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Molecular study on *Senecio fontanicola* (*S. doria* group, *Asteraceae*) and its conservation status

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Key words: alkaline fens, DNA barcoding, conservation status, Senecioneae.

Ključne besede: bazična nizka barja, sistem črtnih kod DNK, ohranitveni status, Senecioneae.

Abstract

Senecio fontanicola is endemic to black-bog-rush fens of southern Austria, north-western Slovenia and north-eastern Italy. It is characterized by oblanceolate leaves, a low number of supplementary bracts and glabrous achenes and it grows in marshy spring areas, fens and reed beds, between elevations from 20 to 850 m. The species was never described with molecular traits and during the last decades, *S. fontanicola* showed a dramatic decline due to land reclamation for agriculture. Therefore, the present study aims to characterize *S. fontanicola* using the molecular barcoding technique and to updated its distribution to propose a global risk category for the species, based on IUCN criteria. The three molecular markers used in this study (*trn*H-*psb*A, *rbc*L, and ITS) clearly distinguished *S. fontanicola* from *S. doria.* s.s. and the revised distribution allowed the definition of the conservation status of the species, that is *Endangered*-EN B2ab(i, ii, iii, iv) following the B criterion of the IUCN guidelines.

Izvleček

Senecio fontanicola je endemit barij s črnikastim sitovcem v južni Avstriji, severozahodni Sloveniji in severovzhodni Italiji. Ima značilne ozko narobejajčaste liste, majhno število dodatnih krovnih listov in golih rožk. Uspeva v močvirnatih povirjih, barjih in trstiščih na nadmorskih višinah med 20 in 850 m. Vrste do sedaj še niso opisali z znaki, pridobljenimi z molekularnimi analizami. V zadnjih desetletjih je *S. fontanicola* doživela dramatičen upad rastišč zaradi melioracij v kmetijska zemljišča. V naši raziskavi smo želeli opisati *S. fontanicola* z molekularno tehniko črtnih kod DNK in prikazati najnovejšo razširjenost vrste. Predlagali smo tudi globalno kategorijo ogroženosti po IUCN kriterijih. Uporabili smo tri molekulske markerje (*trn*H-*psb*A, *rbc*L, and ITS), s katerimi smo jasno ločili vrsto *S. fontanicola* od *S. doria.* s.s. S kritično presojo pojavljanja vrste smo opredelili ohranitveni status kot *Ogrožena vrsta*-EN B2ab(i,ii,iii,iv) v skladu z B kriterijem po navodilih IUCN.

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1. Introduction

Senecio L. (Compositae, Senecioneae) is a genus of approximately 1250 species (Nordenstam 2007) with cosmopolitan distribution and the primary centre of diversity in South Africa and South America (Pelser et al. 2007, Milton 2009). Molecular and phylogenetic investigations clearly showed that this genus is highly complex, listing several critic groups (see e.g. Pelser et al. 2010, Iamonico 2017).

According to Calvo & Aedo (2015) the *S. doria* group is represented in Europe by the following species: *S. altissimus* Mill. (Spain, France, Italy, and Morocco), *S. bithynicus* J. Calvo (Turkey), *S. doria* L. (Austria, Bulgaria, Czech Republic, Hungary, Kazakhstan, Moldova, Russia, Romania, Serbia, Slovakia, and Ukraine; see also Iamonico 2013, Calvo et al. 2014) *S. fontanicola* Grulich & Hodálová (northern Italy, north-western Slovenia and Austria), *S. legionensis* Lange (endemic to north-western of the Iberian Peninsula), *S. morisii* J. Calvo & Bacch. (Sardinia), and *S. umbrosus* Waldst. & Kit. (Central-Eastern Europe and Ukraine).

Senecio fontanicola is an endemic species distributed along north-eastern Italy, Carinthia (Austria) and north-western Slovenia, whose taxonomic position was proposed by Grulich & Hodálová (1994) on the basis of morphological data. Ecologically, *S. fontanicola* is strictly linked to black-bog-rush fens between elevations of 20–850 m, recorded in: i) *Primulo farinosae-Schoenetum ferruginei* Oberd. (1957) 1962 in Carinthia, Austria (Grulich & Hodálová 1994); ii) *Erucastro-Schoenetum nigricantis* Poldini 1973 emend. Sburlino e Ghirelli 1994, in Friuli Venezia Giulia, Italy (Poldini & Oriolo 2002); iii) *Carici paniculatae-Salicetum myrsinifoliae* Dakskobler 2012, in the Zelenci area in Slovenia (Vreš et al. 2012).

The occurrence of *S. fontanicola* in Italy (erroneously identified as *S. doria*) date back to 1855 when Pirona (1855) highlighted the morphological variability of a population occurring in Virco fens (Friuli Venezia Giulia, north-east Italy), although there is no formal publication of a name (the annotation "*S. doria* v. *angustifolium*" occurs in a label of a specimen preserved at FI). About one century later, Zenari (1947: 3–4) described two new varieties for the Friulan populations, i.e. *S. doria* var. *subdecurrens* and *S. doria* var. *golae*. For the *S. doria* var. *subdecurrens*, two subvarieties were described, i.e. the sv. Typicus and the sv. Intermedius, distinguished each other by leaf shape and insertion to the stem, while for the *S. doria* var. *golae* only the sv. Forojuliensis was recorded by Zenari (1947: 3–4) in Italy. These taxa are currently considered heterotypic synonyms of *S. doria* (Poldini et al. 2001) and were recently lectotypified (Calvo & Aedo 2015).

The recent revision of Calvo & Aedo (2015) clarified nomenclature, diagnostic characters, and distribution of the species belonging to *S. doria* group, *S. fontanicola* included. Before this contribution, the distribution of *S. fontanicola* in Italy was rather uncertain because the checklist of the Italian vascular flora (Conti et al. 2005; Scoppola & Spampinato 2005) also wrongly recorded *S. doria*. The lack of solid information entailed that the conservation status of *S. fontanicola* was inaccurate. While the Austrian Red List considered *S. fontanicola* as an endangered (EN) species from 1999 (Niklfeld & Schratt-Ehrendorfer 1999), Pignatti et al. (2001), which resume risk categories of the Italian National Red Lists (Conti et al. 1992; 1997), reported only *S. doria* as vulnerable species (VU). Moreover, *S. fontanicola* still missed in the following updating of the Italian National Red List edited by Rossi et al. (2013).

The present work aimed to characterize *S. fontanicola* from a molecular point of view, using the barcoding technique and including species erroneously attributed to *S. doria* s.l. (in particular to *S. doria*) or recently retrieved (i.e. *S. altissimus*). The amplified loci were *trn*H-*psb*A, *rbc*L and ITS, which are included in the loci recommended in the Consortium for Barcode of Life (CBOL) Plant Working Group (CBOL Plant Working Group 2009). The loci selection took into account i) the high discrimination power of *trn*H-*psb*A (Kang et al. 2017); ii) the great amount of data available in Genbank (Hollingsworth et al. 2016) on both *rbc*L and ITS sequences; and iii) the common use of *trn*H-*psb*A and ITS markers for the genus *Senecio* (Khan et al. 2013). Moreover, the distribution of *S. fontanicola* is updated, especially regarding the Italian populations, and a conservation status proposed using the IUCN criteria (IUCN 2012).

2. Materials and methods

2.1. Plant material

Field sampling was carried out in summer 2009. Three populations of *S. fontanicola* were sampled in Italy and other three in Austria, while *S. doria* samples were provided by prof. Hodálová (Institute of Botany, Bratislava, Slovak Republic) and referred to 2 populations coming from Hungary and 1 population from Slovakia (Table 1). A fresh leaf was collected from at least five individuals for each population. Leaf samples of *S. fontanicola* were frozen in liquid nitrogen within few hours after their collection and stored at -80 °C. Leaf specimens of *S. doria* were dried after sampling and stored in sealed plastic bags with silica gel.

Table 1: Sampling localities, geographic coordinates, and GenBank accession numbers for the three loci sequenced.
Tabela 1: Lokacije vzorčenja, geografske koordinate in pristopne številke iz sekvenčne podatkovne baze (GenBank) za tri sekvencirane lokuse.

Locality	Geographic coordinates (WGS84)	GenBank accession numbers		
		*trn*H-*psb*A	*rbcL*	ITS
Senecio fontanicola				
Virco, Bertiolo, Udine (Italy)	45° 55′ 43”N 13° 03′ 30”E	KU319493 KU319494 KU319495 KU319496 KU319497	KU308436 KU308437 KU308438 KU308439	KU307474 KU307475 KU307476 KU307477 KU307478 KU307479
Gonars, Udine (Italy)	45° 52′ 57”N 13° 13′ 25”E	KU319498 KU319499 KU319500 KU319501 KU319502 KU319503	KU308440 KU308441	KU307480 KU307481 KU307482 KU307483 KU307484 KU307485
Vinchiaruzzo, Cordenons, Pordenone (Italy)	45° 58′ 43”N 12° 43′ 42”E	KU319504 KU319505 KU319506 KU319507 KU319508 KU319509	KU308442 KU308443 KU308444 KU308445	KU307486 KU307487 KU307488 KU307489
Techelweg, Klagenfurt (Austria)	46° 35′ 43”N 14° 06′ 42”E	KU319510 KU319511 KU319512 KU319513 KU319514 KU319515	KU308446 KU308447 KU308448 KU308449 KU308450 KU308451	KU307490 KU307491 KU307492 KU307493 KU307494 KU307495
Trabesing, Klagenfurt (Austria)	46° 33′ 05”N 14° 15′ 00”E	KU319516 KU319517 KU319518 KU319519 KU319520 KU319521 KU319522	KU308452 KU308453 KU308454 KU308455	KU307496 KU307497 KU307498 KU307499 KU307500
Obershütt, Klagenfurt (Austria)	46° 34′ 24”N 13° 45′ 12”E	KU319523 KU319524 KU319525 KU319526 KU319527	KU308456	KU307501 KU307502 KU307503 KU307504 KU307505 KU307506
Senecio doria				
Podunajská nížina Lowland, Ňárad (Topol'ovec, Slovakia)	47° 50′ 08”N 17° 35′ 16”E	KU319474 KU319475 KU319476 KU319477 KU319478 KU319479 KU319480	KU308416 KU308417 KU308418 KU308419 KU308420 KU308421 KU308422	KU307457 KU307458 KU307459 KU307460 KU307461 KU307462
South of Komárom (Újpuszta, Hungary)	47° 40′ 18”N 18° 08′ 00”E	KU319481 KU319482 KU319483	KU308423 KU308424 KU308425	KU307463 KU307464
Slovak Karst, between Turňa nad village (Slovakia) and Tornanádaska (Hungary)	48° 35′ 22”N 20° 51′ 29”E	KU319484 KU319485 KU319486 KU319487 KU319488	KU308426 KU308427 KU308428 KU308429 KU308430	KU307465 KU307466 KU307467 KU307468 KU307469

2.2. DNA extraction, amplification and sequencing

DNA extraction was carried out using the CTAB method (Doyle & Doyle 1997). Primer sequences and amplification conditions of *trn*H-*psb*A, *rbc*L, and ITS were reported by Sang et al. (1997), Hollingsworth et al. (2009), and Stanford et al. (2000) respectively, with slight modifications (Table 2). Loci were amplified by polymerase chain reaction (PCR) on the thermocycler GeneAmp® PCR System 9700 (Applied Biosystem). The quality of the DNA extracted was checked on 0,8% agarose gel. The amplification products were purified and sequenced in both directions with the automatic sequencer Applied Biosystem 3730.

2.3. Data analysis

Forward and reverse sequences were edited, trimmed and assembled with the Staden package software (Staden 1996). The full-length sequences were aligned with Clustal W (Thompson et al. 1994). Sequences were deposited to GenBank (Table 1). A BLASTN search (Altschul et al. 1997) for the three loci was carried out to find homologies with other available sequences from GenBank. Considering the recent revision by Calvo & Aedo (2015), sequences of the so-called *S. doria* specimens of western Europe were labelled as *S. altissimus*. MEGA ver. 7 (Tamura et al. 2013) was used to obtain Kimura two parameter (K2p – Kimura 1980) pairwise distances among the analysed sequences, expressed as average in percentage (%).

Phylogram was inferred using the Bayesian statistics and by adopting the Markov Chain Monte Carlo (MCMC) sampling technique in the BEAUti/BEAST v.1.10.0 software (Bouckaert et al. 2014). Tamura-Nei model was setnas substitution model (Tamura and Nei 1993) and the construction of the dendrogram was computed in FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The maximum clade credibility tree was generated with nodes based on mean heights, where branches were collapsed at the 50% threshold of posterior values.

2.4. Distribution and conservation status

In order to outline the current distribution of *S. fontanicola*, several data were considered: herbarium specimens (see Supplementary material S1), species distribution atlas of Austria (Fisher et al. 2008) and north-eastern Italy (Poldini & Oriolo 2002) verified with field surveys when possible, bibliography (in particular Calvo & Aedo 2015, Vreš et al. 2012, Frattini 2008, Grulich & Hodálová 1994), field observations, and personal communications. The conservation status was assessed following IUCN Red List Criteria version, 3.1 second edition (IUCN 2012). The criterion B was selected for the assessment. The area of occupancy (AOO index) was applied to 2 km² grid cells based on UTM grid.

3. Results

3.1. Molecular analysis

DNA were successfully extracted from all samples. DNA amplification rates differed among loci with the maximum (92%) for the *trn*H-*psb*A locus, 69% for *rbc*L and 85% for ITS, in agreement with literature (Kress et al. 2005, Hollingsworth et al. 2008). The total number of new sequences generated in this study was 145. ITS locus revealed the major variability among sequences, presenting 1 indel and 26 SNPs (18 transitions and 8 transversions) for a total of 27 variable sites. The aligned sequence

Table 2: Primers and reaction conditions used for PCR.
Tabela 2: Oligonukleotidni začetniki (prajmerji) in reakcijski pogoji za verižno reakcijo s polimerazo (PCR).

Locus	Primer name	Primer sequence	Reaction condition
trnH-psbA	psbA3'f	5'-GTTATGCATGAACGTAATGCTC	95°C 5 min
	trnHf	5'-CGCGCATGGTGGATTCACAATCC	94°C 30 s, 58°C 30 s, 72°C 30 s, 38 cycles
			72°C 10 min
rbcLa	rbcLa_f	5'-ATGTCACCACAAACAGAGACTAAAGC	95°C 5 min
	rbcLa_rev	5'-GTAAAATCAAGTCCACCRCG	94°C 30 s, 55°C 1 min, 72°C 1 min, 5 cycles
			94°C 30 s, 54°C 1 min, 72°C 1 min, 30 cycles
			72°C 10 min
ITS	ITS4	5'-TCCTCCGCTTATTGATATGC	95°C 5 min
	ITS5a	5'-CCTTATCATTTAGAGGAAGGAG	94°C 30 s, 63°C 30 s, 72°C 45 s, 8 cycles
			94°C 30 s, 55°C 30 s, 72°C 45 s, 30 cycles
			72°C 10 min

length was 642 bp including indel. For *rbc*L and *trn*H-*psb*A the aligned sequence matrix was 512 and 363 bp in length (no indel) respectively (Table 3).

The total variation among sequences was attributable to inter-specific divergence. No intra-specific variability was observed for *rbc*L and *trn*H-*psb*A loci and only one SNP was found in the ITS locus for 2 specimens of *S. fontanicola* (K2p = 0.20%, Techelweg population, Austria). The range of variation extends from 0.40 to 4.21% of informative positions, with the major presence of SNPs in the nuclear ITS locus (Table 3). This locus clearly distinguished the two species analysed. *Senecio doria* presents 7 diagnostic positions (4 transitions and 3 transversions) and 1 indel, whereas *S. fontanicola* has 8 diagnostic positions (6 transitions and 2 transversions). Plastid loci were very conserved but sufficiently variable to distinguish *S. fontanicola* from *S. doria*, with 2 variable sites for *rbc*L (K2p = 0.39%) and 3 for *trn*H-*psb*A (K2p = 0.83%).

The homology search returned 13 ITS sequences attributed to *S. altissimus* (Calvo et al. 2013). No sequences were found for the other two loci. Table 4 lists species, localities, and GenBank accession numbers. Some variability was noticed within the *S. altissimus* ITS sequences retrieved from GenBank. This variability reflected 15 variable positions (K2p = 1.27%). In any case there was no overlap with the interspecific variability.

S. fontanicola sequences were easily distinguished from *S. altissimus* (K2p = 2.28%). A graphical representation of the relationship of the considered taxa is reported in Figure 1. Three monophyletic clades clearly distin-

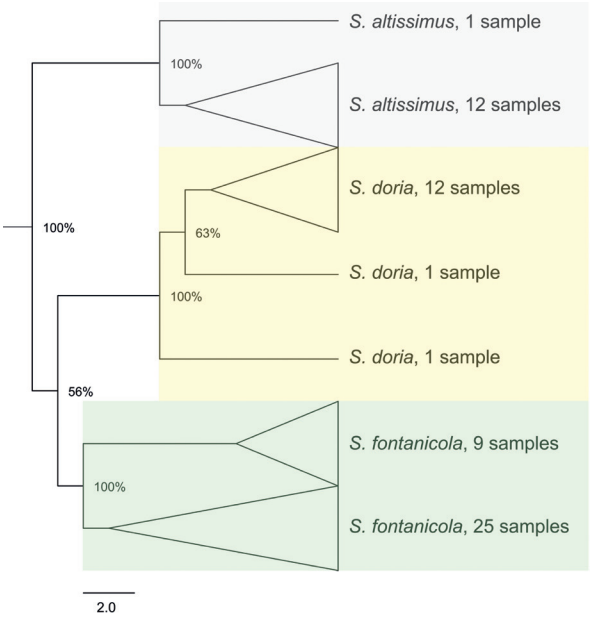

Figure 1: Cladogram obtained from FigTree software after Bayesian interference analysis computed in BEAST for the nuclear data set (ITS locus). Branches corresponding to partitions reproduced in less than 50% of posterior values were collapsed. The three clades corresponded to the three analysed species are highlighted with different colours.

Slika 1: Kladogram, rekonstruiran na osnovi jedrnega markerja (ITS) z uporabo Bayesovega principa v programu BEAST. Za prikaz smo uporabili programsko orodje FigTree. Razvejitve kladograma, ki so imele v pripadajočem kolenu nižjo podporo od 50%, smo strnili (kolapsirali). Trije kladi, označeni z različnimi barvami, pripadajo trem vrstam, vključenim v analizo.

Table 3: Amplification rates (expressed in percentage), number of sequenced specimens, alignment length, transition/transversion rate, informative indels, total number of variable positions compare to the respective loci length and total SNP percentage of the loci ITS, *rbcL* and *trnH-psbA*.

Table 3: Pomnožitvene stopnje (v odstotkih), število sekvenciranih primerkov, poravnava zaporedij, stopnja tranzicije/transverzije, informativne delecije ali duplikacije, skupno število spremenljivih pozicij v primerjavi z dolžino lokusov in skupen odstotek SNP lokusov ITS *rbcL* in *trnH-psbA*.

Locus	Amplification rates (%)	N. sequenced specimens	Alignment length (bp)	Transition/ transversion rate	Informative indels	N. variable positions	SNPs % for total length
ITS	85	50	642	2,28	1	27/642	4,21
rbcL	69	41	512	2,00	0	2/512	0,40
trnH-psbA	92	54	363	0,33	0	3/363	0,83

Table 4: Sampling sites and GenBank accession numbers of BLASTN homology search for the ITS locus (Calvo et al. 2013).

Tabela 4: Vzorčne lokacije in pristopne številke iz sekvenčne podatkovne baze (GenBank) za poizvedovanje BLAST nukleotid-nukleotid homologije za ITS lokus (Calvo et al. 2013).

Locality	GenBank accession
Senecio altissimus	
Close to the Arreo lake, margin of the road (Spain)	JX895272, JX895273, JX895277, JX895275, JX895276, JX895271, JX895270, JX895269, JX895268, JX895266, JX895265, JX895267

guished the three species, i.e., *S. altissimus*, *S. doria*, and *S. fontanicola*. Therefore, these results also confirm the morphological distinction stated by Calvo & Aedo (2015) between *S. doria* and *S. altissimus*; where a total of 3 transitions, 1 transversion and 2 informative indels unambiguously identify *S. altissimus* samples (K2p = 2.20%).

3.2. Distribution update

Senecio fontanicola distribution is about 15580 km², which extends throughout northern Italy, western Slovenia and southern Austria (Figure 2, Ehrendorfer and Hamman 1965).

The species shows a scattered distribution due to its ecological requirements, strictly linked to black-bog-rush fens. The major populations are located in low flats of the Friuli-Venezia Giulia Region (NE-Italy) and in the Gail valley of Carinthia Region (Austria). The species is also present in Lombardy and Veneto (N-Italy) and in the Gorenjska region (Slovenia). The Austrian populations, occurring from Bad Bleiberg up to Bleiburg (Hartl et al. 1992, Fisher et al. 2008), are the largest in terms of both surface area and number of individuals (more than 100 individuals per population). In Slovenia, only one record is reported for the alpine Zelenci wet area (Vreš et al. 2012), placed less than 5 km away from the easternmost Italian locality, in the Scichizza swamp (Fusine wet area, Friuli-Venezia Giulia).

Italian populations are smaller in surface area and number of individuals. In Friuli-Venezia Giulia, *S. fontanicola* is considered a rare taxon, occurring in several localities of lowland wetlands with populations of less than ten individuals (Pavan & Costalonga 2001, Martini & Pavan 2008). The species was also reported for an isolated site nearby Tolmezzo, but its presence there has not been recently confirmed. In the Veneto Region is recorded from the Onara wet area (Padua) and one isolated locality in Lombardy represents the western distribution limit of the species. This last locality is seriously threatened because of drainage activities carried out during the last decade (Frattini 2008). Some historical records e.g. Dimon and Amariana mountains in Friuli (Morassi herbarium, MFU), Euganei hills in Veneto (Ugolini herbarium, pri-

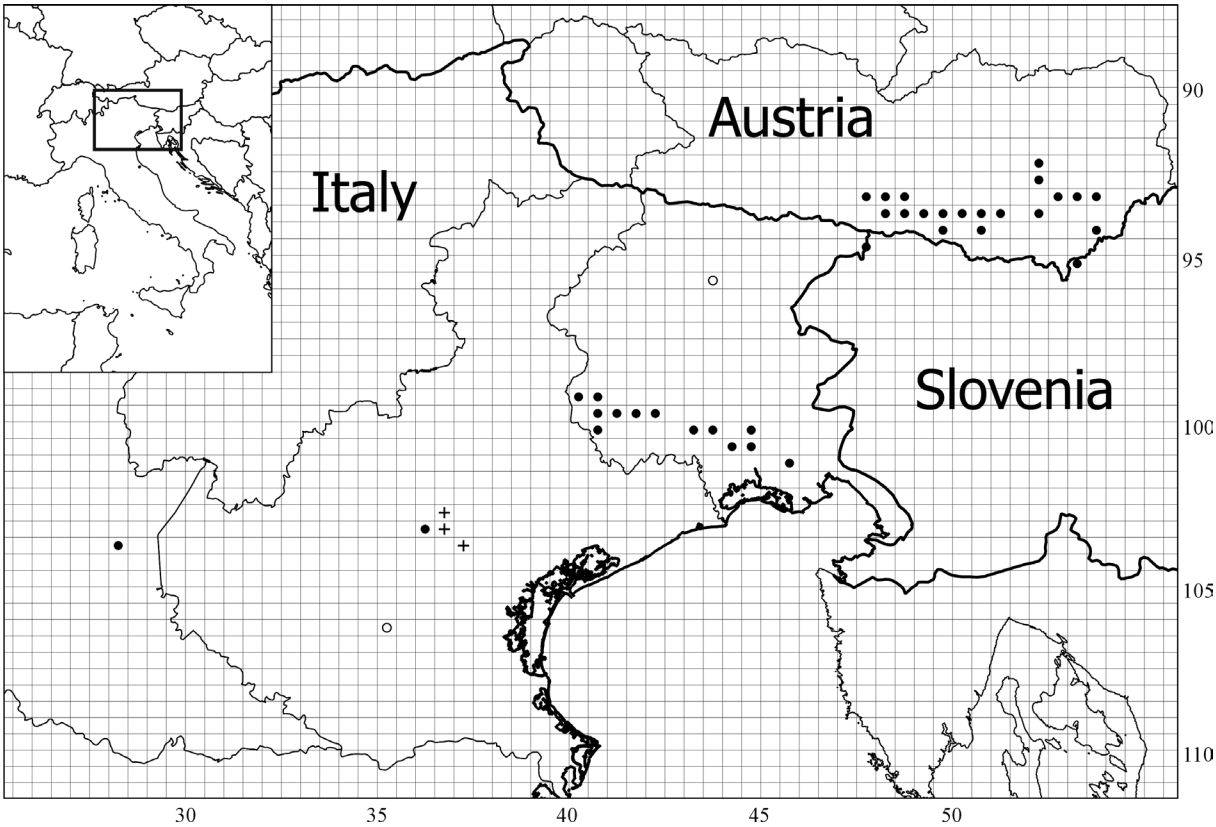

Figure 2: Distribution of *Senecio fontanicola* based on Ehrendorfer & Hamman grid (1965). Legend: ● present, ○ doubtful, + extinct.
Slika 2: Razširjenost vrste *Senecio fontanicola*, prikazana na srednjeveropski mreži (Ehrendorfer & Hamman 1965). Legenda: ● sedanja, ○ dvomljiva, + izumrla.

vate) are questionable because the specification of the locality is generic and the current lack of suitable ecological conditions for the species, but the existence of adequate environmental conditions in the past (at least in the Euganei hills) cannot be excluded.

3.3. IUCN assessment

More than 45% of sites where *S. fontanicola* falls inside protected areas (percentage obtained from the overlapping of *S. fontanicola* populations and areas subject to legal protection, online available at http://irdat.regione.fvg.it/WebGIS/ and https://www.data.gv.at/katalog/dataset?tags=Schutzgebiet). However, the preferential habitat of *S. fontanicola* (7230 alkaline fens, Natura 2000) is considered "unfavourable-inadequate" for the whole continental and alpine bioregion and in particular "unfavourable-bad" for Austria and Italy (EEA 2013). Furthermore, *S. fontanicola* populations frequently have less than 10 plants, entailing the need of management programs for species preservation and the definition of a conservation status.

For the assessment based on IUCN Red List criteria (IUCN, 2012), the geographic criterion B was considered. The extent of occurrence (EOO) of *S. fontanicola* is 16173 km². The calculated area of occupancy (AOO), based on the 2 km² grid cells, is 220 km² (108 km² in Austria, 4 km² in Slovenia, and 108 km² in Italy). Populations are severely fragmented and the habitat suffers reduction and degradation, mainly due to human land-use changes (Bondesan, 1995). A regression is also observed in the number of sites where the species thrives, in the size of populations, and in the extent and quality of the habitat. For these reasons, the global risk category proposed for the conservation status of *S. fontanicola* is *Endangered*-EN B2ab(i,ii,iii,iv).

4. Discussion

In this work, the molecular data supports the taxonomic treatment of *S. fontanicola* at the specific rank. *Senecio fontanicola* plastid sequences were very conserved and clearly separated from *S. doria*. Moreover, the molecular analysis of ITS sequences supported the recent taxonomic decision of recognizing *S. altissimus* as a species distinct from *S. doria* (Calvo & Aedo 2015). This data confirmed the usefulness of ITS as a DNA barcode for the *Asteraceae* family (Gao et al. 2010). Sheth & Thaker (2017) asserted that DNA barcoding not only integrates taxonomy and accomplishes species identification but also stands as a tool for species conservation, especially dealing with endemism and threatened species. In fact, the distribution of *S. fontanicola* leads us to consider this species an endemism that probably underwent allopatric speciation during the Quaternary, driven by geographical isolation from the *S. doria*. In this period, the Austrian territory was covered by ice (Ehlers & Gibbard 2004) while the eastern zone of the North-Italian plain was covered by wet surfaces (Fontana et al. 2014), offering a putative refuge for these populations. The plant probably moved towards the Austrian wet areas, after the glaciation, following the Tagliamento and Fella rivers. This hypothesis is suggested considering the chorology and the speciation of other endemic taxa thriving in the same geographical area, such as *Erucastrum palustre* (Pirona) Vis. (Martini & Poldini 1986) and *Armeria helodes* F. Martini & Poldini (Martini & Poldini 1987). *Senecio fontanicola* appears seriously threatened showing a scattered distribution and small populations (some of them have less than ten individuals). The IUCN attribution as Endangered species leads us to recommend the application of urgent conservation measures for the preservation of this species and its habitat.

5. Conclusion

The genetic characterisation and the monitoring of species distribution are the basis for the conservation of the threatened species, such as *S. fontanicola*. Molecular analysis confirmed the different taxonomic position of *S. fontanicola* from *S. doria* s.s. and the need to extend further surveys on the neighbouring *S. altissimus*. Being *S. altissimus* distribution neighbouring to *S. fontanicola*, it could gather interesting information about species differentiation inside the *S. doria* group, e.g. reconstructing species speciation in time using specific genetic analysis like AFLP. Moreover, the current records of *S. altissimus* in Italy presented in Calvo and Aedo (2015) , are highly unlikely, being based on old herbaria records. The genetic characterisation of the recently described species *S. morisii* (Sardinia – Calvo & Aedo 2015) should also be considered in future studies.

The updated distribution of *S. fontanicola* allowed to define correctly the conservation status for the species. While a risk category was already attributed to the Austrian populations (EN), any active measures for species conservation are being applied in Italy and Slovenia. The definition of the Endangered (EN) risk category points out the critical status of the species, highlighting the need of urgent and effective measures at both European and national level.

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