

Molecular phylogeny and phylogeography reveal recent divergence in the *Iberis simplex* DC. (Brassicaceae) species complex

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Abstract: According to the current taxonomic delimitation, the Mediterranean genus *Iberis* comprises about 30 species. Among them, the taxonomy and species delimitation of *Iberis simplex* and its morphologically close relatives (*I. attica*, *I. carica*, *I. gypsicola*, *I. halophila*, and *I. spruneri*), described here as the *I. simplex* species complex, are controversial and several different names are used for the members of this complex. Past phylogenetic studies did not include the members of this complex and here we present the first comprehensive phylogenetic and phylogeographic study based on nuclear ITS and chloroplast *trnL*-F regions to delimit the boundaries of the species and contribute to the knowledge of Anatolian biogeography. Our analyses clearly showed a monophyletic origin of this complex, polytomic relationships within the clade, and recent divergence of the species of this complex starting from ca. 0.65 million years ago. Finally, a recent population expansion (as a driver of rapid radiation) which is possibly associated with climatic oscillations during the Pleistocene was supported by multiple tests and mismatch distribution analyses.

Key words: Anatolia, Brassicaceae, phylogeography, *Iberis*, Iberideae, pleistocene

1. Introduction

The genus *Iberis* L. (Brassicaceae) consists of approximately 30 species and mostly formed of annual, perennial herbs, and/or evergreen subshrubs worldwide (Marhold, 2011; Al-Shehbaz, 2012; Kiefer et al., 2014). The genus is mainly distributed in the Mediterranean basin, the center of diversity is Europe, and a few species are distributed in Northwest Africa and Southwest and Central Asia but species have been introduced outside their native range, so now the genus has (almost) a worldwide distribution (Gupta, 2009). *Iberis* is placed in the tribe Iberideae together with *Teesdalia* W.T.Aiton and both genera plus *Calepina* Adans are characterized by their monosymmetrical flowers in the mustard family (Busch et al., 2011; Al-Shehbaz, 2012).

Iberis simplex was first described by de Candolle and Lamarck (1815) and its native distribution range is from Crimea to the Caucasus and Turkey (<https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:285660-1>). This species was evaluated under the name *I. taurica* in the first volume of *Flora of Turkey* along with six other species (Hedge, 1965). Since then, *I. attica* Jord., *I. spruneri* Jord., *I. acutiloba* Bertol., and *I. taurica* were synonymized and

one new record (*I. saxatilis* L.), one new subspecies (*I. saxatilis* subsp. *magnesiana* Oskay), and two new species (*I. halophila* Vural & H.Duman and *I. gypsicola* Yild.) were added to the *Flora of Turkey* (Dirmenci et al., 2005; Vural et al., 2012; Oskay, 2017; Yıldırım, 2018). Accordingly, the genus *Iberis* is currently represented by nine species and one subspecies (ten taxa in total) in Turkey of which four taxa (*I. carica*, *I. halophila*, *I. gypsicola*, and *I. saxatilis* subsp. *magnesiana*) are endemic for Turkey. As a result of extensive field, literature, and herbarium studies, along with anatomical (Çilden and Zare, 2019), taxonomical (Çilden, 2022a), fruit-seed macro- and micromorphological studies (Çilden, 2022b), and unpublished palynological data, the list of Turkish *Iberis* taxa has been updated with nomenclature recognitions and listed as follows: *I. attica* Jord., *I. carica* (Bornm.) Prain, *I. gypsicola* Yild., *I. halophila* Vural & H.Duman, *I. odorata* L., *I. saxatilis* subsp. *saxatilis*, *I. saxatilis* subsp. *magnesiana*, *I. sempervirens* L., *I. simplex* DC., and *I. spruneri* Jord. (Hedge, 1965; Yıldırım, 2000, 2001; Dirmenci et al., 2005; Al-Shehbaz et al., 2007; Özhatay et al., 2009; Mutlu, 2012; Oskay, 2017; Yıldırım, 2018; Çilden and Zare, 2019; Yılmaz-Çıtak and Crespo, 2019; Çilden 2022a, 2022b). Among these species, *I. attica*,

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I. carica, *I. halophila*, *I. gypsicola*, *I. simplex*, and *I. spruneri* are considered to be species of the *I. simplex* complex, which is described in this study for the first time, because of their morphological affinities (see Çilden 2022a, 2022b) (Figure 1).

Although many phylogenetic studies have been carried out to shed light on the evolutionary and systematic relationships within Brassicaceae, the members of the genus *Iberis* were represented with only one or two species in these previous studies (e.g., Beilstein et al., 2008; Franzke et al., 2011; Huang et al., 2015; Nikolov et al., 2019). Knowledge of the phylogeny of the genus is limited to its placement within the tribe Iberideae with the genus

Teesdalia (even this relationship seems doubtful in the most current study. For details, see Hendriks et al., 2022) and no detailed phylogenetic or phylogeographic study of the *Iberis* species has been conducted until now (Bailey et al., 2006; German et al., 2009; Couvreur et al., 2010; Warwick et al., 2010; Mandáková et al., 2017; Nikolov et al., 2019).

Anatolia, or the Asian part of Turkey, is considered one of the most floristically diverse regions of the world with approximately 10,000 plant species. It is located at the crossroads of the Mediterranean, Irano-Anatolian, and Euro-Siberian floristic regions and, hosting over 700 crucifer taxa, it is considered the center of the origin of



Figure 1. Representatives of the *Iberis simplex* species complex. A) *I. attica*, B) *I. carica*, C) *I. gypsicola*, D) *I. halophila*, E) *I. simplex*, F) *I. spruneri*. Photos by Emre Çilden (A-D), Barış Özüdoğru (E), and Hasan Yıldırım (F).

the family Brassicaceae as a part of the Irano-Anatolian floristic region (Davis, 1965; Franzke et al., 2009; Güner et al., 2012; Gür, 2016; Özüdoğru et al., 2021). Furthermore, like other Mediterranean refugia, Anatolia was not affected by glaciations except for high mountain peaks during the Pleistocene glaciation periods (Ansell et al., 2011; Şenkul and Doğan, 2013); therefore, the glacial survival of temperate species was possible in most parts of Anatolia (Gür, 2017). Despite these unique features, rapid radiations or sudden population expansion models, which have been tested repeatedly for western Mediterranean refugia, have been rarely tested for Anatolia (Özüdoğru et al., 2020, 2021). Taking into account the distribution of the members of the *I. simplex* species complex covering both the Mediterranean and Irano-Anatolian regions, this species complex is a remarkable candidate to understand the dynamics of Anatolian flora, especially during the Pleistocene.

The present study was designed 1) to test for the presence of distinct evolutionary lineages within the *I. simplex* species complex, 2) to reveal phylogeographic patterns in the study group, and 3) to test whether the *I. simplex* species complex underwent a recent population expansion. To achieve these objectives, we generated molecular phylogenies using nuclear ribosomal internal transcribed spacer region (ITS1, ITS2, 5.8 nrDNA; hereafter ITS) and plastid *trnL*(UAA) intron/*trnL-trnF* intergenic spacer sequence datasets (hereafter *trnL-F*) from 69 herbarium specimens.

2. Materials and methods

2.1. Sampling, DNA extraction, PCR amplification, and sequencing

Silica-dried leaf materials and herbarium specimens of 69 accessions, representing 10 Turkish *Iberis* taxa and nearly their entire geographical distribution area in Anatolia, were used (Table 1, Figure 2). ITS and/or *trnL-F* sequences of *I. amara*, *I. oppositifolia*, *I. spathulata*, *I. umbellata*, and *Teesdalia nudicaulis* from tribe Iberideae (as an outgroup) were obtained from GenBank. Total genomic DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with some modifications to incubation time (30 min vs. 10 min) and final volume (100 µL vs. 200 µL). ITS regions (ITS 1 and ITS 2) and the 5.8S gene of nuclear ribosomal DNA were amplified using primers ITS 1 and ITS 4 (White et al. 1990). The plastid *trnL-F* region was amplified using the *c/f* primer pairs of Taberlet et al. (1991). Amplification of ITS and *trnL-F* followed the protocols of Warwick et al. (2004) and Ansell et al. (2007), respectively. Purification and sequencing were performed in the ECGR Lab (Hacettepe University, Department of Biology, Ankara, Turkey). All new sequences were submitted to GenBank (Table 1).

2.2. Genetic diversity and haplotype analyses

The forward and reverse DNA sequences were edited with the CodonCode Aligner (CodonCode Corporation) to obtain individual consensus sequences. These sequences were aligned with MUSCLE v.3.6 (Edgar, 2004). The best fitting nucleotide substitution model was selected based on the Akaike information criterion (AIC) implemented in MEGA X (Kumar et al., 2018).

For each dataset, nucleotide diversity ($\Theta\pi$), haplotype diversity (HD), and the number of polymorphic (segregating) sites (S) were estimated. To determine the departures from neutrality in the ITS and *trnL-F* dataset, Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) tests were conducted with DnaSP version 5.10 (Librado and Rozas, 2009) and confidence intervals were determined using coalescent simulations. To visualize the relationships between *trnL-F* haplotypes and ITS ribotypes, statistical parsimony networks were constructed with the program POPART (Leigh and Bryant, 2015).

2.3. Phylogenetic analysis and estimation of divergence time

Aligned individual ITS and *trnL-F* datasets were phylogenetically analyzed with the maximum likelihood (ML) and Bayesian inference (BI) approaches. ML analysis was performed with RAxML (Stamatakis, 2006). The program was run using the GTR + G model and the rapid bootstrapping option with 1000 bootstrap iterations. Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012). Two simultaneous Metropolis-coupled Markov chain Monte Carlo (MCMC) runs were sampled for 10,000,000 generations, and one tree was sampled every 1000 generations.

To estimate the time to the most recent common ancestor (t_{mrc}) of the *I. simplex* species complex, a subset of the ITS and *trnL-F* datasets was used. Divergence times were estimated based on an uncorrelated lognormal relaxed clock model in BEAST v.2.6.1 (Bouckaert et al., 2014). Given that there is no reliable fossil for *Iberis* or closely related genera in the literature, a secondary calibration approach was used. The crown group age of the tribe Iberideae was set to 14.6 ± 1.8 (mya), taking into account the variation described by Huang et al. (2020). Normal distribution was used as the most appropriate prior distribution for secondary calibration points as suggested by Ho (2007).

2.4. Demography

In order to understand whether the *Iberis simplex* species complex populations underwent a recent population expansion and associated radiation in response to Pleistocene climatic oscillation, mismatch distribution analyses have been conducted. Historical population expansion in the *Iberis simplex* species complex was evaluated using pairwise differences between individual haplotypes/ribotypes under the sudden expansion model

Table 1. Voucher information (collector and collection number, district, date, habitat, GPS coordinates (if possible), and herbarium acronyms) are given for specimens sequenced for this study. GenBank accession numbers for the sequences (ITS + trnL-F region including trnL(UAA) gene, trnL-F spacer) used in the phylogenetic analyses. A dash (-) indicates missing data.

Voucher number	Taxon name	Location	GenBank accession numbers (trnL-F / ITS)
Özüdoğru 4788	<i>I. sempervirens</i>	Kahramanmaraş: Ahırdağı, 1063 m., <i>Quercus coccifera</i> openings, 08.05.2017 (HUB).	OM735738 / OM669707
Özüdoğru 4001	<i>I. sempervirens</i>	Kahramanmaraş: between Göksun-Geben, Meryemçilbeli, 37,82231 N ; 36,40699 E, 1800 m, 30.04.2016 (HUB).	OM735739 / OM669710
Özüdoğru 4443	<i>I. sempervirens</i>	Kahramanmaraş: south of Baydemirli village, hills, 37,68018 N ; 37,02867 E, 08.06.2016 (HUB).	OM735740 / OM669709
Özüdoğru 4460	<i>I. sempervirens</i>	Adana: Saimbeyli, Naltaş village, Bozoğlan hill, 1830 m, <i>C. libani</i> openings, 38,0573 N ; 36,05485 E, 09.06.2016 (HUB).	OM735741 / OM669708
Özüdoğru 5437	<i>I. sempervirens</i>	Isparta: Dedegöldüğü, Melikler plateau, 37. 69090 N, 31. 29554 E, rocky field, 1815 m, 16.06.2019 (HUB).	OM735742 / OM669706
Duman 4396	<i>I. sempervirens</i>	Kayseri: Sarız, Yalak, Binboğa dağı, 1500-2200 m, 05.07.1991, rocky field, H. Duman & Z. Aytaç (HUB).	OM735743 / OM669711
Sümbül 1752	<i>I. sempervirens</i>	Antalya: Gazipaşa, Sugözü village, Akçal hill, 1900-2000 m, 17.05.1983 (HUB 6884).	OM735744 / OM669713
Erik 3669	<i>I. sempervirens</i>	Konya: Ermenek, Sarıvelliler, Pazarbaşı, Gölümdürüm plateau, calcareous rocks, 1600-1800 m, 18.05.1984 (HUB 6883).	OM735745 / OM669714
Çilden 1892	<i>I. sempervirens</i>	Muğla: Ula, Sandras dağı, in screes, 37°2'11" N, 28°48'3" E, 1960 m, 21.10.2018, (leg. B. Topçuoğlu) (HUB)	OM735746 / OM669712
Koyuncu 8852	<i>I. spruneri</i>	Bursa: Uludağ, 08 vii 1978 (AEF).	OM735747 / OM669737
Doğru Koca 4195	<i>I. spruneri</i>	Denizli: Babadağ, near summit, rocky area, 35°44'22"N, 28°52'19"E, 1995 m, 12.06.2014, ADK & G. Zare (HUB).	OM735748 / OM669736
Yıldırım 3794	<i>I. spruneri</i>	Denizli: Çameli district, Karabayır, marny soil, 36°55'59.5"N, 29°8'55.3"E, 1610 m, 30.04.2016 (HUB).	OM735749 / OM669734
Çilden 1334	<i>I. spruneri</i>	Aydın: Dağeymiri village, Karlık hill, rocky hills, 1515 m, 24.04.2010 (HUB).	OM735750 / OM669735
Çilden 1922	<i>I. odorata</i>	Denizli: between Çardak-Denizli, 500 m to Kaklık district, roadside, 562 m, 37°50'12.6" N, 29°26'4.34" E, 09.05.2019, E. Çilden & Şeyda Çilden (HUB).	OM735751 / OM669718
Altınözlü 5877	<i>I. odorata</i>	Mardin: Dargeçit, between Temelli-Kartalkaya villages, 739 m, steppe, 37,4161454 N, 37,7535578 E, 12.04.2008 (HUB).	OM735752 / OM669717
Güner 8446	<i>I. odorata</i>	Hatay: Belen, 700 m, maquis, calcareous field, 06.04.1991, A. Güner & H. Karaca (HUB 6874).	OM735753 / OM669716
Çilden 1829	<i>I. carica</i>	Aydın: Söke, above Güllübağçe village, Priene ruins, rocky slopes, 160 m, 37°39'39,16" N, 27°17'52,1" E, 02.05.2018 (HUB).	OM735754 / OM669741

Table 1. (Continued).

Çilden 1766	<i>I. halophila</i>	Aksaray: Eskiil, Tuz gölü yanı, tuzcul alan, yıkanmış toprak, 38°26'33.762" N, 33°26'52.60 E, 930 m, 31.03.2018 (HUB).	OM735755 / OM669740
Dirmenci 2516a	<i>I. saxatilis</i> subsp. <i>saxatilis</i>	Balıkesir: Kazdağı (İda), Nanekırı, calcareous, 1500 m, 19.05.2004 (HUB).	OM735756 / -
Çilden 1895	<i>I. saxatilis</i> subsp. <i>magnesiata</i>	Manisa: Soma, Kocasıvri hill, 850 m, 23.04.2019 (HUB).	OM735757 / OM669715
Güner 5066	<i>I. gypsicola</i>	Eskişehir: 18 km from Polatlı to Sivrihisar, near military zone, 750 m, steppe, calcareous fields, 29.06.1983 (HUB 6875).	OM735758 / OM669739
Özüdoğru 3805	<i>I. simplex</i>	Antalya: Serik, exit of Haciosmanlar village, 160 m, roadside, 37.16641 N; 30.94884 E, 31.03.2016 (HUB).	OM735759 / -
Özüdoğru 4012	<i>I. simplex</i>	Hatay: Kızıldağ, Maden, Radar road, 1418 m, open fields, 36.30389 N; 36.05105 E 02.05.2016 (HUB).	OM735760 / OM669758
Özüdoğru 3599	<i>I. simplex</i>	Niğde: Çamardı, Emni valley entrance, 1400 m, steppe, rocky slopes, 37.782246 N; 35.07461 E, 27.06.2015 (HUB).	OM735761 / -
Özüdoğru 3626	<i>I. simplex</i>	Osmaniye: south of Yarpuz village, Daz road, 1275 m, mixed forest, 37.02202 N; 36.45902 E, 29.05.2015 (HUB).	OM735762 / -
Özüdoğru 3672	<i>I. simplex</i>	Erzincan: Karadağ crest way, 2750 m, rocky places at alpine zone, 39.90662 N; 39.12456 E, 16.07.2015 (HUB).	OM735763 / -
Özüdoğru 3449	<i>I. simplex</i>	Kayseri: Between Burhaniye and Kapuzbaşı, 1435 m, 37°49'51" N, 35°30'28" E, 09.06.2013 (HUB).	OM735764 / -
Özüdoğru 3609	<i>I. simplex</i>	Niğde: between Hamidiye and Büyüksokurlu villages, 37.53602 N; 35.01011 E, 1250 m, 27.06.2015 (HUB).	OM735765 / -
Özüdoğru 3543	<i>I. simplex</i>	Kayseri: between Yahyalı and Kapuzbaşı, roadside, exit of Delialıuşağı village, 1123 m, 37°56'712"N, 35°32'16"E, 12.06.2014 (HUB).	OM735766 / -
Özüdoğru 3426	<i>I. simplex</i>	Ankara: Ayaş, calcareous steppe, 1200 m, 40°05'19"N, 32°26'01"E, 17.05.2013 (HUB).	OM735767 / OM669753
Özüdoğru 3474	<i>I. simplex</i>	Erzincan: Sakaltutan passage, 2040 m, in scree and slopes, 13.07.2013 (HUB).	OM735768 / -
Özüdoğru 3517	<i>I. simplex</i>	Mersin: between Anamur and Ermenek, towards Kaşaylaya, mountain road, 36°10'8"N, 32°54'35"E, roadside, 730 m, 25.05.2014 (HUB).	OM735769 / -
Vural 4777	<i>I. simplex</i>	Neşehir: Ortahisar, 1200 m, volcanic tuff, border of gardens, slopes, 20.05.1989 (HUB 24298).	OM735770 / OM669742
Özüdoğru 4693	<i>I. simplex</i>	Antalya: Elmali, Kızılarsivri, 2500 m, rocky scree, 36.59413 N; 30.0966 E, 08.07.2016 (HUB).	OM735771 / OM669757
Özüdoğru 4770	<i>I. gypsicola</i>	Ankara: Polatlı, between Sazlar and Ömerler villages, gypseous steppe, 04.05.2017 (HUB).	OM735772 / OM669756
Özüdoğru 3665	<i>I. simplex</i>	Erzincan: Sakaltutan passage, slopes at the entrance of Karadağ mountain road, 2170 m, rocky scree, 39.88215 N; 39.13458 E, 16.07.2015 (HUB).	OM735773 / -

Table 1. (Continued).

Özüdoğru 5209	<i>I. simplex</i>	Çankırı-Tosya road, 37 km to Tosya district, damp places under <i>P. nigra</i> forest, 1370 m, 579674 N; 4519651 E, 22.06.2017 (HUB).	OM735774 / OM669755
Özüdoğru 5400	<i>I. simplex</i>	Sinop: Durağan, around Ilıksu dam, fractures of rocks, 230 m, 41.357154 N; 35.023796 E, 20.04.2019 (HUB).	OM735775 / OM669747
Özüdoğru 5418	<i>I. simplex</i>	Sivas: Kangal, Düşek area, 1125 m, gypseous area, 39.239005 N; 37.351054 E, 14.06.2019 (HUB).	OM735776 / OM669754
Özüdoğru 5430	<i>I. simplex</i>	Adana: Pozanti, Börücek village, roadside, <i>Pinus</i> forest, 37.85036 N; 34.83395 E, 1217 m, 22.06.2019 (HUB).	OM735777 / OM669752
Erik 3859	<i>I. simplex</i>	Neşehir: between Göreme and Ürgüp, calcareous hills, 17.06.1986, S. Erik 3859, J. Venter (HUB 6896).	OM735778 / -
Özüdoğru 5449	<i>I. simplex</i>	Kayseri: between Pınarbaşı and Sarız, 15. km, 1650 m, serpentine field, 39.866606 N; 39.550770 E, 05.07.2019 (HUB).	OM735779 / OM669751
Çilden 1809	<i>I. simplex</i>	Mersin: Mersin to Gülnar, Çamlıca (Çaltı) village entrance, 36°20'35.18" N; 33°43'36.17" E, 950 m, 21.04.2018 (HUB).	OM735780 / OM669746
Çilden 1782	<i>I. simplex</i>	Niğde: Pozantı-Kamışlı road, Alpu village, 37°28'18.152" N; 34°52'30.46" E, 19.05.2018 (HUB).	OM735781 / OM669744
Erik 1886	<i>I. simplex</i>	Konya: Ereğli, Aydos dağı, Kayasaray, steppe, 1600 m, 16.05.1977 (HUB 6900).	OM735782 / OM669743
Özüdoğru 5481	<i>I. simplex</i>	Sivas: Ulaş-Kangal road, around DŞİ dam, 39.201250 N; 37.247840 E, 2560 m, gypseous-marn field, 07.07.2019 (HUB).	OM735783 / OM669750
Çilden 1887	<i>I. simplex</i>	Eskişehir: Alpu, Kireçköy, gypseous field, 30°96'58"N, 44°04'38.5" E, 25.05.2018 (HUB).	OM735784 / OM669745
Özüdoğru 5496	<i>I. simplex</i>	Erzincan: Above Sakaltutan passage, Karadağ, 39.897650 N; 39.131600 E, 2600 m, 07.07.2019 (HUB).	OM735785 / OM669749
Özüdoğru 5524	<i>I. simplex</i>	Erzincan: Keşiş mountain, 39.821630 N, 39.764810 E, 2670 m, roadside, 09.07.2019 (HUB).	OM735786 / OM669748
Çilden 1759	<i>I. attica</i>	Muğla: between Marmaris and Datça, roadside, Hisarönü village, 36°47'29,05"N, 28°3'18,97" E, 33 m, 17.03.2018 (HUB).	OM735787 / -
Doğru Koca 4201	<i>I. attica</i>	Manisa: Spiladağı, around Atalını, 1250 m, 13.06.2014, ADK & G. Zare (HUB).	OM735788 / OM669730
Çilden 1806	<i>I. attica</i>	Mersin: between Gözne and Arslanköy, Yavca village, 100 m, roadside, calcareous soil, 36°1'6.74" N, 34°31'59.27" E, 1200 m, 20.04.2018, E. Çilden & Y.E. Özdemir (HUB).	OM735789 / OM669725
Çilden 1841	<i>I. attica</i>	Muğla: Köyceğiz, Hamitköy, roadside, 36°54'43.29"N, 28°37'12.8" E, 80 m, 02.05.2018; EÇ & Şınası Çilden (HUB).	OM735790 / OM669731
Çilden 1849	<i>I. attica</i>	İzmir: Nif dağı, 38°23'9.59"N, 27°21'57.47" E, 1290 m, 03.05.2018, E. Çilden & H. Yıldırım & A.F. Pirhan (HUB).	OM735791 / OM669727
Çilden 1885	<i>I. attica</i>	Muğla: between Ören and Maşta (Balık village, Burdur), roadside, 1380 m, 20.05.2018, (leg. R. Özdemir) (HUB).	OM735792 / OM669732

Table 1. (Continued).

Özüdoğru 4982	<i>I. attica</i>	Adana: Feke, above Gürümze village, 38.059625 N; 35.792996 E, 1700 m, roadside, 06.06.2017 (HUB).	OM735793 / OM669720
Çilden 1888	<i>I. attica</i>	Antalya: Alanya, İbradi mountain road, 36°30'46"N, 32°12'42"E, 903 m, 01.06.2018, (leg. A. Tıraş) (HUB).	OM735794 / OM669729
Çilden 1889	<i>I. attica</i>	Antalya: Alanya-Hadim road, 36°34'30"N, 32°22'4"E, 1330 m, 01.06.2018, (leg. A. Tıraş) (HUB).	OM735795 / OM669726
Çilden 1893b	<i>I. attica</i>	Antalya: Alanya-Sarveliler road, 36°30'53"N, 32°12'48"E, 916 m, 25.07.2018, (leg. A. Tıraş) (HUB).	OM735796 / OM669724
Çilden 1898	<i>I. attica</i>	Muğla: Yılanlı dağı, 37°13'36"N, 28°23'30"E, 920 m, 25.04.2019, (leg. B. Topçuoğlu) (HUB).	OM735797 / -
Çilden 1899	<i>I. attica</i>	Antalya: Akseki, Murtıçı village, Kapuz area, north hills, river side, forest road, 36.862758 N, 21.745709 E, 400-450 m, 28.04.2019, (leg. S. Uysal) (HUB).	OM735798 / OM669721
Çilden 1928	<i>I. attica</i>	Muğla: entrance of Muğla province, roadside, calcareous soil, 37°12'17.495"N, 28°20'37.081"E, 650 m, 10.05.2019 (HUB).	OM735799 / OM669728
Çilden 1929	<i>I. attica</i>	Muğla: between Muğla and Kale, roadside, 800 m, 10.05.2019 (HUB).	OM735800 / OM669723
Çilden 1937	<i>I. attica</i>	Muğla: Köyceğiz, Toparlar waterfall, Toparlar area, 36°59'31"N, 28°37'53"E, 10 m, (leg. B. Topçuoğlu) (HUB).	OM735801 / OM669719
Çilden 1938	<i>I. attica</i>	Karaman: Tarlaören, 36S 0523921, 4100333, 1363 m, 20.05.2019, (leg. Ö. Koçak) (HUB).	OM735802 / OM669722
Çilden 1939	<i>I. attica</i>	Muğla: Çameli-Fethiye road, near Tuzlabeli, 900-1200 m, (leg. R. Özdemir) (HUB).	OM735803 / OM669733

of Rogers and Harpending (1992). The significance of the sudden expansion model was assessed by calculating the raggedness index (r) (Harpending, 1994) and Ramos-Onsins and Rozas's test statistic (R_2) (Ramos-Onsins and Rozas, 2002). Analyses were conducted in DnaSP version 5.10 (Librado and Rozas, 2009).

3. Results

Basic statistics of *I. simplex* and closely related species obtained from ITS and *trnL-F* datasets are given in Table 2. As seen in Table 2, sequencing success is lower in ITS dataset compared to *trnL-F* dataset (40 vs. 52). On the other hand, the number of the parsimony informative sites are conspicuously higher in the ITS dataset. A total of 13 ITS ribotypes were obtained from 40 individuals. One ribotype (R1) appeared at higher frequency, observed in 21 individuals. The remaining ribotypes were separated from common ribotypes generally by one or two mutational

steps, with the exception of R3 (5 steps) and R13 (3 steps) (Figure 3). In the *trnL-F* dataset, 11 haplotypes were detected from 52 individuals. One haplotype (H1) was observed in 42 individuals and was found at higher frequencies, as observed in the ITS dataset. The haplotype network is typically star-shaped, in which a common haplotype is placed in the center and the remaining haplotypes are separated from it by one or two mutational steps (Figure 4). Tajima's D and Fu's F_s values were negative in both ITS and *trnL-F* datasets and statistically significant. In general, both haplotype and nucleotide diversities are higher in the ITS dataset, although the *trnL-F* dataset includes more individuals.

3.1. Phylogenetic analyses and time estimation

The ITS and *trnL-F* datasets were concatenated because the tree topologies obtained from individual gene trees were almost the same (The members of *I. simplex* species complex are monophyletic, *I. amara* is close to this

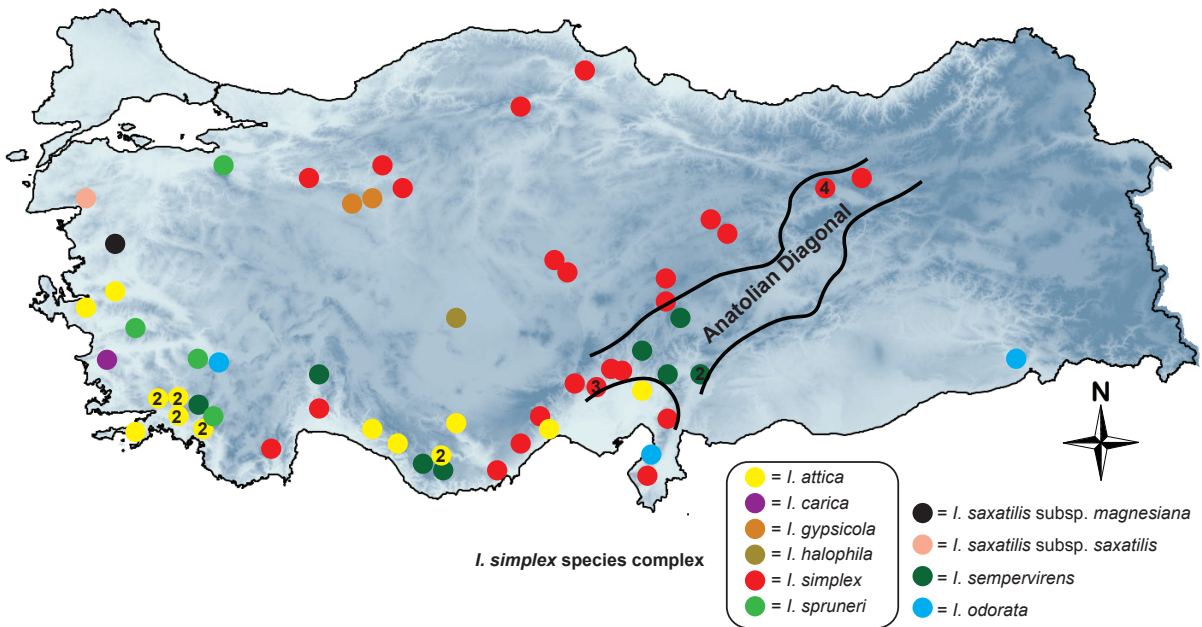


Figure 2. Distribution of the *Iberis* species used in this study throughout Anatolia. Close accessions are given with a single circle and accession numbers are given in the circle. The members of the *I. simplex* species complex are indicated in the square below the map.

Table 2. Summary of genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F_s) for nuclear ITS data. N, number of sequences; SL, sequence length (bp); S, number of segregating sites; Pi, parsimony informative sites; H, number of haplotypes; Hd, haplotype diversity; π , nucleotide diversity. *: $p < 0.05$, **: $p < 0.01$.

	N	SL	S	Pi	H	Hd	π	D	F_s
ITS	40	632	18	7	13	0.717	0.00265	-1.97574*	-6.831
<i>trnL-F</i>	52	716	11	1	11	0.351	0.00064	-2.34523**	-12.598

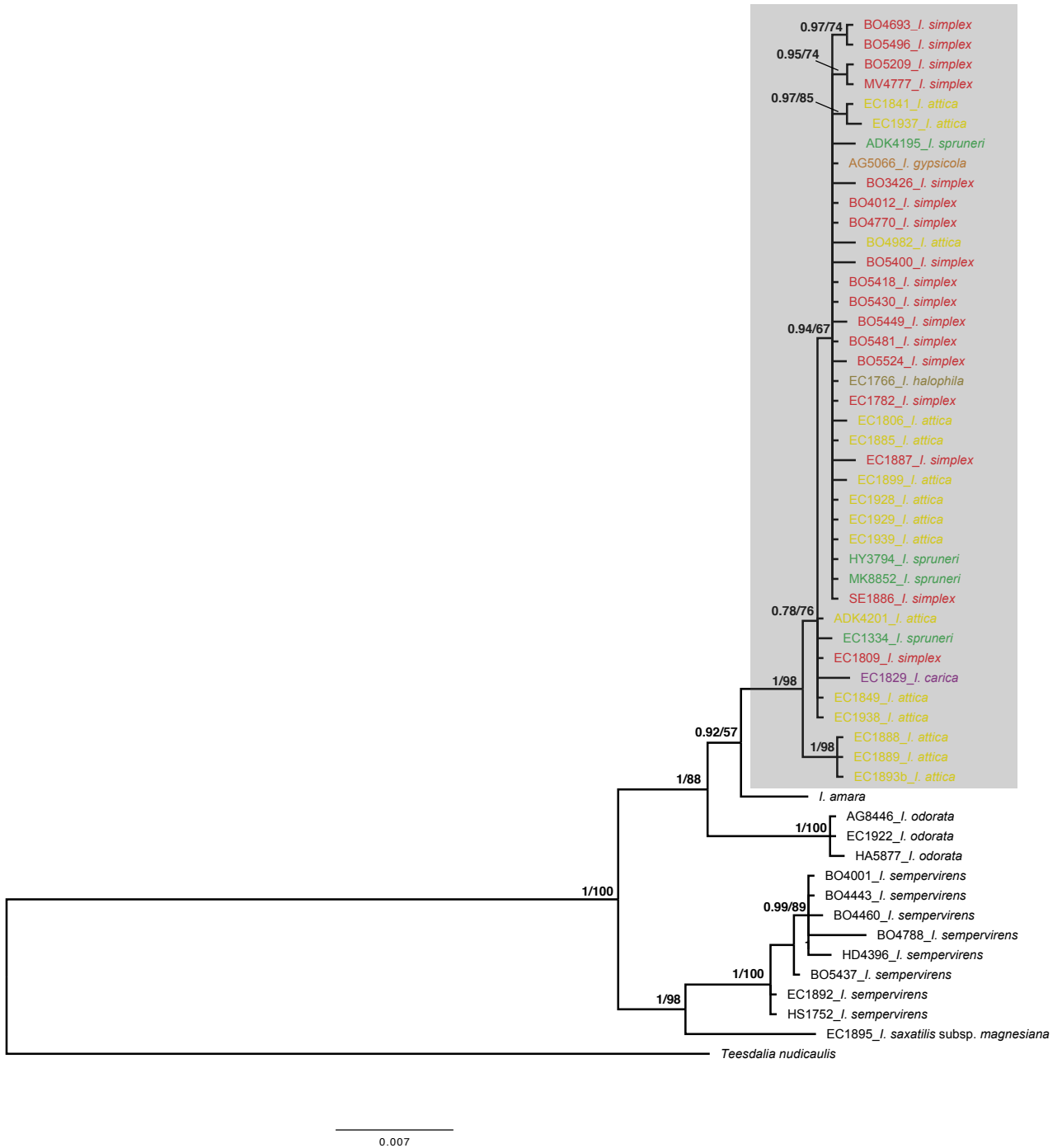


Figure 3. Phylogenetic relationships of the *I. simplex* species complex and available *Iberis* species based on concatenated ITS and *trnL-F* datasets. The *I. simplex* species complex is highlighted in gray on the tree.

complex, *I. sempervirens* and *I. saxatilis* are sister etc.). The results of concatenated analyses are presented in Figure 3 and individual ITS and *trnL-F* trees are given in Figures S1 and S2.

The monophyly of the *I. simplex* species complex was strongly supported by both BI and ML analyses with 1

posterior probabilities (pp) and 98 bootstrap (bp) supports, respectively (Figure 5). However, the relationships of these six species remain unknown because of the several polytomies of the clade. Although another weakly supported clade (pp = 0.94, bp = 67) appeared within the main clade, it does not correspond to any taxonomic

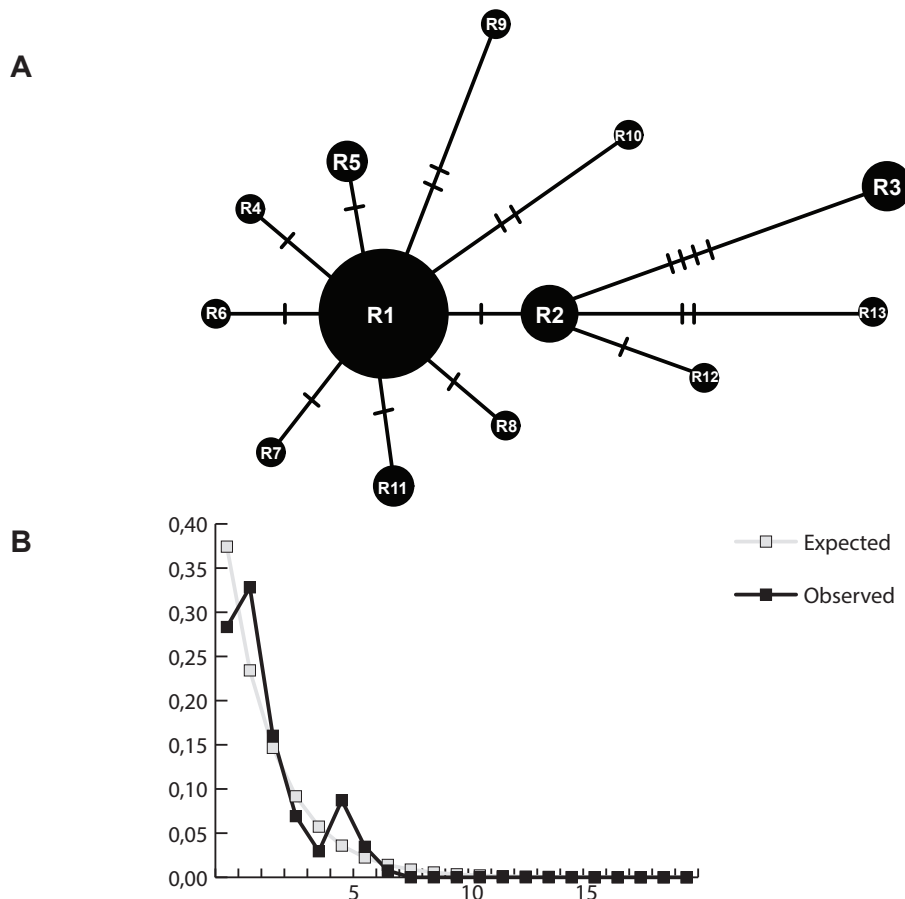


Figure 4. Ribotype network and mismatch distribution analyses of pairwise ribotype differences of *I. simplex* species complex: **A)** haplotype network of the obtained ITS ribotypes, **B)** distribution of pairwise differences among ITS ribotypes.

treatment of the members of the *I. simplex* species complex and it includes individuals from five of the six species, excluding some individuals belonging to *I. attica* and *I. simplex*. Additionally, the phylogenetic positioning of the remaining Anatolian *Iberis* species outside the *I. simplex* species complex (*I. odorata*, *I. sempervirens*, and *I. saxatilis*) seems to be robust.

The BEAST chronogram of the ITS and *trnL-F* datasets is presented in Figure 6. All parameters had an effective sample size (ESS) of >200. Divergence time estimates indicated that the crown age of the members of the *I. simplex* species group dates back to 0.65 mya (95% HPD: 0.27–1.12), which suggests that the species complex originated during Pleistocene.

A significant population expansion in the *I. simplex* species complex was supported by multiple tests. Both ITS and *trnL-F* data and Tajima's D and Fu's F_s values were significantly negative for the *I. simplex* species complex, indicating population expansion. Whereas mismatch distributions of ITS data showed bimodal distribution,

trnL-F data were unimodal, indicating a population expansion in the history of the species complex.

4. Discussion

There have been several crucifer genera subjected to phylogeographical studies (*Aubrieta*, *Bornmuellera*, *Microthlaspi*, and *Ricotia*, among others) in recent years (Ali et al., 2016; Koch et al., 2016; Özüdoğru and Mummenhoff, 2020; Özüdoğru et al., 2021). Among these studies, *Iberis* species have never been tested phylogenetically or biogeographically with the exception of family-wide phylogenetic studies that only included one or two *Iberis* species (e.g., Warwick et al., 2010; Huang et al., 2020). Therefore, to the best of our knowledge, the present study is the first attempt to elucidate the phylogenetic relationships and phylogeographic history of the six *Iberis* species, which are considered for the first time within the *I. simplex* species complex. Phylogenetic analyses of the *I. simplex* species complex, with the inclusion of the remaining Anatolian species not encompassed by this

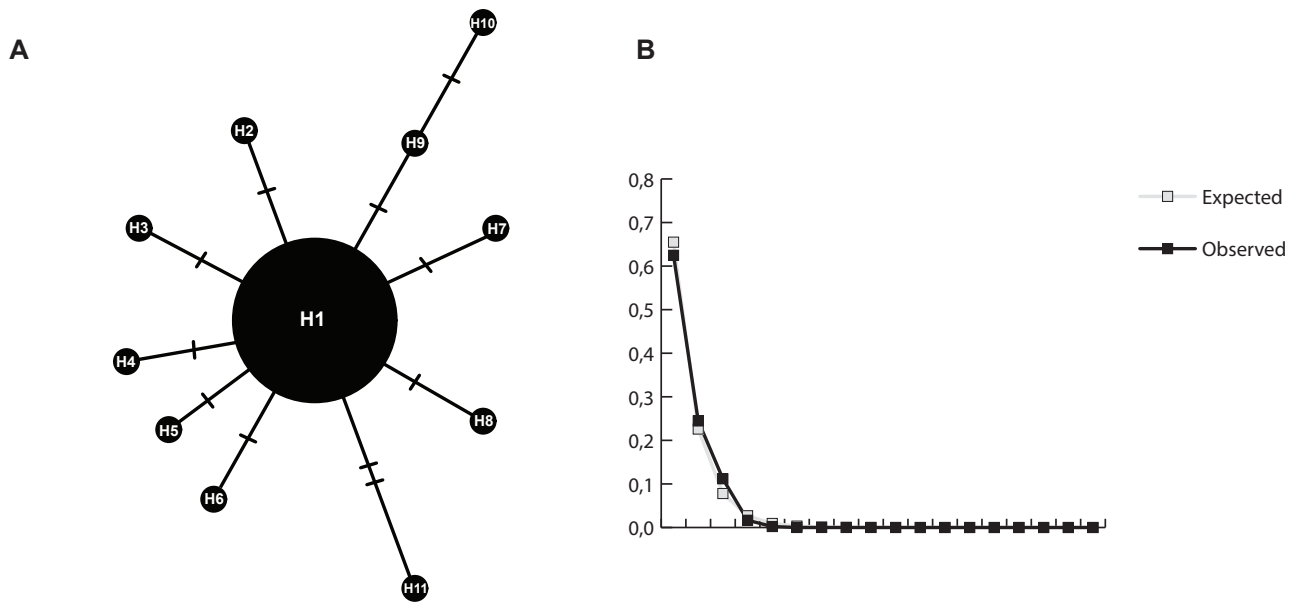


Figure 5. Haplotype network and mismatch distribution analyses of pairwise haplotype differences of the *I. simplex* species complex: A) haplotype network of the obtained *trnL-F* haplotypes, B) distribution of pairwise differences among *trnL-F* haplotypes.

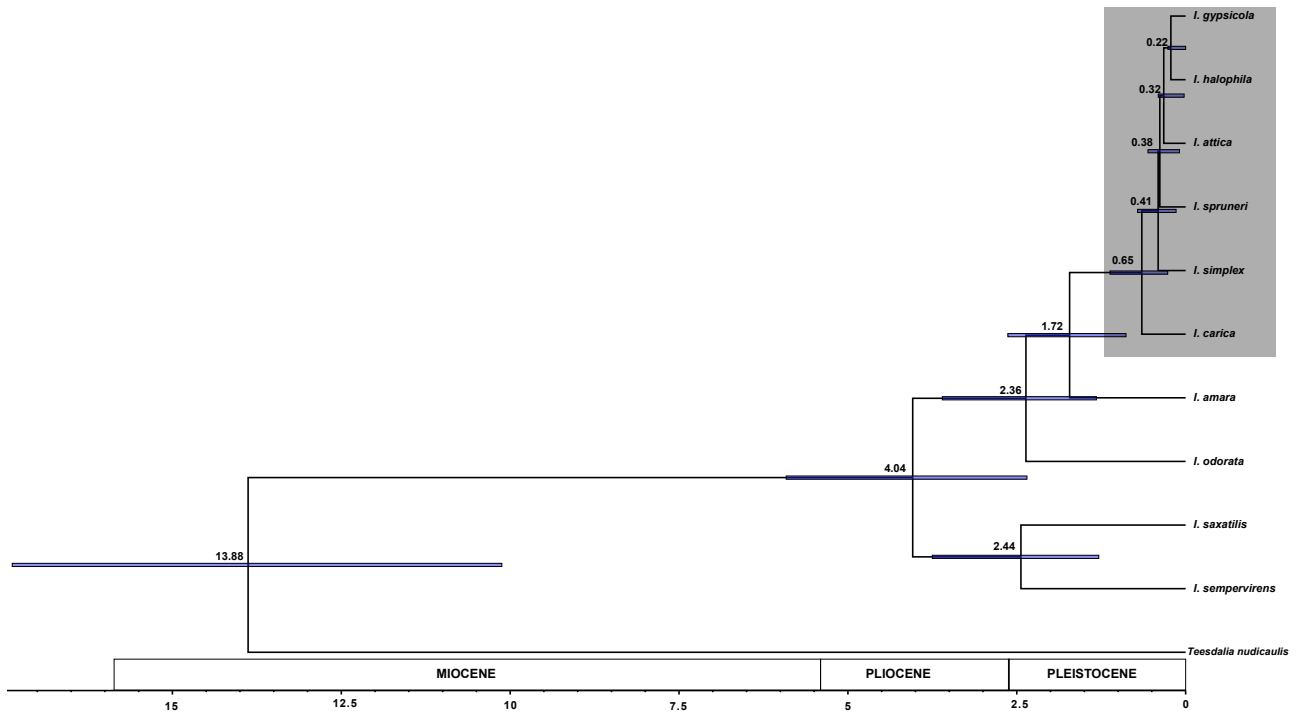


Figure 6. BEAST-derived chronogram of *Iberis*. The members of the *I. simplex* species complex are highlighted in gray on the tree. Blue bars on the nodes indicate lower and upper bands of the estimated times, whereas numbers indicate mean values.

complex (*I. odorata*, *I. sempervirens*, and *I. saxatilis*) along with accessible species from beyond Anatolia, clearly showed the monophyletic origin of the *I. simplex* species complex. Although species outside the complex seem to be distinct with high pp/bp supports, the relationships

among the species of the *I. simplex* species complex remain unresolved as the clade collapses into polytomy (Figure 3).

According to coalescent theory, recently diverged species with large effective population size (N_e) might have such polytomic relationships in their gene trees

because of incomplete lineage sorting (Kingman, 1982; Hudson et al., 1990; Nichols, 2001; van Velzen et al., 2012). Our diverging time analysis estimates the time to the most recent common ancestor (t_mrca) of the *I. simplex* species complex to be ca. 0.65 mya and this result corresponds with the recent divergence of this species considering the t_mrca of the remaining *Iberis* species in the analyses at more than 1 mya (ranging from 1.72 to 2.44 mya, Figure 6).

Some organisms in Anatolia have been tested for sudden population expansion. A rapid population expansion after the Last Interglacial (LIG; ~140-120 kya) has been shown for the alpine plant *Noccaea iberidea* (Boiss.) Al-Shehbaz & Menke, whereas demographic equilibrium over time and stable population size over time have both been demonstrated for the Mediterranean genus *Ricotia* L. (Özüdoğru et al., 2020, 2021). Given the fact that most of the *Iberis* species are Mediterranean, the most likely scenario is a rapid expansion of the populations from Mediterranean parts of Anatolia to steppic areas of the Irano-Turanian floristic region in Anatolia. Similar or opposite scenarios have been observed in other plant groups in Anatolia and adjacent regions. For example, the genera *Haplophyllum* Rchb. (Rutaceae) and *Hesperis* L. (Brassicaceae) were reported to have evolved in the Irano-Turanian region and subsequently invaded the Mediterranean, whereas the opposite was reported for the genus *Ricotia* (Manafzadeh et al., 2014; Eslami-Farouji et al., 2021; Özüdoğru et al., 2021). However, it is not possible to test these hypotheses with the available data and, for such a study, a larger number of *Iberis* species from outside Anatolia should be included.

The rapid population expansion of the *I. simplex* species complex was supported by haplotype and ribotype networks. One haplo/ribotype (H1/R1) was found at a higher frequency, and the other haplotypes were generally separated from this haplotype by one or two mutation steps in both networks with the exception of R3 (5 steps) and R13 (3 steps) (Figures 3 and 4). As a consequence, both networks were more or less star-like and indicated a recent population expansion. A rapid historical population expansion was also supported by neutrality tests (negative and significant values in Tajima's D and Fu's F_s tests) and the unimodal mismatch distribution of *trnL-F* data. The unimodal distributions of pairwise differences between individuals indicate that the population recently experienced demographic expansion, whereas bimodal or multimodal distributions suggest that the population is stable or declining (Harpending, 1994).

From a taxonomic perspective, *I. attica* and *I. spruneri* have been reduced to synonyms of *I. carnosa* recently and this treatment is being followed by some databases listing these names under the name *I. carnosa* (<https://powo.science.kew.org/taxon/285494-1>; <http://www.theplantlist.org/tpl/record/kew-2859707>;

<https://powo.science.kew.org/taxon/285665-1>; <http://www.theplantlist.org/tpl/record/kew-2859930>; Mutlu, 2012). However, there are several micromorphological and anatomical features to distinguish these two species, e.g., bifacial/dorsiventral leaf anatomy is characteristic of *I. attica*, whereas *I. spruneri* has isolateral leaves (Çilden and Zare, 2019). *Iberis attica* is distributed in the Mediterranean phytogeographic region from İzmir to Hatay including some parts of inner Anatolia, such as Karaman, Denizli, and Burdur, and altitude from 10 to 1300 m (Çilden & Zare 2019, Çilden 2022a). *I. spruneri* is a morphologically small-sized species up to 10 cm and generally grows at high altitudes from 1500 to 2800 m (Çilden 2022a). These two taxa differ from each other by habit, plant height, basal leaf shape, petal color and dimension, mature fruit color, sinus width, and wing width (Çilden 2022a, b). Similarly, the taxonomic relationships between *I. attica* and *I. carica* seem to be confusing based on the identification of herbarium specimens, and most *I. attica* specimens were identified as *I. carica* in several herbaria (Çilden and Zare, 2019; Çilden, 2022a, 2022b). However, our unpublished pollen data together with published anatomical, fruit/seed macro- and micromorphological data indicate several differences between the two species.

Although there are several morphological similarities between *I. simplex* and *I. halophila*, we have observed at least one morphological difference (succulent leaves) and one ecological difference (adaptation to halophytic areas) between these two species to distinguish *I. halophila* from its close relatives and the widely distributed species *I. simplex*. Although this species resembles *I. attica* and *I. simplex* by its petal dimensions and color, and it grows geographically at the intersection of the distribution areas of these species, there have been no cytogenetic data obtained yet in order to evaluate a possible hybridization event between these species.

The only exception in the *I. simplex* species complex in terms of morphological distinctiveness is *I. gypsicola*, as mentioned before. This species is endemic to Inner Anatolia and is distinguished from *I. simplex* by its densely racemose inflorescence, fruit dimensions, fruit shape, and habitat preferences (gypseous habitats) (Yıldırım, 2018). However, detailed field and herbarium studies showed that some populations of *I. simplex* (e.g., Sivas-Kangal, Kayseri-Kapuzbaşı, Eskişehir-Sivrihisar/Kepen, and Ankara-Ayaşbeli) have the same morphological characters (Çilden, 2022b). Furthermore, individuals of *I. gypsicola* are found not only in gypseous but also in limestone soil (e.g., the Eskişehir-Oğlakçı, Sivas-Kangal, or Kayseri-Kapuzbaşı populations). Therefore, we tentatively conclude that *I. gypsicola* represents a group of *I. simplex* population which adapted to harsh environmental conditions.

This conclusion may also appear to be applicable to *I. halophila*, but contrary to *I. gypsicola*, it is known from only one location and there have been no observations in nonhalophilous habitats. For this reason, to achieve a final taxonomical conclusion about *I. halophila* seems to be harder than *I. gypsicola*.

For two reasons, we ignore the possibility of subsuming all six species within one species, at least for now. Firstly, our study was based on two markers and we believe that including more markers will facilitate the obtaining of a more robust phylogenetic tree and delimited species boundaries. Secondly, apart from *I. gypsicola* (see above), there are several micro-/macromorphological, anatomical, and palynological characters that distinguish these species.

Taking into account the available data, it can be tentatively argued that the members of the *Iberis simplex* species complex underwent a rapid population expansion in the Pleistocene and some key innovations (e.g., succulent leaves to adapt to saline environments) evolved for them to colonize different habitats, but they did not accumulate sufficient mutations.

Although the impact of the hybridization and polyploidy on the diversification of the crucifers have long been documented by several studies (Mandáková et al., 2018; Guo et al., 2020; Dogan et al., 2021 among others), we have very little knowledge about these processes in the genus *Iberis* except for *I. umbellata* which has undergone whole genome duplication (WGD) event (Mandáková et al., 2017). Various base chromosome numbers ($n = 7, 8, 9, 11$) have been known for the genus *Iberis* and diploid and polyploid cytotypes have been reported for some *Iberis* species (e.g., *I. sempervirens*, $2n = 22, 66, 70$) until

now (Ančev, 2007), but there are no data from Anatolian *Iberis* species with the exception of the reported base chromosome numbers of *I. attica* and *I. spruneri* (Oskay, 2016). Therefore, this limited amount of information about the Anatolian population of *Iberis* restricts our understanding of the possible role of polyploidization in the diversification of *Iberis*.

In conclusion, our study represents the first step in reconstructing the phylogeographic history of the *I. simplex* species complex and it also draws the boundaries among these six species. However, the demographic hypothesis presented here needs to be tested with the inclusion of nonmolecular data (e.g., climate or soil data) to test for niche differentiation between the members of the *I. simplex* species complex.

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