP (Bioron, Germany), 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany). The following thermocycler parameters were used: 95°C for 2 min, followed by 33 cycles at 95°C for 30s, 56°C for 60s, and 72°C for 2 min, followed by one final extension step at 72°C for 7 min.

The plastid intergenic spacer psbA-trnH^{GUG} was amplified and sequenced with universal primers following the methodology of SHAW *et al.* (2005) and TIMME *et al.* (2007). The psbA-trnH^{GUG} forward primer was trnH^{GUG} (5´-CGC GCA TGG TGG ATT CAC AAT CC-3´) and, the reverse primer was psbA (5´- GTT ATG CAT GAA CGT AAT GCT C-3´) (Table 2). Each 20 µl PCR reaction contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of Taq DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng μl^{-1}. The amplifications were performed in a Techne thermocycler (Germany) with the following program: 2 min initial denaturation step 94°C, 1 min at 94°C; 1 min at 58°C and 1 min at 72°C. The reaction was completed by a final extension step of 6 min at 72°C. The

PCR products were electrophoresed on 2.5% agarose gels and visualized through GelRed™ Nucleic Acid Gel Staining. Fragment size was estimated using a 100 bp size ladder (Thermo-Fisher Scientific, Waltham, MA USA).

Table 2. Primers used in PCR reactions.

Fragment to be Amplified	Primer Name (and Direction)	Primer Sequence
trnH-psbA(Chloroplast)	psbA (forward)	GTTATGCATGAACGTAATGCTC (SHAW *et al*., 2005)
trnH-psbA(Chloroplast)	H (reverse)	CGCGCATGGTGGATTCACAATCC (SHAW *et al*., 2005)
ITS (Nuclear)	ITS5 (forward)	5'-GGA AGT AAA AGTCGTAACAAG G-3' (WHITE *et al*., 1990)
ITS (Nuclear)	ITS4 (reverse)	5'-TCC TCC GCT TATTGA TAT GC -3' (WHITE *et al*., 1990)

Data analysis

Morphological analyses

Grouping of the species was obtained by using UPGMA (Unweighted Paired Group Method with Arithmetic mean) and Ward (Minimum spherical cluster method) as well as PCoA (Principal Coordinate Analysis) (PODANI, 2000). Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distances among pairs of taxa (PODANI, 2000; SHEIDAI *et al.*, 2014). The obtained distances were used for clustering. Morphological difference of the studied species was investigated by ANOVA (Analysis of Variance) and CVA (Canonical Variance Analysis). PCA (Principal Components Analysis) was performed to identify the most variable morphological characters (PODANI, 2000). Morphometric analyses were performed by PAST ver. 2.17 (HAMMER *et al.*, 2012).

ISSR analysis

Significances of genetic differences among the studied species was determined by AMOVA (Analysis of Molecular Variance) with 1000 permutations for dominant molecular markers as implemented in GenAlex v.6.4 (PEAKALL and SMOUSE, 2006). Nei's genetic distances were determined among the studied species and used for clustering. For grouping of the plant specimens, Neighbor Joining (NJ) clustering and PCoA were used (PODANI, 2000). GenAlex 6.4, and PAST v.2.17 (HAMMER *et al.*, 2012), programs were used for these analyses. The genetic structure of species was studied by STRUCTURE (PRITCHARD *et al.*, 2000), for dominant markers (FALUSH *et al.*, 2007), using the admixture model. The Markov chain Monte Carlo simulation was run 20 times for each value of K for 106 iterations after a burn-in period of 105. All other parameters were set at their default values.

Cp-DNA and nuclear gene sequence analyses

The intergenic chloroplast spacer psbA-trnH^{GUG} as well as the ITS region nuclear sequences obtained for all the studied species. The sequences obtained were aligned by MUSCLE program implemented in MEGA ver. 5 (TAMURA *et al.*, 2011), and used to study the species relationship by different phylogenetic reconstruction methods like: Neighbor Joining, UPGMA clustering (Unweighted paired group using average), and maximum parsimony and

maximum likelihood using MEGA v.5 (TAMURA *et al.,* 2011). The proper model for sequence evolution was determined by the same option provided in MEGA program.

The molecular clock test was performed as implemented in MEGA v.5. The test was done by comparing the Maximum likelihood value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model. Genetic differentiation versus species gene flow was checked by DnaSP v.5 (LIBARDO and ROZAS, 2009). For this, samples of each species or species placed in one cluster can be compared with the other species or group of species.

The occurrence of gene flow or the presence of shared alleles for ITS sequences was determined by horizontal gene transfer detection (HGT) method as emplimented in T-REX program (BOC *et al.,* 2012). In this program species tree and gene tree (ITS tree in this case) are compared and based on the least square method, HGT is determined to occur in the species involved.

Time of species divergence

For rooting in estimating divergence time, we first carried out phylogenetic analysis based on ITS sequences of the studied *Senecio* species along with the other sections representatives, as the outgroups. BEAST v1.6.1 (DRUMMOND *et al.,* 2010a, b) was used for the Bayesian MCMC inferred analyses of the nucleotide sequence data (DRUMMOND and RAMBAUT, 2007). BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (DRUMMOND *et al.,* 2010a, b) was utilized to generate initial xml files for BEAST. A Yule process of speciation ('a pure birth' process) was used as a tree prior for all the tree model analyses. The Yule tree prior is widely recognized as giving the best-fit model for trees describing the relationships between different species. The parameter can be regarded as explaining the net speciation rate (NEE, 2006). For the MCMC posterior analyses, the length of chain was 10000000. After 100 trees burn-in processing, 10000 trees were used for the analyses. The BEAUti xml file (DRUMMOND *et al.*, 2010a, 2010b) was run in the BEAST v1.6.1 program (DRUMMOND *et al.,* 2010a, 2010b) and the maximum clade credibility (MCC) chain generations were repeated five times for each molecular clock model with independent runs to ensure suitable convergence and adequate mixing. The MCC tree was generated under the relaxed clock model (HKY substitution). Because no fossils are available for the studied species, we used a rate of evolution of the ITS sequence ($u = 4.13 \times 10^{-9}$ substitutions per site per year; KAY *et al.,* 1984).

Tracer v1.5 software (RAMBAUT and DRUMMOND, 2007) was used for the output of the model parameters to examine the sampling and convergence results obtained from BEAST. TreeAnnotator v1.6.1 software (RAMBAUT and DRUMMOND, 2007) was used to annotate the phylogenetic results generated by BEAST as a form of single 'target' tree. On the target trees are shown summary statistics of posterior probabilities of the nodes: the 95% highest posterior density (HPD) limits of the node heights, rates and the posterior estimates. For the annotated BEAST MCC tree output analyses, the FigTree v1.3.1 (RAMBAUT, 2009) program was also used. The posterior probability was set to 0.5. This is equivalent to the bootstrapping value in PAUP (Phylogenetic Analysis Using Parsimony analysis) analyses. (WAN-PYO HONG and JURY, 2011).

Biogeography

For biogeography, we used RASP (Reconstruction Ancestral State in Phylogenies) program. In this program two methods are available to carry on the analysis, 1- S-DIVA

(Statistical Dispersal-Vicariance Analysis) and, 2- BBM (Bayesian Binary Method) analyses (YU *et al.*, 2010). In these methods, the frequencies of an ancestral range at a node in ancestral reconstructions are averaged over all trees (YAN *et al.*, 2011). To account for uncertainties in phylogeny, we used the tree obtained from BEAST analysis. The possible ancestral ranges at each node on a selected tree were obtained.

Phylogenetically based historical biogeographical reconstructions have been used to illuminate the evolutionary history of organisms in space and time (SYED SHUJAIT *et al.*, 2012). RASP (Reconstruct Ancestral State in Phylogenies) (YAN *et al.*, 2011) is a useful tool to reconstruct evolutionary histories in phylogeny, by using three different methods, S-DIVA, Bayesian binary MCMC (BBM) and maximum-parsimony (MP) analysis (YAN *et al.*, 2011). These methods obtain the ancestral ranges at each node. Therefore, we used RASP to illustrate the ancestral nodes in *Senecio* species diversification in Iran.

RESULTS

Morphometry

Different clustering methods like UPGMA and Ward produced similar results and both had high cophenetic correlation values (>0.85). Therefore, only the Ward dendrogram is presented here (Fig. 1). The specimens studied in each *Senecio* species were placed in species-specific clusters, separating the studied species from each other (Species 9 had only one specimen). The ANOVA showed significant differences ($p < 0.05$) for the quantitative morphological characters among the *Senecio* species (data not given). Moreover, the CVA revealed significant differences among the studied species.

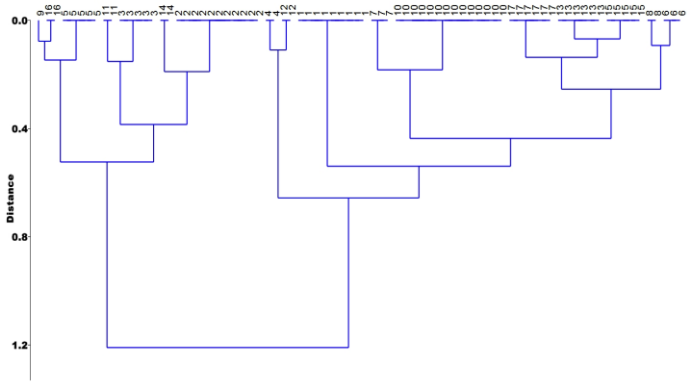

Fig. 1. Ward dendrogram of morphological characters among *Senecio* species (Species code 1-17 are according to Table 1).

PCA analysis revealed that the first four components comprised about 88% of the total morphological variability. The PCA plot of both quantitative and qualitative morphological characters separated the studied species into four groups that corresponded to the four sections recognized by NORDENSTAM (1989) (Fig. 2). *Senecio vulgaris*, *S. glaucus*, *S. leucanthemifolius*

subsp *vernalis*, *S. iranicus*, *S. vulcanicus*, *S. kotschyanus*, *S. kraschenininkovii* from section *Senecio* (blue box) were placed close to each other. Within this section, *S. iranicus* (sample 8) showed morphological similarity with *S. glaucus* (sample 13), while, *S. kotschyanus*, *S. kraschenininkovii* and *S. leucanthemifolius* subsp. *vernalis* were grouped together. Similarly, *S. mollis* and *S.erucifolius* subsp. *grandidentatus* of section *Jacobaea* (orange box) were placed close to each other, while *S. paulsenii* subsp. *khorasanicus*, *S. pseudo-orientalis*, *S. doriiformis* subsp. *orientalis* and *S. joharchii* of section *Crociseris* (pink box) were placed together. Within this section, *S. joharchii* (sample 14) showed morphological similarity with *S. doriiformis* subsp. *orientalis* (sample 11), while *S. paulsenii* subsp. *khorasanicus* and *S. pseudo-orientalis* were placed closer to each other. *Senecio taraxacifolius*, *S. lipskyi* and *S. davisii* of section *Quadridentati* (green box) were placed close to each other. Therefore, morphological characters used in this study, almost separated the studied sections.

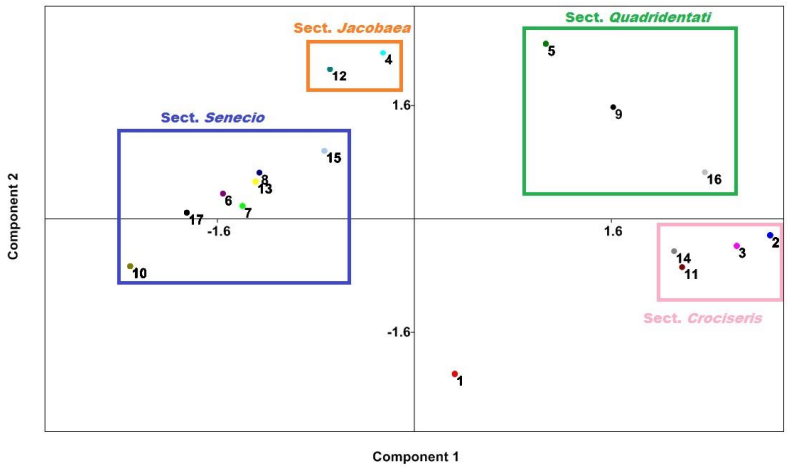

Fig. 2. PCA plot of morphological characters (Species code 1-17 are according to Table 1).

Morphological characters like corolla length, anther length, style length in disc floret, life-history strategy, petiole length of basal leaf and status of cauline leaf lobes showed the highest positive correlation (> 0.80) with the first PCA component, while the number of stamen, base of anther, number of lobes of disc florets, showed the highest positive correlation (> 0.50) with the second PCA component. These characters may be focused on in the taxonomy of the genus and the delimitation of *Senecio* species.

ISSR markers

We obtained 105 reproducible ISSR bands from almost all ISSR primers used. These bands formed our initial data matrix. AMOVA revealed significant genetic difference among the studied species (PhiPT = 0.83, $P = 0.01$). It also revealed that 16% of total variance occurred due within species genetic variability, while 84% was due to among species genetic difference.

The reconstructed NJ tree (Fig. 3) separated the individual samples studied from the 17 studied species in species-specific clusters, except for species 4 and 5, and 6 and 7. Thus, the ISSR makers are perhaps suitable for species delimitation in the genus *Senecio*, but more number of species should be studied.

Senecio paulsenii subsp. *khorasanicus*, *S. pseudo-orientalis*, *S. doriiformis* subsp. *orientalis* and *S. joharchii* of the section *Crosiceris* were placed in a single cluster, together with *S. davisii* of section *Quadridentati*. Very close genetic affinity was observed between *S. kotschyanus* and *S. kraschenininkovii*, while *S. iranicus* joined them with some distance. Similarly, the close genetic affinity observed between *S. leucanthemifolius* subsp *vernalis* and *S. glaucus*. It also holds true for *S. paulsenii* subsp. *khorasanicus* and *S. pseudo-orientalis* of the sect. *Crociseris*. The STRUCTURE plot revealed that the studied species differed in their allele composition (Fig. 4). The occurrence of similarly colored segments in all the studied species indicated the presence of shared alleles. However, the proportion of these shared alleles differed in these species. Such differences might have occurred during species diversification. The genetic affinity revealed by STRUCTURE analysis was almost in agreement with the NJ tree result.

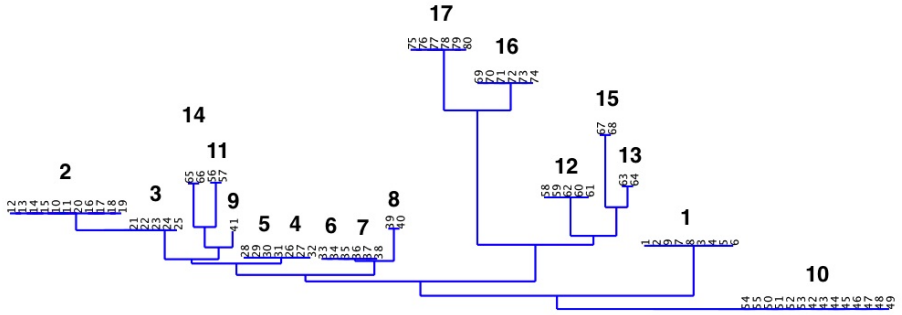

Fig. 3. Neighbor Joining tree of ISSR data using Nei's genetic distance of the studied Senecio species. (Species numbers are according to Table 1).

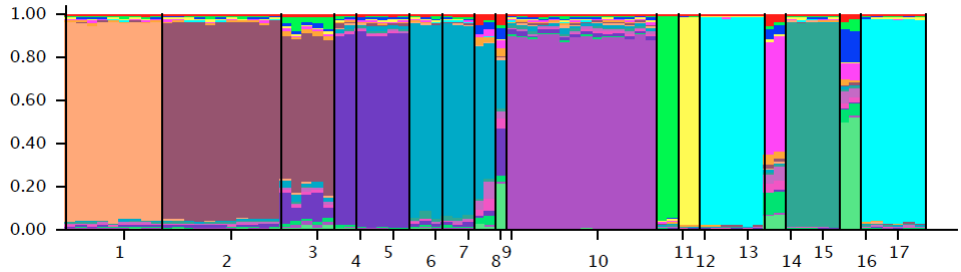

Fig. 4. STRUCTURE plot of Scenecio species based on ISSR data (Species code 1-17 are according to Table 1).

ITS and Cp-DNA sequences

Individual phylogenetic trees of Cp-DNA and ITS, had some disagreement, therefore, combined ITS and Cp-DNA sequences were used for phylogenetic analysis. NJ, Maximum parsimony and maximum likelihood trees produced similar results and therefore MP tree is presented and discussed (Fig. 5). The species of the sect. *Senecio* were placed close to each other and formed a single clade with high bootstrap value (89%). *Senecio mollis* and *S. erocifolius* of the sect. *Jacobaea* formed a single clade with high bootstrap value (95%). Also, *S. taraxacifolius* and *S. davisii* of the sect. *Quadridentati* were placed in a single clade with a good bootstrap value (87%), while *S. lipskyi* of the same section has evolved in a distinct way.

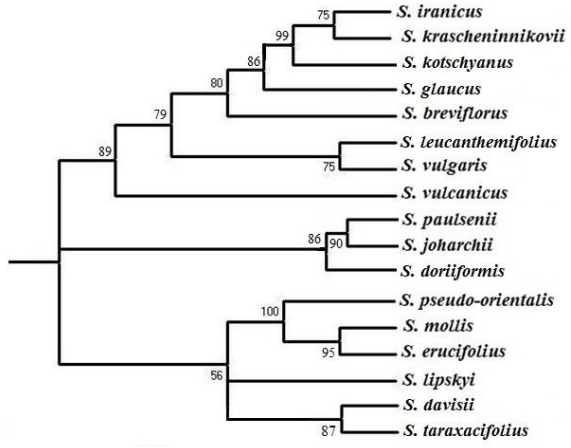

Fig. 5. Maximum parsimony tree of the combined ITS and cp-DNA data (values above branches are bootstrap values).

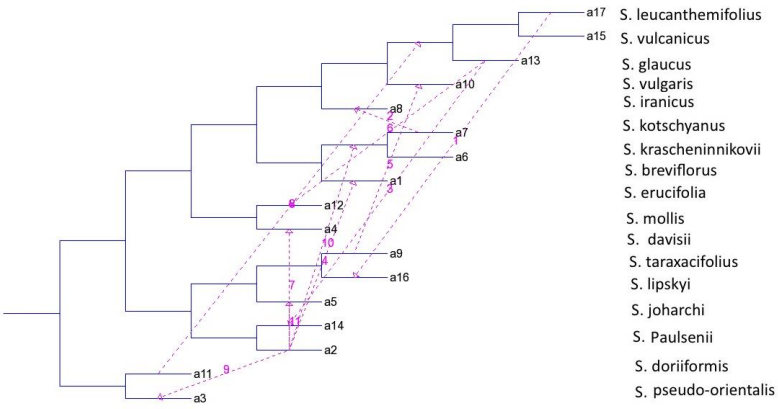

Fig. 6. HGT tree of T-REX showing gene flow and shared alleles (dashed lines) among the studied Senecio species.

DnaSP produced no significant genetic differentiation between ITS clusters, and revealed high level of gene flow among the studied species (Fst: 0.12, Nm: 1.85). This is supported by HGT tree produced by T-REX (Fig. 6). HGT tree revealed the presence of gene flow and shared alleles in most of the species and even between those species that were placed in different clusters. For example, gene flow occurred between *S. leucanthemifolius* and *S. lipskyi* that belong to two different clusters. This is in agreement with STRUCTURE plot of ISSR data presented before.

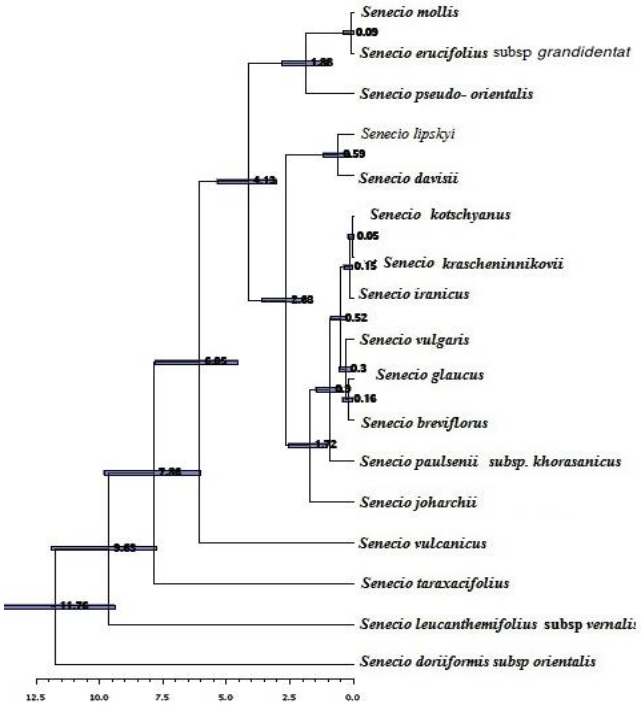

Fig. 7. The BEAST tree for Senecio species based on ITS data (Node bars are 95% posterior probability values).

Species divergence time

Pylogenetic tree obtained from ITS analysis of the studied *Senecio* species and the species representative from the other sections, put *Senecio doiiformis* and *S. leucanthemifolius* at the basal node of the sect. *Senecio* studied (Figure not given). For ITS data, the molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Kimura 2-parameter model (+G) and the null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P = >0.05). Therefore, relaxed molecular clock model was used in BEAST analysis. The oldest node of *Senecio doiiformis* appeared about 11 Mya, followed by *S. leucanthemifolius* in about 9 Mya. However, active radiation occurred from 2-5 Mya (Pliocene Epoch; 5.3 to 2.58 mya) (Fig. 7). This is in agreement with the study of PELSER *et al.* (2010).

Biogeography

S-DIVA and Bayesian binary MCMC methods of RASP produced similar results (Fig. 8). S-DIVA suggests a complex biogeographical history in which 27 global dispersal, 13 vicariance and 1 extinction were vital in the shaping of the current distribution pattern in *Scenecio* species in Iran.

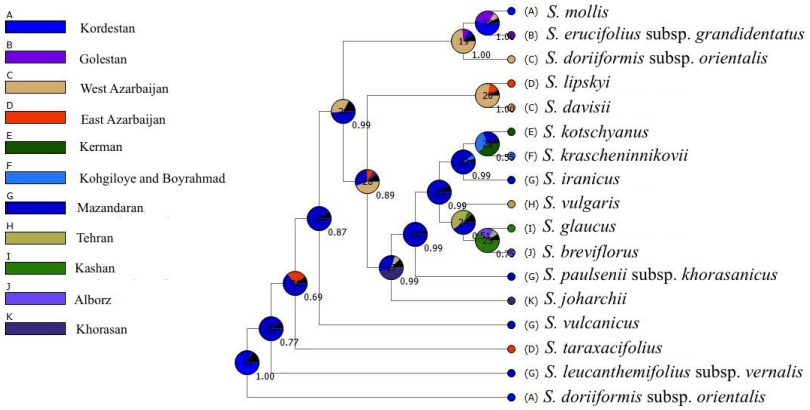

Fig. 8. RASP diagram showing ancestral distribution area of Senecio species in Iran.

DISCUSSION

Species delimitation is the first step toward understanding the evolution of plants and mechanisms of their divergence. However, it is a difficult task in plant species with recent speciation events and the complex species that faced hybridization and reticulate evolution in their history (MEDRANO *et al.*, 2014). Species delimitation in the genus *Senecio* is considered to be of taxonomic and phylogenetic importance that can be achieved through molecular studied (PELSER *et al.*, 2007). Moreover, molecular phylogenetic methods can be utilized to revise the morphology-based infra-generic classifications in plant taxonomy and help to achieve a sectional classification reflecting the evolutionary history of the group. This in turn can provide insight about the process of speciation in plants including the genus *Senecio* (PELSER *et al.*, 2005; LANGEL *et al.*, 2011).

In our study, morphological characters and ISSR data could delimit the studied *Senecio* species. Use of multilocus molecular markers in species delimitation of different plant groups were reported by other workers too (see for example, MEUDT *et al.*, 2009; SHEIDAI *et al.*, 2012, 2013).

We studied three species from the sect. *Quadridentati*, the phylogenetic tree placed two species of *Senecio lipskyi* and *S. davisii* of this sect. in a single clade, but the third species was placed in another clade. Similarly, the studied species of the sect. *Senecio* were scatted in two different clades. *Senecio vulcanicus* and *S. leucanthemifolius* subsp. *vernalis* were placed far from the other species in this sect. showed closer affinity with *S. taraxacifolius* of the sect. *Quadridentati.*

Disagreement between morphological and molecular phylogenetic trees is frequently observed in different sections of *Senecio*. For example, based on molecular data, the section

Jacobeae has been considered to be a distinct genus (PELSER *et al.*, 2006) by some authors (GREUTER, 2008; NORDENSTAM *et al.*, 2009; HAMZAOĞLU *et al.*, 2011). However, some others do not recognize it due to the inability to establish a morphological delimitation of *Jacobaea* accompanied by an morphological overlap shared with several species of *Senecio* that grow in similar habitats and within overlapping distribution areas (HULBER *et al.*, 2009; BLANCA and QUESADA, 2009).

The contradiction between morphological and molecular phylogeny has been noticed in many plant groups even by molecular barcoding methods (using the combination of matK and rbcL; CBOL Plant Working Group 2009), especially for taxa in which molecular variability does not match with morphological differences (FEDERICI *et al.*, 2013). Controversies and difficulties in *Senecio* phylogeny and species delimitation rise possibly due to rapid evolutionary diversification and/or hybridization within the genus (KIRK *et al.*, 2004; PELSER *et al.*, 2010, 2012).

Based on morphological similarity (GHAHREMANINEJAD *et al.*, 2010) considered *S. joharchii* closely related to *S. paulsenii*. The present study supports this suggestion and shows close affinity between *S. joharchii* and *S. paulsenii* in combined ITS and cp-DNA tree. High degree of gene flow may correspond to ancient as well as ongoing events among these species (SHEIDAI *et al.*, 2014). In general it seems that the molecular markers used could delimit the species, particularly ISSR markers grouped plant specimens of each species in separate clusters. However, all molecular markers used in this study inter-mixed the species from different sections together, which indicates that the present taxonomic treatment of the genus *Senecio* is not compatible with the species relationship as evidenced my molecular markers. This result is in agreement with the idea of polyphyletic nature of the genus *Senecio*. The ITS and cp-DNA sequencing of 6 species of *S. iranicus*, *S. vulcanicus*, *S. kotschyanus*, *S. paulsenii subsp. khorasanicus* and *S. joharchii* were done for the first time and used for species delimitation.

The oldest node of *Senecio doiiformis* appeared about 11 mya, followed by *S. leucanthemifolius* in about 9 mya. However, active speciation seems occurred from <1 mya. This is in agreement with the study of PELSER *et al.* (2010). These authors also revealed some degree of incongruence between Cp-DNA and ITS-ETS divergence estimation time estimation possibly due to wide occurrence of ancient hybridization events in *Senesio*. The studied *Senecio* species mostly occur in mountains and regions of high elevations in Iran. The climatic oscillations during Pleistocene imposed important range shifts on the paloarctic biota and shaped the demographic history and genetic diversity of the European flora. The ice sheets of the Northern Hemisphere first developed about 2.5 My ago, and a series of major climatic oscillations occurred during last 700 Kyr with dominant 100 Kyr glacial-interglacial cycles causing the European biota to experience marked and repeated climatic change. Cooling of the climate during glaciation forced temperate species to retreat into fragmented distribution ranges in Caspean/Caucasus region. These areas acted as refugia, and many temperate species now show taxonomic and genetic diversity within and among these regions. Conversely, alpine plants exhibited a more continuous distribution during glaciation in Mediterranean Basin and were isolated in high-altitude mountains during the Pleastocene (PEREDO *et al.*, 2009). Many *Senecio* species exhibit altitude differentiation and adaptaion (BARRY COX and MOORE, 2010).

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REFERENCES

ABBOTT, R. J., J.K. JAMES, J.A. IRWIN, H.P. COMES (2000). Hybrid origin of the Oxford ragwort, *Senecio squalidus* L..Watsonia, *23*: 123-138.

BAIN, J. F., J.L. GOLDEN (2000). A phylogeny of *Packera* (Senecioneae; Asteraceae) based on internal transcribed spacer region sequence data and a broad sampling of outgroups. Mol. Phylogenet. Evol., *16*: 331-338.

BARRY, COX C., P.D. MOORE (2010). Biogeography: An Ecological and Evolutionary Approach, eighth ed. John Wiley & Sons, Eugene, OR, U.S.A.

BELAUNDE, A.J., J.G. SANDOVAL, L.D. MARTINO, F. SENATORE, V.D. FEO (2007). Chemical composition and antibacterial activity of *Senecio nutans* essential oil. J. Essent. Oil. Bear. Pl., *10*: 332-338.

BLANCA, G., C. QUESADA (2009). Senecio L.: 316–326. In: Blanca, G., Cabezudo, B., Cueto, M., Fernandez Lopez, C. & Morales Torres, C. (eds.), *Flora vascular de Andalucia Oriental*: Consejeria de Medio Ambiente, Junta de Andalucia, Sevilla, Spain.

BOC, A., B. DIALLO ALPHA, V. MAKARENKOV (2012). T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks, Nucleic Acids Research, *40*(W1), W573-579.

BREMER, K. (1994). Asteraceae: cladistics and classification. Timber Press, Portland, Oregon,USA.

CALVO, J., I. ÁLVAREZ, C. AEDO (2015). Systematics of *Senecio* section *Crociseris* (Compositae, Senecioneae). Phytotaxa, *211*: 001-105.

COLEMAN, M., A. LISTON, J.W. KADEREIT, R.J. ABBOTT (2003). Repeat intercontinental dispersal and Pleistocene speciation in disjunct Mediterranean and desert *Senecio* (Asteraceae). Am. J. Bot., *90*: 1446–1454.

COMES, H. P., R.J. ABBOTT (1999). Reticulate evolution in the Mediterranean species complex of *Senecio* sect. *Senecio*: uniting phylogenetic and population-level approaches: 171–198. In: Hollingsworth P.M., R.M. Bateman, R.G. Gornall (eds.). Molecular systematics and plant evolution. CRC Press.

COMES, H.P., R.J. ABBOTT (2001). Molecular phylogeography, reticulation and lineage sorting in the Mediterranean species complex of *Senecio* sect. *Senecio* (Asteraceae). Evolution, *55*: 1943–1962.

COUVREUR, T. L. P., L.W. CHATROU, M.S.M SOSEF, J.R. RICHARDSON (2008). Molecular phylogenetics reveal multiple tertiary vicariance origins of the African rain forest trees. BMC Biology, *6*:54.

DRUMMOND, A.J., A. RAMBAUT (2007). BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol., *7*: 214. http://dx.doi.org/10 .1186/1471-2148-7-214.

DRUMMOND, A.J., A. RAMBAUT, M.A. SUCHARD (2010a). BEAST version 1.6.1 (computer program). http://beast.bio.ed.ac.uk.

DRUMMOND, A.J., A. RAMBAUT, W. XIE (2010b). BEAUti version 1.6.1 (computer program). http://beast.bio.ed.ac.uk .

FALUSH, D., M. STEPHENS, J.K. PRITCHARD (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol. Ecol. Notes**,** *7*:574-578.

FEDERICI, S., A. GALIMBERTI, F. BARTOLUCCI, I. BRUNI, F. DE MATTIA, P. CORTIS, M. LABRA (2013). DNA barcoding to analyse taxonomically complex groups in plants: the case of Thymus (Lamiaceae). Bot. J. Linn. Soc., *171*: 687-699.

GHAHREMANINEJAD, F., A. EZAZI, N. RAHCHAMANI, F. ATTAR (2010a). A new *Senecio* L. (*Asteraceae*: *Senecioneae*) from Northeast Iran. Feddes Repert., *121*: 27–31.

GREUTER, W. (2008). *Senecio* L, pp. 711–720. In: Greuter W., E. von. Raab-Straube (Eds.), Med-Checklist, Dicotyledones (Compositae), vol. 2. Conservatoire et Jardin botaniques de la Ville de Geneve, Switzerland.

HAMER, Ø., D.A. HARPER, P.D. RYAN (2012).PAST: Paleontological Statistics software package for education and data analysis. Palaeontol. Electron., *4*: 9.

HAMZAOĞLU, E., U. BUDAK, A. AKSOY (2011). A new genus, Turanecio, of the Asteraceae (tribe Senecioneae). Turk. J. Bot. *35*: 479–508.

HONG, S.W.P., S.L. JURY (2011). Phylogeny and divergence times inferred from rps16 sequence data analyses for *Tricyrtis* (Liliaceae), an endemic genus of north-east Asia. AoB PLANTS plr 025.

HULBER, K., M. SONNLEITNER, R. FLATSCHER, A. BERGER, R. DOBROVSKY, S. NIESSNER, T. NIGL, G.M. SCHNEEWEISS, M. KUBEŠOVA, J. RAUCHOVA, J. SUDA, P. SCHONSWETTER (2009). Ecological segregation drives fine-scale cytotype distribution of *Senecio carniolicus* in the Eastern Alps. Preslia, *81*: 309–319.

JEFFREY, C. (1978). Compositae. In: Heywood V.H. (ed), Flowering plants of the world: 263-268. Mayflower Books, New York, New York, USA.

KIM, S., D.J. CRAWFORD, R.K. JANSEN, A. SANTOS-GUERRA (1999). The use of a non-coding region of chloroplast DNA in phylogenetic studies of the subtribe Sonchinae (Asteraceae:Lactuceae). Plant. Syst. Evol., *215*: 85-99.

KIRK, H., M. MÁČEL, P.G.L. KLINKHAMER, K. VRIELING (2004). Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: Molecular and chemical evidence. Mol. Ecol., *13*: 2267–2274.

LANGEL, D., D. OBER, P.B. PELSER (2011). The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the Senecioneae. Phytochem. Rev., *10*: 3–74.

LIBARDO, P., J. ROZAS (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphic data. Bioinformatics, *25*: 1451-1452.

MEDRANO, M., E. LÓPEZ-PEREA, C.M. HERRERA (2014). Population genetics methods applied to a species delimitation problem: Endemic trumpet daffodils (*Narcissus* section *Pseudonarcissi*) from the Southern Iberian Peninsula. Int. J. Plant Sci., *175*: 501-517.

MEUDT, H. M., P.J. LOCKHART, D. BRYANT (2009). Species delimitation and phylogeny of a New Zealand plant species radiation. BMC Evol. Biol., *9*: 111.

NEE, S. (2006). Birth-death models in macroevolution. Annu. Rev. Ecol. Evol. Syst., *37*: 1–17.

NORDENSTAM, B. (1989). Tribus Senecioneae. In: Rechinger K.H. (ed.), Flora Iranica. Akademische Druck- und Verlagsanstalt, Graz.

NORDENSTAM, B., P.B. PELSER, J.W. KADEREIT, L.E. WATSON (2009). Senecioneae. In: Funk V.A., A. Susanna, T. Stuessy, R. Bayer (eds.), Systematics, evolution and biogeography of the Compositae. Vienna, Austria.

PEAKALL, R., P.E. SMOUSE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes., *6*: 288-295.

PELSER, P. B., B. GRAVENDEEL, R. VAN DER MEIJDEN (2002). Tackling speciose genera: species composition and phylogenetic position of *Senecio* sect.*Jacobaea* (Asteraceae) based on plastid and nrDNA sequences. Am. J. Bot., *89*: 929–939.

PELSER, P.B., H. DE VOS, H.C. THEURING, T. BEUERLE, K. VRIELING, T. HARTMANN (2005). Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* sect. *Jacobaea* (Asteraceae). Phytochemistry, *66*: 1285–1295.

PELSER, P.B., J.F. VELDKAMP, R. VAN DER MEIJDEN (2006). New combinations in *Jacobaea* Mill. (Asteraceae: Senecioneae). Compositae Newslett., *44*: 1–11.

PELSER, P.B., B. NORDENSTAM, J.W. KADEREIT, L.E. WATSON (2007). An ITS phylogeny of tribe Senecioneae (Asteraceae) and a new delimitation of *Senecio* L. Taxon, *56*: 1077– 1104.

PELSER, P.B., A.H. KENNEDY, E.J. TEPE, J.B. SHIDLER, B. NORDENSTAM, J.W. KADEREIT, L.E. WATSON (2010). Patterns and causes of incongruence between plastid and nuclear Senecioneae (Asteraceae) phylogenies. Am. J. Bot., *97*: 856–873.

PEREDO E. L., M.A. REVILLA, B. JIMÉNEZ-ALFARO, A. BUENO, J.A. FERNÁNDEZ PRIETO, R.J. ABBOTT (2009). Historical Biogeography of a Disjunctly Distributed, Spanish Alpine Plant, Senecio boissieri (Asteraceae). Taxon, *58*: 883-892.

PODANI, J. (2000). Introduction to the Exploration of Multivariate Data. Backhuys Publ, Leiden.

PRITCHARD J.K., M. STEPHENS, P. DONNELLY (2000). Inference of population structure using multilocus genotype Data. Genetics, *155*: 945-959.

RAMBAUT, A. (2009). FigTree v1.3.1. http://tree.bio.ed.ac.uk/ software/figtree.

RAMBAUT, A., A.J. DRUMMOND (2007). Tracer, version 1.5. http:// beast.bio.ed.ac.uk/Tracer.

SHAW, J., E.B. LICKEY, J.B. BECK, S.B. FARMER, W, LIU, J. MILLER, K.C. SIRIPUN (2005). The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot., *92*: 142–166.

SHEIDAI, M., E. SEIF, M. NOUROOZI, Z. NOORMOHAMMADI (2012). Cytogenetic and molecular diversity of *Cirsium arvense* (Asteraceae) populations in Iran. J. Jap. Bot., *87*: 193-205.

SHEIDAI, M., S. ZANGANEH, R. HAJI-RAMEZANALI, M. NOUROOZI, Z. NOORMOHAMMADI, S. GHASEMZADEH-BARAKI (2013). Genetic diversity and population structure in four *Cirsium* (Asteraceae) species. Biologia, *68*: 384-397.

SHEIDAI, M., F. AFSHAR, M. KESHAVARZI, S.M. TALEBI, Z. NOORMOHAMMADI, T. SHAFAF (2014). Genetic diversity and genome size variability in *Linum austriacum* (Lineaceae) populations. Biochem. Sys. Ecol., *57*: 20-26.

SWENSON, U., U. MANNS (2003). Phylogeny of *Pericallis* (Asteraceae): a total evidence approach reappraising the double origin of woodiness. Taxon, *52*: 533–546.

SYED SHUJAIT, A., Y. YAN, P. MARTIN, W. WOLFGANG (2012). Inferences of biogeographical histories within subfamily Hyacinthoideae using S-DIVA and Bayesian binary MCMC analysis implemented in RASP (Reconstruct Ancestral State in Phylogenies). Ann. Bot., *109*: 95–107.

TAMURA, K., M. NEI (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol., *10*: 512-526.

TAMURA, K., D. PETERSON, N. PETERSON, G. STECHER, M. NEI, S. KUMAR (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol., *28*: 2731-2739.

TIMME, R., E.J. KUEHL, J.L. BOORE, R.K. JANSEN (2007). A comparative analysis of the *Lactuca* and *Helianthus* (Asteraceae) plastid genomes: identification of divergent regions and categorization of shared repeats. Am. J. Bot., *94*: 302-313.

WHITE, T.J., T. BRUNS, S. LEE, J. TAYLOR (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., D.H. Gelfand, J.J. Sninsky, T.J. White (eds.), PCR Protocols: 315–322. Academic Press, London.

YAN, Y., A.J. HARRIS, H. XINGJIN (2011). RASP (Reconstruct Ancestral State in Phylogenies) 1.1. Available at: http://mnh.scu.edu.cn/soft/blog/RASP.

YU, Y., A.J. HARRIS, X.J. HE (2010). S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. Mol. Phylogenet. Evol., *56*: 848-850.

MOLEKULARNO I MORFOLOŠKO PROUČAVANJE RODA *Senecio* L. (ASTERACEAE: SENECIONEAE) U IRANU

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Izvod

Rod *Senecio* (Asteraceae, Senecioneae) sa oko 1250 vrsta je jedan od najvećih rodova u familiji. Zbog istorijske i sadašnje inter-specifične hibridizacije i retikulacije evolucije u rodu, morfološka i molekularna filogenetska evolucija su disjunktni. Rod sadrži 17 vrsta koje sapadju u četiri sekcije: *Crociseris*, *Kuadridentati*, *Jacobaea* i *Senecio*, u Iranu, i od kojih su šest endemične vrste. Zbog toga je ovo istraživanje obavljeno sa ciljem da pruži podatke o gore navedenim pitanjima. Generalno, ISSR molekularni markeri mogu razgraničiti proučene *Senecio* vrste i otkriti vezu sa vrstama, ali nisu podržavali nijednu od sekcija. ITS i cp-DNA sekvence šest vrsta *S. iranicus, S. vulcanicus, S. kotschyanus, S. paulsenii subsp. khorasanicus* i *S. joharchii* su prvi put dobijene. S-DIVA ukazuje na tri moguća pretka: Kordestan (A), Mazandaran (G) i Zapadni-Azerbejdzan (D), za vrste *Sececio* u Iranu. Ove oblasti se nalaze u zapadnom delu Irana. Pokrajina Mazandaran je odigrala važnu ulogu u procesu koji je doveo do stvaranja endemičnih vrsta *Sececio* u zemlji, dok su Kordestan i Zapadni-Azerbejdžan glavni putevi za ulazak *Sececio* vrsta iz Evrope preko susednih zemalja. Filogenetska stabla zasnovana na ISSR, cp-DNK i nuklearnim genima prikazala su drugačiju povezanost između vrsta od dendrograma zasnovanog na morfološkim osobinama.

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