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DNA barcoding of *Campanula choruhensis* Kit Tan & Sorger Endemic to Artvin (Türkiye)

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Abstract

DNA barcoding is the method of description of species based on gene diversity. In current studies, registration, genetic identification and protection of especially endemic plant species are carried out by DNA barcoding techniques. Molecular studies are based on the amplification and sequencing of the barcode gene regions by the PCR method. *Campanula choruhensis* Kit Tan & Sorger is endemic and widespread in Artvin and around Çoruh valley passing through it. Roots of this species are used in medicinal and aromatic studies. Intense roadworks and dam constructions are carried out in and around the distribution area of this species. This situation harms the habitat of the species and comes across with its extinction. In this study, the plastid barcode gene regions (trnK-rps16 and trnL-rpl32) of the *C. choruhensis* was sequenced from five populations. To make the identification of this species quickly and accurately, gene sequence compared with sequences of other related *Campanula* L. species. In addition, gene sequences (in Genbank Library) and phylogenetic relations were given.

Keywords: Artvin, *Campanula*, DNA barcoding, medicinal and aromatic, molecular, Türkiye

Artvin (Türkiye) Endemiği *Campanula choruhensis* Kit Tan & Sorger'in DNA Barkodlaması

Özet

DNA barkodlama türlerin gen çeşitliliğine dayalı olarak tanımlanması yöntemidir. Son güncel çalışmalarda, özellikle endemik bitki türlerinin tür teşhislerinin doğruluğu, genetik tanımlanmaları ve korunması çalışmaları DNA barkodlama teknikleri ile yapılmaktadır. Moleküler çalışmalar DNA barkod gen bölgesinin PCR yöntemi ile çoğaltılması ve dizilenmesi esasına dayanmaktadır. Endemik bir türümüz olan *Campanula choruhensis*, Artvin, ve bu iller içerisinden geçen Çoruh vadisi ve çevresinde yayılış göstermektedir. Bu bitkinin kökleri tıbbi ve aromatik çalışmalarda kullanılmaktadır.Bu türün yayılış alanında ve çevresinde yoğun yol ve baraj inşaatları gerçekleştirilmektedir. Bu durum türün habitatına zarar vermekte ve neslinin tükenmesine neden olmaktadır. Bu çalışmada, *C. choruhensis* türünün plastit barkod gen bölgeleri (trnK-rps16 and trnL-rpl32) dizilenmiştir. Doğru ve hızlı tanımlanması için bu türe ait gen dizisi *Campanula* cinsinin diğer yakın türleriyle karşılaştırılmıştır. Ayrıca türün gen dizisi (Genbank Kütüphanesinde) ve filogenetik ilişkisi de verilmiştir.

Anahtar Kelimeler: Artvin, *Campanula*, DNA barkodlama, moleküler, tıbbi ve aromatik, Türkiye

Introduction

Campanulaceae is a family of Angiosperm containing more than 700 herbaceous plants and few shrub forms (Banki et al. 2023). The most concentrated areas of *Campanula* species start from the Mediterranean countries and continue to the Caucasus. The hottest spots in terms of endemism are Eastern Mediterranean Countries, Balkan Countries, Caucasus and Turkey (Borsch et al. 2009; Khansari et al. 2011). The genus *Campanula* generally includes edaphically and microclimatically differentiated 'casmophytes' and mostly locally distributed endemic species (Damboldt 1965; Kovanda 1970; Park et al. 2006). According to the Flora of Turkey records, the genus *Campanula* comprises 127 species (139 taxa in total) in our country. Of these, 66 (74 taxa) are endemic with an endemism rate of 52% (Damboldt 1978; Davis et al. 1988; Güner 2000; Özhatay and Kültür 2006; Özhatay et al. 2009, 2011; Güner et al. 2012; Yıldırım 2013; Mutlu and Karakuş 2015; Yıldırım 2018; Behçet and İlçim, 2018; Yıldırım et al. 2019).

Artvin is one of the most richest province of Turkey in terms of plants diversity, with a total of 2727 natural plant taxa belonging to 137 families and 761 genera. A total of 500 plants, 198 of which are endemic and 302 are rare, and under risk. Dams built in the Çoruh valley have narrowed the distribution area of endemic *Campanula* species (Eminağaoğlu et al. 2015; Eminağaoğlu et al. 2018; Özcan and Eminağaoğlu 2018). Within the borders of Artvin province, 26 *Campanula* taxa are distributed and 6 of them are endemic (Güner et al. 2012). Among these endemic species, *Campanula troegera* Damboldt, *C. seraglio* Kit Tan & Sorger., *C. betulifolia* K.Koch. and *C. choruhensis* Kit Tan & Sorger show morphologically similar features and inhabit in similar areas in Artvin. They are taxonomically complex species. The diagnosis of plant species in our country is generally based on morphological distinctions but there are difficulties in the identification of these species, especially in herbarium samples. These species have not been studied much yet. Eminağaoğlu et al. (2015) gave general information about this species. Özcan and Eminağaoğlu (2018) evaluated anatomically the two closely related endemic species (*C. betulifolia* C.Koch and *C. choruhensis* Kit Tan & Sorger), in detail and their IUCN risk categories were given.

Studies on plants have not been limited to systematic studies in recent years, but have also included molecular studies. The relationships of many systematically problematic taxa were resolved by DNA studies and sequence analyses especially DNA barcoding studies. DNA barcoding has been used in many areas, especially taxonomy of species, identification of forensic cases, population genetic studies and enable accurate and rapid species identification (Hebert et al. 2004; Cywinska et al. 2006; Rolo et al. 2013; DiPinto et al. 2015). In DNA barcoding applications, barcode sequences are obtained from various living things and these DNA sequences are used in variations of phylogenetic trees (Dasmahapatra and Mallet 2006). Ultimately, it is envisaged that closely related individuals will congregate in the same groups, and each separate group is considered a different species. Naturally, plastid (cpDNA) and nuclear genome (nrDNA) emerge as candidates as plant barcode sources (Hollingsworth et al. 2009). The CBOL plant working group recommended plastid gene regions consisting of the rbcL, matK, trnK, trnL, rps16 combinations with additional markers as plant barcode candidates. The combination of these barcode regions gives accurate results in the identification of species (CBOL Plant Working Group 2009; Hollingsworth et al. 2011).

C. choruhensis has a wide distribution area around Çoruh valley and Artvin, but has not been sufficiently addressed in molecular studies on differentiation from other species. Although matK is the most commonly used gene region in DNA barcoding studies, there are studies showing that it does not provide sufficient discrimination in some plant species. For this reason, it was decided to use 2 different barcode regions in our study, which were stated to be more distinctive. Therefore, we apply molecular

techniques, specifically a DNA barcoding (trnK-rps16 and trnL-rpl32 regions) approach, to better understand the species relationships and delimitation for endemic *C. choruhensis* from other releated species.

Materials and Methods

Endemic *C. choruhensis* collected from five localities in Altıparmak, Barhal (Yusufeli), Zeytinlik, Çevreli and Ardanuç of Artvin district, located in the northeast of Türkiye during in the summer of 2020-2021. This specimens were sampled at an altitude of approximately 1800-2500 m. Collected specimens were dried and stored at ARTH herbarium, giving a collection number to each specimen (*Akyil.* 218, 219, 220, 232, 239). For identification of the specimens, the identification key in the *Flora of Turkey and the East Aegean Islands* were used (Davis et al. 1988). Morphologically confirmed species were labelled and 1-3 leaf (40-50g) of this species dried with silica gel at room temperature for DNA extraction.

DNA barcoding studies carried out in 3 stages, DNA isolation, matK amplification and data collection and cluster of phylogenetic analysis. Total DNA from leaf tissues (50–75 mg) was extracted by using a commercial DNA extraction kit (DNeasy Plant Mini Kit, Qiagen), following the manufacturer's protocol. Total DNA concentrations were determined by Nanodrop (Thermo Microvolume UV-Vis) and visualised with 2% agarose gel electrophoresis. Obtained DNA was used in PCR amplification. matK region of studied taxa were amplified using trnK-rps16 gene region, rps16-2F2 (5' AAA GTG GGT TTT TAT GAT CC 3') and trn $K^{(UUU)}$ (5' TTA AAA GCC GAG TAC TCT ACC 3') (Shaw et al., 2007) and rpl32-trnL gene region $trnL^{(UAG)}$ (5' CTGCTTCCTAAGAGCAGCGT 3') and rpl32-F (5' CAGTTCCAAAAAAA CGTACTTC 3') (Shaw et al. 2007) primers PCR reaction mixture contained a volume of 50 µl; 10 × Taq Buffer with (NH4)2SO4: 5 µl, 10 mM dNTP mix: 0.5 µl, 5u/ul Taq DNA polymerase (Thermo Scientific): 0.20 µl, 25 mM MgCl2: 5 µl, 10 mg/ml BSA: 4 µl, 10 uM each primer: 0.5 µl, and genomic DNA extract: 2.5 µl, dH2O: 30.8 µl). PCR condition is 95 °C for 5 min initial denaturation, 34 cycles of 94 °C for 1min denaturation, 48 °C for 1.30 s anneling, and 72 °C for 1 min extension, 72 °C for 10 min final extension. Sequence analysis both directions (Forward and Reverse) were completed by ABI 3100 Genetic Analyzer, BM Labosis, Ankara, Turkiye.

Phylogenetic Analyses

trnK-rps16 and trnL-rpl32 gen region sequences of the taxa were aligned via BioEdit version 7.0.9.0. (Bioedit 2023) and were used to construct phylogenetic trees by using Mega7.0. (Tamura et. al. 2023). The related trnK-rps16 and trnL-rpl32 sequences of *Campanula* species (9) were retrieved from the GenBank (Genbank 2023) database (Table 1). *Triodanis perfoliata* (L.) Nieuwl., *Edraianthus graminifolius* (L.) A.DC. ex Meisn. and *Adenophora lamarckii* Fisch. were used as the outgroup for the phylogenetic analysis. Phylogenetic analysis with Maximum likelyhood and Neighbour-Joining methods were applied by MEGA 7.0. (Tamura et. al. 2023).

Results

Studies on revealing the genetic diversity of plants continue to be popular. Determining and preserving the genetic diversity of endemic species, especially those distributed in limited areas, is important in terms of transferring them to future generations (CBOL 2009). In this study, endemic *Campanula choruhensis* (Campanulaceae) was studied with two barcoding gen regions. The DNA sequence of the trnK-rps16 and TrnL-rpl32 gene region sequences of the narrow endemic *C. choruhensis* was created and its relationships with similar species were compared with the sequences obtained from GenBank (Table 1).

Figure 1. Habitus of endemic *Campanula choruhensis*

Table 1. Accession numbers of related *Campanula* species available in GenBank database.

	Taxon	Gen Region	GenBank Accession Number
1	*Campanula medium*	trnK-rps16	KP014307.1
2	*C. alpina*	trnK-rps16	EU287572.1
3	*Triodanis perfoliata*	trnK-rps16	KP014308.1
4	*Adenophora lamarckii*	trnK-rps16	LT706725.1
5	*C. choruhensis*	trnK-rps16	PP430154
6	*Edraianthus graminifolius*	TrnL-rpl32	KJ684490
7	*C. trachelium*	TrnL-rpl32	KY034640.1
8	*C. occidentalis*	TrnL-rpl32	KU867524.1
9	*C. kremeri*	TrnL-rpl32	KU867384.1
10	*C. rotundifolia*	TrnL-rpl32	KY034632.1
11.	*C. choruhensis*	TrnL-rpl32	PP430155

The phylogenetic tree was created by Maximum Likelihood approach using Tamura-Nei model based on chloroplast DNA. The two barcoding loci specific primers resulted in robust amplification in the 7 *Campanula* species. The phylogenetic tree constructed from trnK-rps16 region sequences was clustered into the two main groups. *C. choruhensis* were seperated from other *Campanula* species. As a result of phylogenetic analysis, it was determined that *C. choruhensis* was close to *C. medium* and *C. alpina* with a brightness value of 76% (Figure 2).

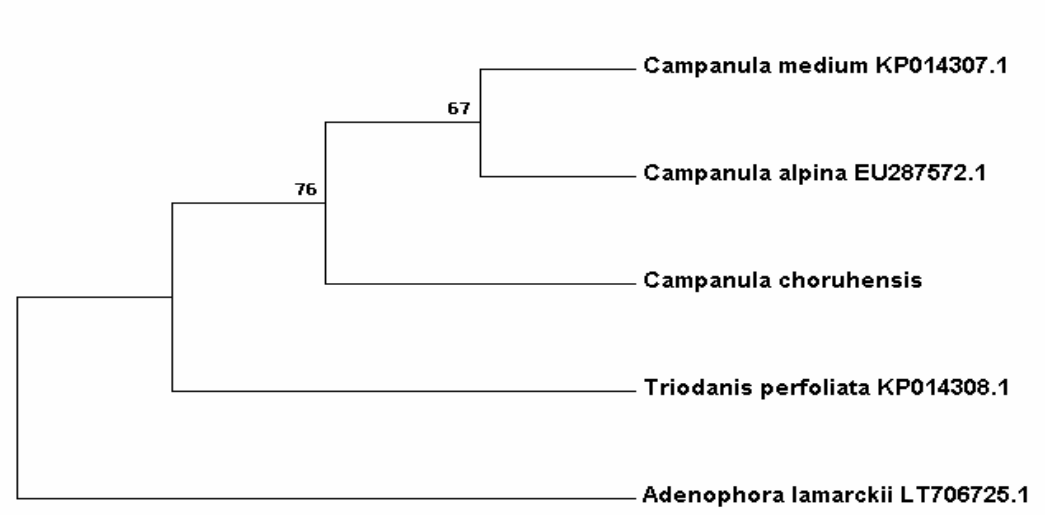

Figure 2. Phylogenetic tree using Maximum Likelihood approach using Tamura-Nei model based on chloroplast DNA (trnK-rps16 region)

The phylogenetic tree constructed from trnL-rpl32 region sequences was clustered into the three main groups. *C. choruhensis* showed a separate cluster like a outgroup. As a result of the analysis, it was clearly observed that it differs from the others as a species. Therefore, this two DNA barcoding region (trnK-rps16 and trnL-rpl32) were useful to solve taxonomic problems (Figure 3).

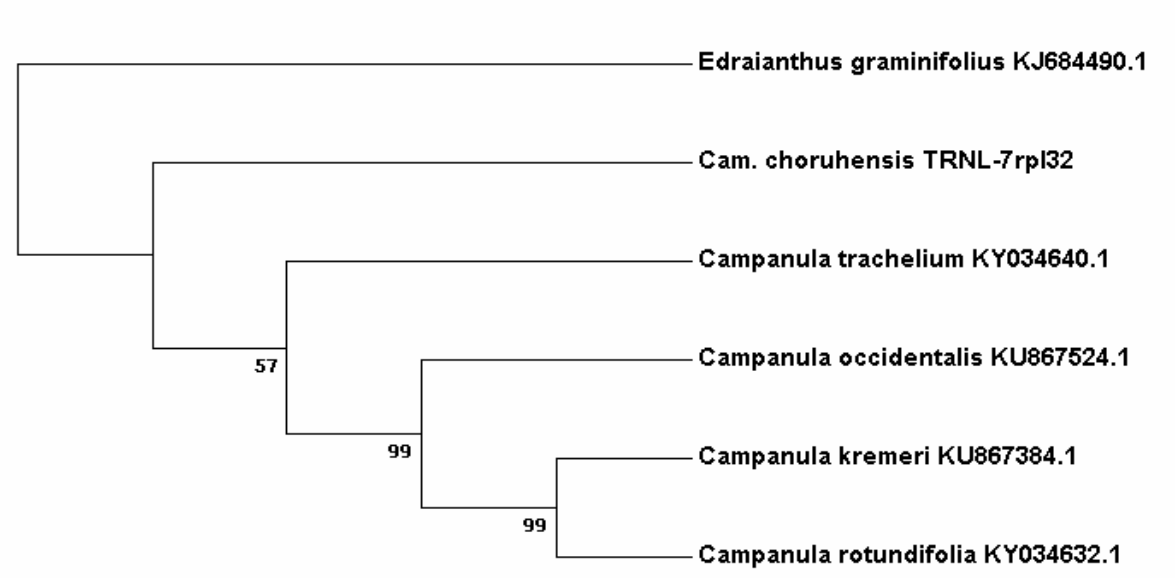

Figure 3. Phylogenetic tree using MEGA 7 based on chloroplast DNA (trnL-rpl32 region)

According to the phylogenetic tree constructed from the aligned nucleotide sequences of the trnK-rps16 and trnL-rpl32 gene regions (Fig. 2,3), the phylogenetic relationships of taxa belonging to the *Campanula* genus are strongly supported.

Discussion and Conclusion

Recent advancements in molecular biology and DNA sequencing techniques have empowered scientists to comprehensively characterize the genomes of various organisms. Ongoing genome projects across diverse species are now yielding valuable insights into their taxonomy, genetic composition, and functional traits. In this particular study, we analyzed nucleotide sequence polymorphisms of the chloroplast gene regions (trnK-rps16 and trnL-rpl32).

Many morphological and molecular studies have been carried out on *Campanula* species in Türkiye. Most of the studies are on morphological taxonomy of *Campanula* species (Karakısa 1997; Tatlı 2003; Aytaç and Duman 2005; Duru 2013; Alçıtepe 2015). In addition to taxonomic evaluations on the taxa belonging to the genus *Campanula*, studies have been carried out on many subjects such as phylogenetic polymorphism and biogeography. Liveri et al. (2020) found that *Campanula* sect. *Quinqueloculares* is polyphyletic, as traditionally circumscribed. Eddie et al. (2003) gave information about the phylogenetic relationships of some taxa in this family through sequencing studies of the ITS region on many Campanulaceae members. Park et al. (2006) presented phylogenetic and biogeographic data about some *Campanula* taxa found in the Mediterranean region. In the present study, two gene regions of endemic *C. choruhensis* have been determined and its relationship to some closely related species is given on the phylogenetic tree. This barcoding regions are most frequently used regions in the chloroplast DNA genome because it contains the most nucleotide sequence differences (CBOL 2009). It shows that the species are quite well separated on the basis of section, that is, the variations are meaningful for this barcoding region in distinguishing the species according to their common morphological characteristics.

C. choruhensis species is also in EN categories according to IUCN Red List (IUCN 2023). This species has a very narrow distribution throughout the eastern black sea region. As a result in the study, the phylogenetic relationships of endemic *C. choruhensis* were determined by looking at the trnK-rps16 and trnL-rpl32 gene regions and their kinship with some close species was given. The data obtained from this study will serve as a resource for other studies on *Campanula* species, which are widely distributed in almost every region of our country. In this respect, it will be extremely important to collect and protect genetic resources from the significant genetic variation of these endemic species.

In the Çoruh Valley, there are approximately 1000 plant taxa with natural distribution. In the area that will be submerged under the Yusufeli Dam reservoir, 582 plant taxa, including 49 endemics, are distributed. Of these 49 endemic plant species, 26 are globally endangered, 20 are endangered at the European scale, and 3 are rare species endangered at the national scale. Additionally, one species (*Orchis punctulata*) is subject to the Bern Convention, and two taxa (*Cyclamen coum* subsp. *coum* and *Anacamptis pyramidalis*) are subject to the CITES convention, all having natural distribution in this area. Artvin province, where a significant portion of the Çoruh River valley, including the area on which the Yusufeli Dam is being constructed, is located, is one of the most important and richest centers of plant resources in our country due to its geographical location, topographic structure, water resources, microclimate diversity, geological structure, and plant geography, being influenced by the European-Siberian, Iranian-Turanian, and Mediterranean plant geographies and its position as a gene center (Eminağaoğlu and Tilki, 2015).

In the area where dam and road construction activities are intense, out of the 26 endemic species present, 14 species (*Acer cappadocicum* subsp. *divergens*, *Alyssum artvinense*, *Bupleurum schistosum*, *Campanula troegerae*, *Centaurea pecho*, *Alkanna cordifolia*, *Convolvulus pseudoscammonia*, *Haplophyllum armenum*, *Chesneya elegans*, *Clypeola raddeana*, *Iris taochia*, *Iris nezahatiae*,

Micromeria elliptica, Reseda globulosa) must be conserved ex-situ due to the destruction of their habitats in other parts of Artvin (Eminağaoğlu and Tilki, 2015).

A future molecular study that encompasses all taxa of the genus and explores more genetic regions will allow the phylogenetic relationships of all taxa in this genus to be elucidated. Therefore, it is necessary to revise the morphology and molecular characteristics of this genus, including karyology, using morphological and molecular methods.

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