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Original Article

Molecular Insight on Phylogeny and Systematics of the Narrowly Endemic *Centaurea bingoelensis* (Asteraceae)

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ABSTRACT

Centaurea L. is a genus that is systematically discussed and whose taxonomic issues need to be clarified in the light of molecular techniques developed in recent years. This study aims to contribute taxonomically in molecular terms by studying the morphological and phylogenetic relationships of *Centaurea bingoelensis*. This limited, endemic species grows naturally in the Bingöl province of Türkiye. A phylogenetic analysis based on the molecular marker ITS, which is the most preferred in evolutionary studies and found in nuclear DNA, has been proposed as a molecular marker of *C. bingoelensis* , an endemic plant in Türkiye, and has not yet been molecularly identified. For this purpose, PCR amplification of the fragment mentioned above was performed, followed by direct sequencing, analysis of nucleotide variation, and phylogenetic analysis with different species. The *Centaurea* species identified so far have been shown to have three main distinct major clades and the specific phylogenetic methods used (NJ, ML, MP) and the ITS nucleotide sequences analyzed revealed a close phylogenetic relationship between *C. bingoelensis* and *C. spectabilis*. For a more accurate phylogenetic classification of this species within the Asteraceae family, their close relationships were probed using similar sequences from the GenBank Database. Phylogenetic analysis of *C. bingoelensis* specimens using ITS sequences reveals their position in the Asteraceae family and molecular support for these specimens being new species as proposed. Although morphologically similar to *C. fenzlii* and *C. obtusifolia*, the remarkable point of our study is that *C. bingoelensis* is phylogenetically closer to *C. spectabilis*. This work has exciting implications for understanding the evolution and diversification of the genus *Centaurea*. In addition, the results of this study show that the samples identified as *Centaurea* species in Türkiye are still not entirely determined genetically.

Key words: Endemic, Compositae, ITS, Systematic, Phylogeny

Dar Yayılışlı Endemik *Centaurea bingoelensis'in* (Asteraceae) Filogenisi ve Sistematiğine Moleküler Bakış

ÖZ

Centaurea L. sistematik olarak tartışılan ve son yıllarda gelişen moleküler teknikler ışığında taksonomik sorunların açıklığa kavuşturulması gereken bir cinstir. Bu çalışma *Centaurea bingoelensis* Behçet & İlçim türünün morfolojik ve filogenetik ilişkilerini inceleyerek moleküler açıdan taksonomik katkı sağlamayı amaçlamaktadır. Bu sınırlı endemik tür, Türkiye'nin Bingöl ilinde doğal olarak yetişmektedir. Türkiye'de endemik bir bitki olan ve henüz moleküler olarak tanımlanmamış olan *C. bingoelensis* için evrimsel çalışmalarda en çok tercih edilen ve nükleer DNA'da bulunan ITS moleküler markörüne dayalı bir filogenetik analiz önerilmiştir. Bu amaçla, yukarıda

bahsedilen parçanın PCR amplifikasyonu gerçekleştirilmiş, ardından doğrudan dizileme, nükleotid varyasyonunun analizi ve farklı türlerle filogenetik analiz yapılmıştır. Bu türün Asteraceae familyası içinde daha doğru bir filogenetik sınıflandırması için, Gen Bank veri tabanındaki benzer diziler kullanılarak yakın ilişkileri araştırılmıştır. Şu ana kadar tanımlanan *Centaurea* türlerinin üç ana belirgin klada sahip olduğu ve kullanılan spesifik filogenetik yöntemlerin (NJ, ML, MP) ve analiz edilen ITS nükleotid dizilerinin *C. bingoelensis* ve *C. spectabilis* arasında yakın bir filogenetik ilişki olduğunu ortaya koyduğu gösterilmiştir. *C. bingoelensis* örneklerinin ITS dizileri kullanılarak yapılan filogenetik analiz sonuçları, bu türün Asteraceae familyasındaki konumunu ve yeni tür olma olasılığını moleküler açıdan desteklemektedir. Morfolojik olarak *C. fenzlii* ve *C. obtusifolia* türüne benzemekle birlikte, çalışmamızın dikkat çekici noktası *C. bingoelensis* türünün filogenetik olarak *C. spectabilis* türüne daha yakın olmasıdır. Bu çalışma, *Centaurea* cinsinin evrimini ve çeşitlenmesini anlamak için heyecan verici çıkarımlara sahiptir. Ayrıca bu çalışmanın sonuçları, Türkiye'de *Centaurea* türü olarak tanımlanan örneklerin genetik olarak hala tam olarak belirlenemediğini göstermektedir.

Anahtar kelimeler: Endemik, Compositae, ITS, Sistematik, Filogeni

INTRODUCTION

The genus *Centaurea*, one of the important genera of the Asteraceae family, is a systematically problematic genus distributed worldwide and includes approximately 700 species in Asia, North Africa, America, and Europe (Güner et al., 2012). Türkiye is one of the centers of genetic differentiation for the genus *Centaurea*. *Centaurea*, the third largest genus of the flora of Türkiye, has 219 species and 131 of them are endemic (Ozbek, 2021; Uysal, 2012). It is one of the genera with the highest number of endemic taxa. The endemism rate is approximately 59%. The systematics of the genus *Centaurea* has changed, especially with the development of molecular techniques, and thus some problems have been solved. In the light of these studies, it is known that Türkiye is an essential speciation center of the genus *Centaurea*, with many rare endemic species as evidenced by ITS-based molecular data (Médail and Diadema, 2009). For this reason, *Centaurea* constitutes an excellent model for analyzing speciation and diversification processes in the region (López-Pujol et al., 2016). *Centaurea* is also a genus that can be useful for determining gene flow between species and providing insights into the history of the species (Hilpold et al., 2014; Garcia-Jacas et al., 2009).

As a result of ongoing developments in the field of plant molecular systematics, DNA sequence analysis studies have been started to reveal the spectrum of all taxonomic levels and to provide solutions (Soltis et al., 1992). In the last decade, several molecular studies based on the ITS barcode or microsatellite marker for taxonomic identification have been published to investigate genetic diversity and relationships among *Centaurea* (Atia et al., 2021). More recently, Doğan et al. (2015) used ISSR markers to resolve genetic relationships within taxonomic entities of *Centaurea* species, including those considered taxonomically incorrect, such as *C. ptosimopappoides* Wagenitz and *C. straminicephala* Hub.-Mor. (Syn. *Psephellus straminicephalus* (Hub.-Mor.) Wagenitz). They concluded that it was a powerful tool. In addition, Yıldırım et al. (2009), in a study examining genetic relationships among 16 *Centaurea* species, concluded that the RAPD marker system was appropriate when compared to fatty acid methyl ester (FAME) profiles in species in the Eastern Anatolia Region of Türkiye.

As the number of species increases, it becomes increasingly difficult to classify them. *Centaurea* is a taxonomically complex genus, as it contains many species that differ significantly in morphology. In their study, researchers defined *Centaurea bingoelensis* as a new species. They stated that it was similar to *C. fenzlii* and especially to *C. obtusifolia* in terms of leaf structure (Behçet et al., 2017). As a result of their detailed systematic studies, they definitively revealed that it is a distinct species. However, this study dealt with the relationships between taxa based on morphology. Considering the distribution areas of the taxa, *C. fenzlii* is distributed in eastern Anatolia, *C. obtusifolia* is distributed in east-southeast Anatolia, *C. spectabilis* is distributed in eastern Anatolia, while *C. bingoelensis* is found in an area where the distributions of these taxa intersect. In addition, studies have shown that this species, which lives on rocky slopes, is among those at risk of extinction if not protected due to grazing and erosion. For this reason, molecular analyses to be conducted for the first time on this species are important for understanding the systematics of both the genus and species. These analyses also aim to support and complement the existing morphological data. The absence of a comprehensive, well-resolved, and well-supported phylogeny for *Centaurea* is primarily due to the fact that most existing studies are based on relatively small subsets of species and rely on only one or, at most, a few genes or other character sets (e.g., morphology or karyology). Studies using modern systematic methods are critical in determining the degree of relatedness among species and in identifying endemic taxa, if present. To overcome these limitations, all available DNA sequence data of species in this family were pooled and analyzed. *C. bingoelensis* is a rare, narrowly distributed local endemic species found in a limited region of eastern Anatolia (Bingöl province, from

which it is named) in Türkiye. So far, no studies have been conducted on the molecular taxonomy and variation of *C. bingoelensis* , apart from basic ITS-based morphological characterization and identification. In addition, according to Uysal et al. (2021), it is a plant with strong potential for new phyto-pharmacological studies. This study aims to evaluate the phenotypic variation of *C. bingoelensis*, a local endemic species with high pharmacological potential and endangered status, and to evaluate the position of the species in the genus based on the nucleotide sequences of the internal transcribed spacer (ITS) region, which includes nuclear ribosomal DNA.

MATERIALS AND METHODS

Plant specimens

The specimens of thestudied species were collected in the Genç district of Bingöl province (Türkiye) during the flowering period (Figure 1). The collected specimens were prepared as herbarium material for morphological research and are kept in the laboratories of Munzur University for further studies. The studied specimen of *C. bingoelensis* were collected from B8 Bingöl (Türkiye), Genç district, Çötele (çotla) highland roadsides, on 10 June 2020, at an altitude of 1900-2050 m (collector no: UC 20-15).

Figure 1. Photographs from the natural habitat of *C. bingoelensis* (Photo: M.Maruf Balos)

Isolation of DNA

Genomic DNA was isolated from young leaves by the hexadecyltrimethylammonium bromide (CTAB) method as modified by Doyle and Doyle (1990). For each individual, 100 mg of ground leaf tissue was suspended in 500 µl extraction buffer (20 mM EDTA, 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 2% (w/v) CTAB and 5 µl β-mercaptoethanol). The suspension was mixed well and incubated at 60°C for 30 minutes, followed by chloroform-isoamyl alcohol (24:1) extraction and precipitated with 0.75 volumes of isopropanol at -20°C. After centrifugation at low speed for 5 minutes, the resulting pellet was washed with 70% (v/v) ethanol and 10 mM ammonium acetate. DNA was then suspended in TE buffer. The resulting DNA concentration was assessed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

PCR amplification and sequencing

PCR amplification of the samples was carried out using the primers ITS5a_F (5’-CCTTATCATTTAGAGGAAGGAG-3’) and ITS4_R (5’-TCCTCCGCTTATTGATATGC-3 (White et al. 1990; Stanford et al. 2000). These primers amplify the ITS region of approximately600-700 bp in length. The PCR reaction was performed in a thermal cycler (BIO-RAD). The reaction mixture consisted of 40 µl of 500 ng genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of 10 X Taq buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200 µM dNTP, 10 pmol of each primer. Amplification included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, , and extension at 72°C for 1 minute. PCR products were visualized under a transilluminator after electrophoresis on a 1.5% agarose gel-stained ethidium bromide, using 5 µl of the amplified product.

DNA sequencing and phylogenetic analyses

PCR products were submitted to Iontek Laboratories (Istanbul, Türkiye) for Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, Austin, TX,

USA).Bidirectional sequencing was conducted using primers specific to the ITS1-5.8S-ITS2 regions. Sequence alignment and data analysis were performed using ChromasPro V1.34 and Clone Manager 10, respectively. Similarity analysis was carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). In addition to the sequences obtained in this study, reference taxa used in the phylogenetic analysis were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), and the original publications and accession numbers of these sequences were provided in Supplementary material for sequence.

The nucleotide sequences of the ITS1-5.8S-ITS2 regions were aligned using BioEdit v7.2.5 software (Hall, 1999) and the CLUSTAL-W algorithm (Thompson et al. 1994). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), and phylogenetic trees were constructed using the Neighbor-Joining (NJ) (Saitou and Nei, 1987), Maximum Likelihood (ML) and Maximum Parsimony (MP) methods in MEGA X (Kumar et al. 2018). Tree topologies were evaluated using bootstrap analysis with 1,000 replications (Felsenstein, 2004), and *Astragalus mongholicus* (AF359750.1) was used as the outgroup. The resulting trees were visualized using the Interactive Tree of Life (iTOL) webserver (http://itol.embl.de/) (Letunic and Bork, 2016). All other parameters followed default settings.

RESULTS AND DISCUSSION

The molecular and phylogenetic analyses conducted in this study aimed to clarify the taxonomic status and evolutionary relationships of the local endemic species *C. bingoelensis.* Analyses were based on ITS region sequences obtained from two individual samples (T1 and T2) collected from the natural habitat of species. The resulting sequence data were submitted to the NCBI GenBank databases and assigned accession numbers OM905073.1 (for T1) and OM905074.1 (for T2).

Phylogenetic analyses produced similar topologies using the NJ, ML, and MP methods, therefore the results were presented as a single consensus tree (Figure 2). All trees generated by the NJ, MP, and ML methods are showed bootstrap values exceeding 40%, and to improve reliability, only branches with support values above 50% were considered. Bootstrap values greater than 50% are indicated in the Figure 2 and 3. The sequence analyzes of Centaurea-T1 and T2 revealed high similarity to *C. spectabilis* (GenBank Access. No: DQ319164). Both Centaurea-T1 and T2 formed clades strong support, as indicated by bootstrap values exceeding 50% across multiple phylogenetic models: 61% under the Jukes-Cantor(JC69) model, 63% under the Tamura-Nei model with a gamma distribution (TN93+G) and 89% under the MP method (Figure 2-3).

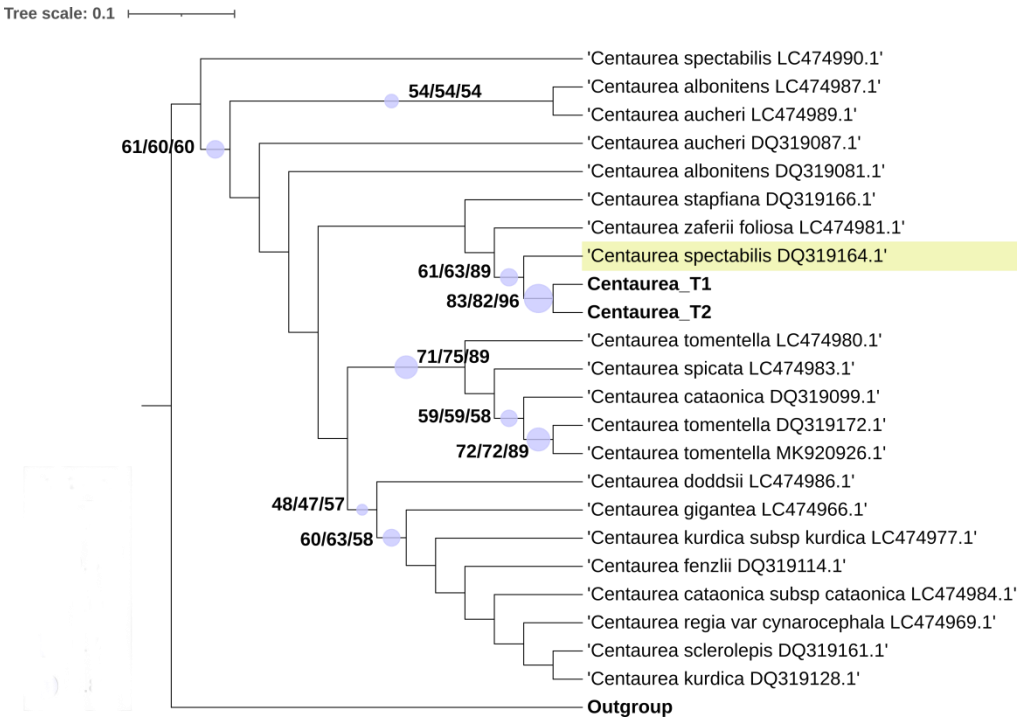

Figure 2. Phylogenetic tree of *Centaurea* species Türkiye, including two samples of *C. bingoelensis* (T1 and T2) analyzed in this study. Bootstrap support values are indicated by the size of the purple circles along the branches, with values ranging from 0.46 to 0.83. The tree was constructed based on ITS region sequences, and branch lengths represent genetic distance. Numbers along branches indicate confidence percentages in neighbor-joining (NJ-JC69), maximum likelihood (ML-TN93+G) and maximum parsimony (MP) analyses (Examples

identified in the study are marked in bold, branching points with Bootstraps values above 40% are shown with purple dots).

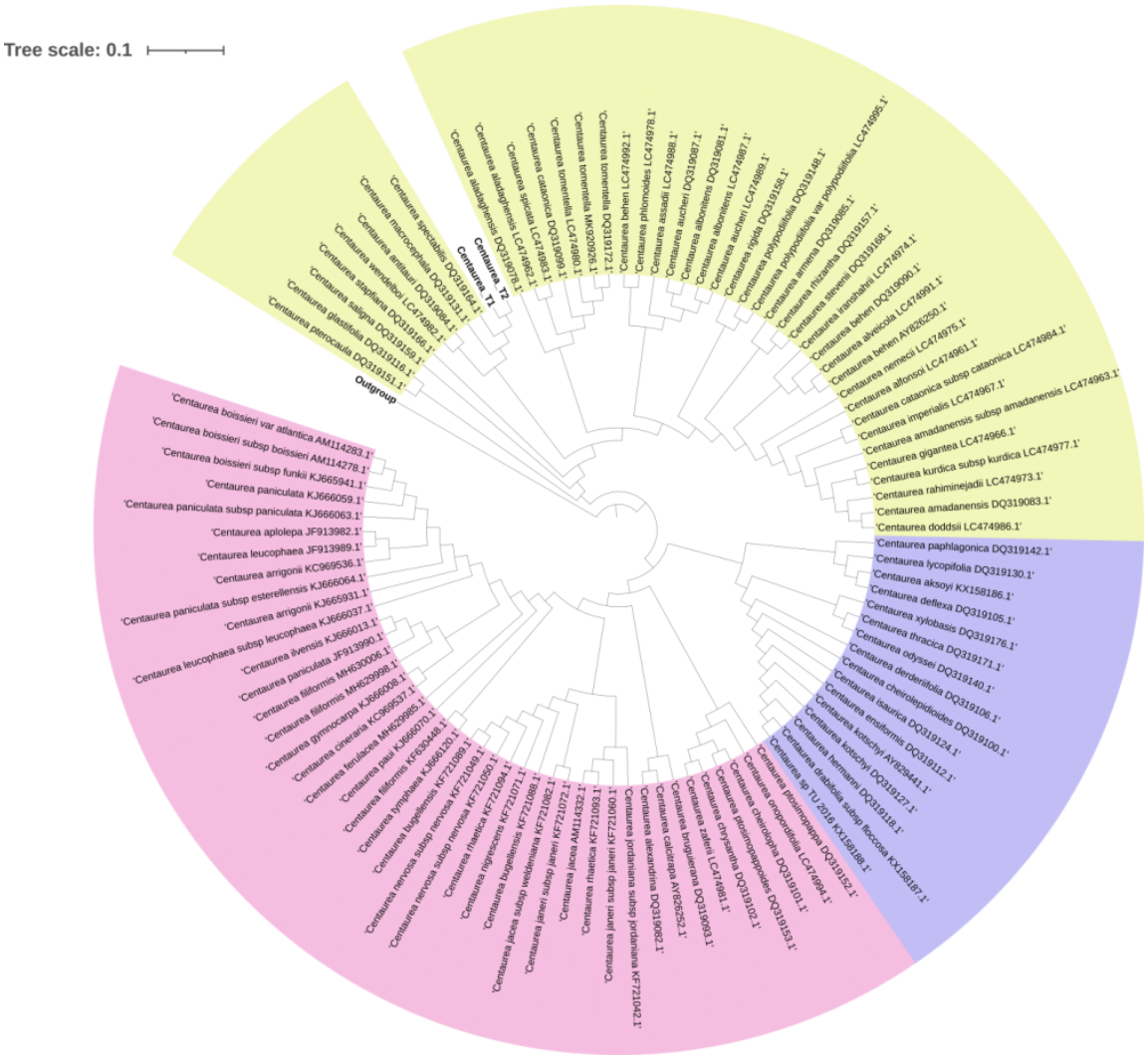

Figure 3. Phylogenetic relationships among identified Centaurea species distributed in Türkiye. The tree is based on ITS region sequences, and taxa are grouped by color to indicate major phylogenetic clades. Bootstrap values (from JC69, ML-TN93+G, and MP methods) were used to construct the consensus tree but are not displayed here.

In this study, phylogenetic analysis was performed by combining the DNA sequences obtained from the endemic species *C. bingoelensis* with the DNA sequences of other *Centaurea* species available in the NCBI database. Two ITS nucleotide sequences derived from two plant samples collected in the study area were used to assess interspecific and intraspecific variation, based on Maximum Likelihood (ML) analysis using the Tamura-Nei 1993 model with gamma distribution (TN93+G). Samples from different locations showed similar morphological features, and these results were consistent with DNA sequencing data. Furthermore, no genetic differences were observed in the ITS region among *C. bingoelensis* specimens collected from different sites Phylogenetic analysis revealed three major clades (Figure 3). A notable finding was that *C. bingoelensis*, which is morphologically similar to *C. fenzlii* and *C. obtusifolia,* clustered with *C. spectabilis,* supported by a high bootstrap value (89%) (Figure 2). However, *C. fenzlii* and *C. obtusifolia* could not be included in the phylogenetic comparison due to the absence of ITS data in NCBI database. With the advancement of phylogenomic approaches, numerous taxa previously misclassified have been reassigned, enabling a clearer understanding of species boundaries. These methods help distinguish between intraspecific variation, introgression, and true speciation, and allow for deeper taxonomic reassessment of evolutionary units, particularly in complex taxa such as *Centaurea* (Wagner et al., 2013; Baumsteiger et al., 2017).

All analyses generated slightly different tree topologies, differing only in poorly supported internal nodes. Therefore, only the Neighbor-Joining (NJ) tree is presented (Figure 2), while notable differences with other topologies are discussed where relevant. All topologies supported the close association among *Centaurea* species. This study represents the first phylogenetic analysis of *C. bingoelensis* that incorporates nearly all currently available ITS sequences of the genus *Centaurea*. Phylogenetic trees were rooted using *Astragalus mongholicus* (AF359750.1) as the outgroup. Despite low bootstrap and posterior probability values in some branches (Figure 3), the general tree topologies were consistent. Significant genetic variation was observed between *Centaurea* species and subspecies, confirming the need for a more comprehensive taxonomic revision. Phylogenetic relationships among European taxa also conformed to their geographic distribution, and ITS region was shown to be a reliable marker for taxonomic clarification. Nonetheless, a complete revision of *Centaurea* taxonomy and systematics will require broader sampling and more detailed morphological, karyological, and molecular analyses.

Both DNA sequence and morphological data revealed that the *C. bingoelensis* specimens analyzed in this study were closely related. This raises questions about their phylogenetic placement within the genus, especially in relation to phytogeographic lineages of *Centaurea*. Phylogenetic analysis using multiple methods, including Maximum Likelihood (under the Jukes-Cantor 1969 and Tamura-Nei 1993+G models) and Maximum Parsimony with1000 bootstrap replicates, consistently placed *C. bingoelensis* outside the clades formed by previously described species. These results support its recognition as a distinct taxon, as originally proposed by Behçet et al. (2017) (Figure 2). Interestingly, although *C. bingoelensis* is a narrow endemic, our results showed a relatively high level of genetic diversity in this species. This was supported by various statistical analyses based on NJ, ML, and MP methods, all indicating genetic variance among species (Saitou and Nei, 1987). According to genetic theory, narrowly endemic species are expected to have low genetic diversity due to small population sizes, population isolation, inbreeding, and ecological specialization (Babbel and Selander 1974; Frankham 2005; López-Pujol et al., 2016). Indeed, meta-analyses have shown a consistent association between endemism and low genetic variation (Nybom, 2004). However, our findings suggested that the ITS region can be a useful molecular marker in evaluating genetic diversity and resolving taxonomic complexity within the difficult genera such as *Centaurea*. Garcia-Jacas et al. (2006) reported that ITS-based phylogenies divided *Centaurea* into three main groups:(1) Mediterranean/European-Siberian, (2) Eastern Mediterranean, and (3) Eastern Mediterranean and Iran-Turanian. Consistent with this, C. bingoelensis clustered within Group 2, along with other *Centaurea* species native in Türkiye. This group is the largest lineages within *Centaurea*, comprising approximately 200 species distributed across the Caucasus, Greece, Iran, and Türkiye (Garcia-Jacas et al., 2006).

Furthermore, López-Pujol et al. (2016) noted a lack of correlation between morphological and genetic classification in *Centaurea* due to the high allopatric (speciation) diversity of genus. Our findings support this, as *C. bingoelensis*, while morphologically similar to *C. obtusifolia* and *C. fenzlii*, clustered phylogenetically with *C. spectabilis*. In terms of geographic distribution, *C. bingoelensis* occurs in an area that overlaps with the ranges of *C. obtusifolia* (South), *C. spectabilis* and *C. fenzlii* (East). Based on phylogenetic relationships, it is likely that *C. bingoelensis* originated from Eastern Anatolia rather than Southeastern Anatolia.

CONCLUSION

This study provides the first molecular phylogenetic assessment of the narrowly endemic species *C. bingoelensis* based on ITS region sequences. The results reveal that *C. bingoelensis*, despite its morphological similarity to *C. spectabilis* and *C. fenzlii.* No significant genetic variation was observed between individuals of *C. bingoelensis* from different collection sites, supporting its status as a genetically coherent species. These findings underscore the importance of integrating molecular data into taxonomic studies, particularly for morphologically complex and geographically overlapping taxa. A broader phylogenetic and morphological reassessment is recommended to fully resolve the evolutionary relationships within the genus *Centaurea.*

DNA availability

The ITS sequences generated in this study, ITS1-5.8S-ITS2, have been deposited in GenBank under the accession numbers OM905073.1 and OM905074.1. Reference sequences used for phylogenetic analysis, along with their GenBank accession numbers and original sources, are listed in Supplementary Table 1.

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Declaration of interests
The authors of this article declare that there are no conflicts of interest.

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The authors declare that they have contributed equally to this article.

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Supplementary Material

The GENBANK accession numbers are given in table.

Taxa	Ac. No
C. aksoyi	KX158186.1
C. aladaghensis	DQ319078.1, LC474962.1
C. albonitens	DQ319081.1, LC474987.1
C. alexandrina	DQ319082.1
C. alfonsoi	LC474961.1
C. alveicola	LC474991.1
C. amadanensis	DQ319083.1, LC474963.1
C. antitauri	DQ319084.1
C. aplolepa	JF913982.1
C. armena	DQ319085.1
C. arrigonii	KC969536.1, KJ665931.1
C. assadii	LC474988.1
C. aucheri	DQ319087.1, LC474989.1
C. behen	AY826250.1, DQ319090.1, LC474992.1
C. boissieri* subsp. *boissieri	AM114278.1
C. boissieri* subsp. *funkii	KJ665941.1
C. boissieri* var. *atlantica	AM114283.1
C. bruguierana	DQ319093.1
C. bugellensis	KF721088.1, KF721089.1
C. calcitrapa	AY826252.1
C. cataonica	DQ319099.1, LC474984.1
C. cheirolepidioides	DQ319100.1
C. cheirolopha	DQ319101.1
C. chrysantha	DQ319102.1
C. cineraria	KC969537.1
C. deflexa	DQ319105.1
C. derderiifolia	DQ319106.1

C. doddsii	LC474986.1
C. drabifolia* subsp. *floccosa	KX158187.1
C. ensiformis	DQ319112.1
C. ferulacea	MH629985.1
C. filiformis	KF630448.1, MH629998.1, MH630006.1
C. gigantea	LC474966.1
C. glastifolia	DQ319116.1
C. gymnocarpa	KJ666008.1
C. hermanni	DQ319118.1
C. ilvensis	KJ666013.1
C. imperialis	LC474967.1
C. iranshahrii	LC474974.1
C. isaurica	DQ319124.1
C. jacea	AM114332.1
C. jacea* subsp. *weldeniana	KF721082.1
C. janeri* subsp. *janeri	KF721060.1, KF721072.1
C. jordaniana* subsp. *jordaniana	KF721042.1
C. kotschyi	AY829441.1, DQ319127.1
C. kurdica* subsp. *kurdica	LC474977.1
C. leucophaea	JF913989.1
C. leucophaea* subsp. *leucophaea	KJ666037.1
C. lycopifolia	DQ319130.1
C. macrocephala	DQ319131.1
C. nemecii	LC474975.1
C. nervosa* subsp. *nervosa	KF721049.1, KF721050.1
C. nigrescens	KF721071.1
C. odyssei	DQ319140.1
C. onopordifolia	LC474994.1
C. paniculata	JF913990.1, KJ666059.1
C. paniculata* subsp. *esterellensis	KJ666064.1
C. paniculata* subsp. *paniculata	KJ666063.1
C. paphlagonica	DQ319142.1
C. paui	KJ666070.1
C. phlomoides	LC474978.1
C. polypodiifolia	DQ319148.1
C. polypodiifolia* var. *polypodiifolia	LC474995.1
C. pterocaula	DQ319151.1
C. ptosimopappa	DQ319152.1
C. ptosimopappoides	DQ319153.1
C. rahiminejadii	LC474973.1
C. rhaetica	KF721093.1, KF721094.1
C. rhizantha	DQ319157.1
C. rigida	DQ319158.1
C. saligna	DQ319159.1
C. spectabilis	DQ319164.1
C. spicata	LC474983.1
C. stapfiana	DQ319166.1
C. stevenii	DQ319168.1
C. thracica	DQ319171.1
C. tomentella	DQ319172.1, LC474980.1, MK920926.1
C. tymphaea	KJ666120.1
C. wendelboi	LC474982.1
C. xylobasis	DQ319176.1
C. zaferii	LC474981.1
Outgroup	
Astragalus mongholicus	AF359750.1