LEPL SCIENTIFIC-RESEARCH CENTER OF AGRICULTURE

DNA barcoding of tree endemic *Campanula* species from Artvin, Türkiye

Campanula troegera

Campanula betulifolia

Campanula choruhensis

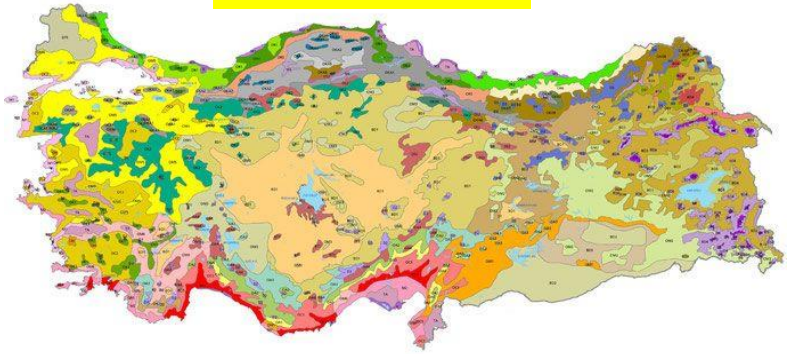
Hayal AKYILDIRIM BEĞEN[1], Özgür EMİNAĞAOĞLU[2], Melahat ÖZCAN[2]

[1]Health Services Vocational School, Artvin Coruh University, Artvin, Turkey

[2]Department of Forestry, Artvin Coruh University, Artvin, Turkey

h.akyildirim@artvin.edu.tr

TÜRKİYE



- 13.701 plant taxa
- 4.319 Endemic plants

ARTVİN

- 2727 plant taxa
- 500 Endemic and rare plants

	CR	EN	VU	LC	NT	DD	NE	Total
Endemic	23	32	20	83	16	22	2	198
Non-Endemic	3	6	76	190	4	23	0	302
Total	26	38	96	273	20	45	2	500

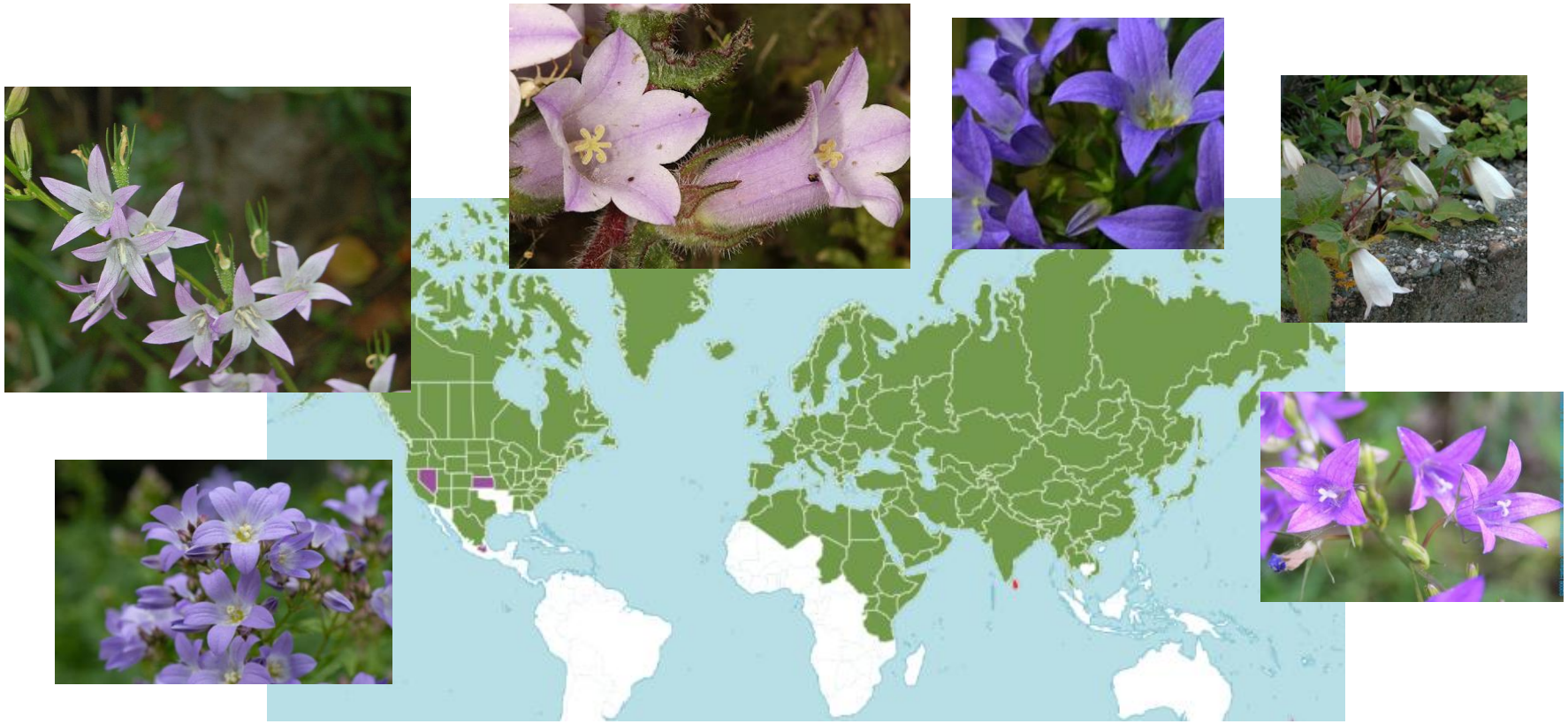
Of the 500 rare plants with natural distribution in Artvin:

- 75 are globally endangered,
- 123 are endangered on a European scale,
- 302 are at risk on a national scale.

Introduction

Campanula L., belonging to the family Campanulaceae, is a genus that encompasses around 90 genera and 2,500 species worldwide. It is particularly prevalent in the subtropical and temperate regions of the Northern Hemisphere, with approximately 420 species represented there. This genus encompasses both annual, biennial, and perennial species, with varying lifespans.

Perennial *Campanula* species are particularly noteworthy for their captivating beauty and remarkable size variation. These long-lived plants serve as valuable representatives of the genus's rich genetic diversity, having been extensively cultivated for ornamental purposes.



Campanula in Türkiye: A Rich Diversity of Endemic Species

In Türkiye, the genus *Campanula* is divided into six subgenera: *Campanula, Megalocalyx, Sicyodon, Roucela, Brachycodonia,* and *Rapunculus*. These subgenera encompass a remarkable 125 species within the country's borders.

Notably, the vast majority (approximately 66%) of *Campanula* taxa distributed in Türkiye are chasmophytes, plants adapted to growing in rocky crevices or cliffs. Among these chasmophytic taxa, an impressive 67 species are endemic to Türkiye, representing an endemism rate of 52%.

Introduction

Artvin: A Haven for Endemic *Campanula* Species

Within the borders of Artvin province, a remarkable 26 *Campanula* taxa (taxonomic groups) are distributed, with six of them holding the distinction of being endemic. These endemic species, namely *Campanula trogera, C. betulifolia, and C. choruhensis*, exhibit striking morphological similarities and share similar distribution patterns.

Taxonomically Complex *Campanula* Species: Challenges in Identification

The *Campanula* species mentioned, namely *Campanula trogera, C. betulifolia,* and *C. choruhensis*, present significant taxonomic complexities. This intricate nature of their classification prompted Özcan and Eminağaoğlu (2018) to undertake a comprehensive study titled "Anatomical Characteristics and Conservation Status of Endemic *Campanula betulifolia* and *C. choruhensis* (Campanulaceae)" to shed light on their unique characteristics and conservation needs.

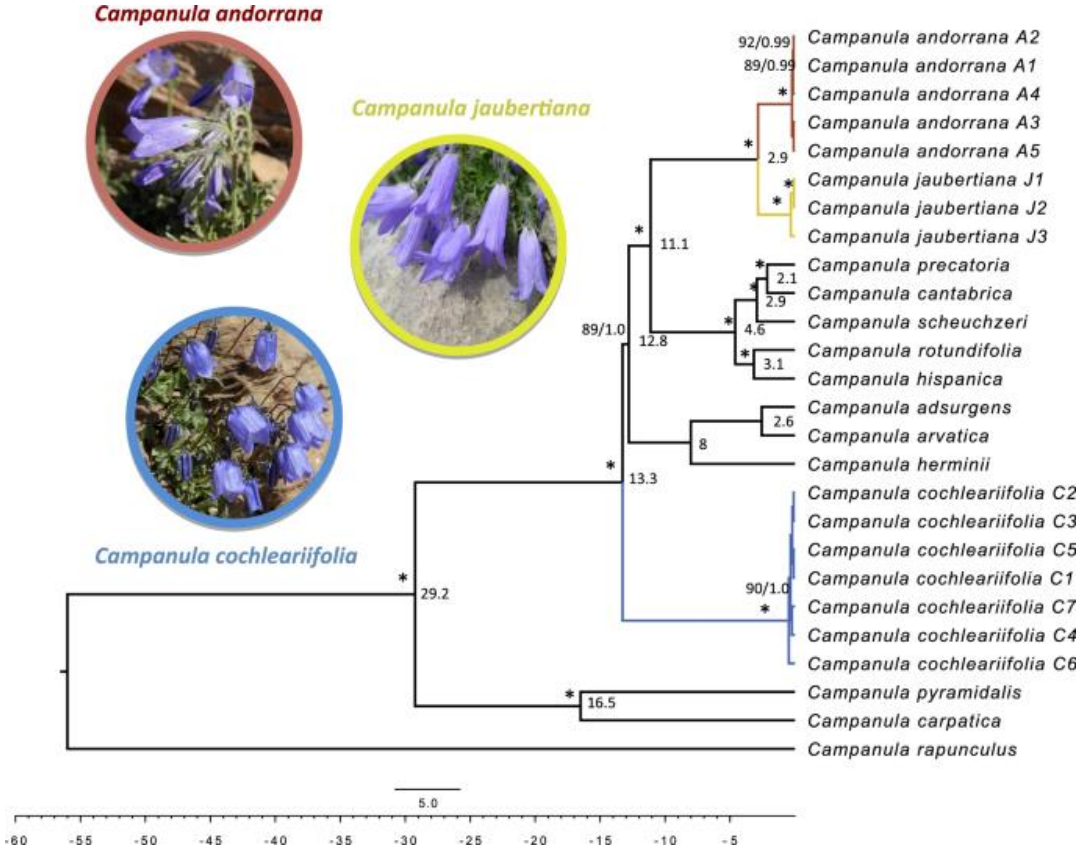
Campanula tomentosa

Campanula troegera

Campanula lyrata

Molecular Insights in to *Campanula* Species: Unraveling Relationships and Conservation

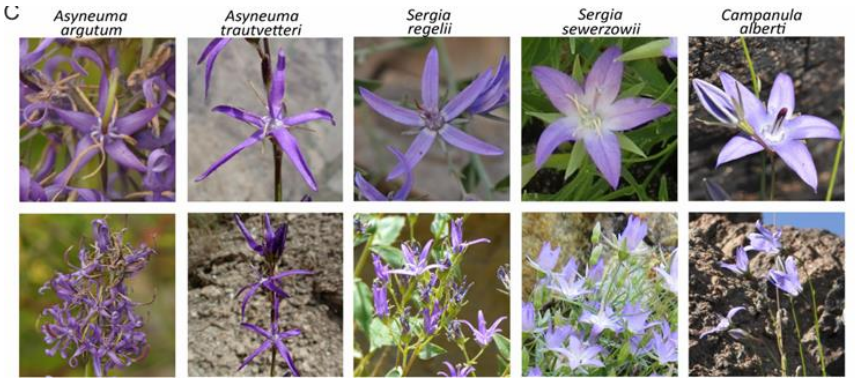
- In recent years, research on *Campanula* species has expanded beyond systematic studies to encompass molecular approaches.
- **DNA sequencing and analysis have proven invaluable in resolving the complex relationships among taxonomically challenging taxa.**



Identification studies with molecular methods

Molecular Plant Systematics: Unveiling Evolutionary Relationships and Advancing Botanical Knowledge

- Molecular plant systematics has emerged as a transformative field, particularly in the last two decades, revolutionizing our understanding of plant relationships and evolutionary histories.
- The advent of DNA and amino acid sequence analysis, coupled with the development of novel phylogenetic analysis methods, has fueled this remarkable progress.
- In instances where morphological characteristics fall short in providing comprehensive phylogenetic information, sequence analysis steps in as an invaluable tool. This stems from the fundamental principle that DNA and amino acid sequences closely mirror the evolutionary relationships of organisms.
- The applications of sequence analysis methods span a wide spectrum, ranging from elucidating the geographical origins of organisms to establishing molecular proofs of their evolutionary relationships. In studies delving into the molecular phylogenies of angiosperms, researchers have predominantly employed chloroplast (plastid) and mitochondrial genes, along with highly repetitive nuclear ribosomal DNA (nrDNA) genes.



Identification studies with molecular methods

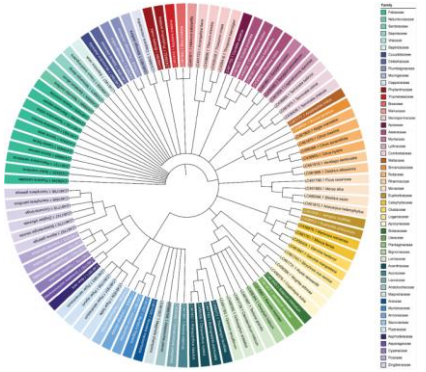
DNA Barcoding: Unveiling Species Identities with Molecular Precision

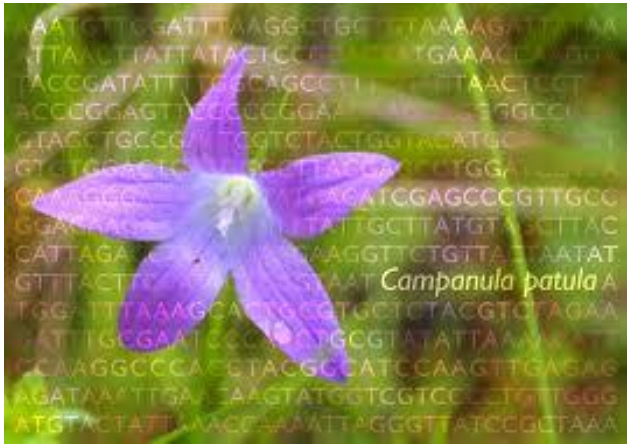
DNA barcoding has emerged as a powerful and reliable technique for species identification, revolutionizing the field of taxonomy. This approach utilizes short (600-1500 base pair) DNA sequences from predetermined or well-characterized regions of the genome to uniquely identify organisms. The beauty of DNA barcoding lies in its simplicity and effectiveness, making it a valuable tool for a wide range of applications, including:

- **Rapid and accurate species identification:**
- **Unveiling cryptic species:**
- **Monitoring biodiversity:**
- **Combating illegal wildlife trade:**

Common DNA Barcode Regions in Plants:

- **Chloroplast Organellar Genes:**
  - atpF-atpH
  - trnL
  - rbcL
  - ycf5
  - rpoC1
  - rpoB
  - psbA-trnH
  - psbK-psbI
- **Nuclear (Core) Genome:**
  - Internal Transcribed Spacer (ITS) regions



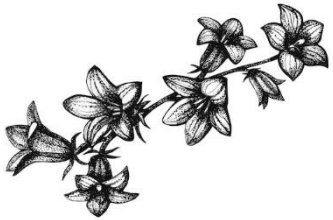


MatK and trnH-psbA: Versatile Tools for Plant Molecular Systematics

In the realm of molecular plant systematics, two regions have gained prominence as valuable tools for DNA barcoding and phylogenetic analysis: the MatK gene and the trnH-psbA intergenic spacer.

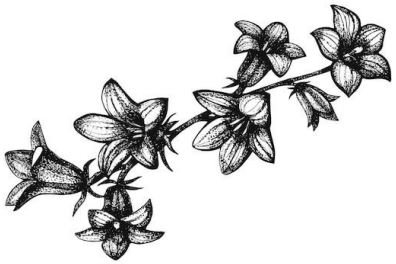
MatK: A Gene with Remarkable Versatility

- **MatK Gene:** The MatK gene, with its 841 nucleotide length, stands as a highly versatile and widely employed coding region within the chloroplast genome. Its extensive application in molecular plant systematic studies is a testament to its effectiveness (Hilu and Liang, 1997; Hochbach et al., 2018).
- **High Sequence Variability:**
- **Uniform Substitution Rates:**
- **Informative Parsimonious Sites:**
- **Strong Phylogenetic Resolution:**



Aim of this study

- In this study, the plastid matK barcode gene regions (650 bp) of three *Campanula* species were created.
- To make the identification of this species quickly and accurately, gene sequence compared with sequences of other *Campanula* L. species.
- To further clarify the distinctions between the three *Campanula* species mentioned (*C. trogera, C. seraglio, C. betulifolia,* and *C. choruhensis*), this study employed PCR (polymerase chain reaction) to generate DNA sequences from the chloroplast rbcL and matK regions.
- Additionally, morphological characteristics, including leaves, flowers, and fruits, were thoroughly examined.



Material and Methods

- **Gathering Specimens of Endemic *Campanula* Species in Artvin**
- The study has meticulously collected samples of four endemic Campanula species from three distinct districts within Artvin province: Artvin Merkez (Zeytinli), Yusufeli (Demirkent, Dereiçi, and Sarıgöl), and Ardanuç (Merkez). These precious specimens were primarily found growing on rocky surfaces.

	Taxon	Locality	Date	Collection number
1	*Campanula trogera*	Yusufeli	18.07.2020	*H.Akyil 200*
2	*Campanula trogera*	Yusufeli	18.07.2020	*H.Akyil 201*
3	*Campanula trogera*	Yusufeli	18.07.2020	*H.Akyil 202*
4	*Campanula trogera*	Yusufeli	18.07.2020	*H.Akyil 203*
5	*Campanula trogera*	Yusufeli	18.07.2020	*H.Akyil 204*
6	*Campanula betulifolia*	Demirkent	21.08.2020	*H.Akyil 205*
7	*Campanula choruhensis*	Yusufeli	21.08.2020	*H.Akyil 206*
8	*Campanula choruhensis*	Çevreli	21.09.2020	*H.Akyil 207*
9	*Campanula trogera*	Kaleköy	22.09.2020	*H.Akyil 208*
10	*Campanula choruhensis*	Merkez-Zeytinli	26.08.2020	*H.Akyil 209*
11	*Campanula choruhensis*	Merkez-Zeytinli	15.08.2021	*H.Akyil 210*
12	*Campanula betulifolia*	Demirkent	17.08.2021	*H.Akyil 211*
13	*Campanula trogera*	Ardanuç-Merkez	22.09.2021	*H.Akyil 212*

Herbarium material

Preserving Campanula Specimens for Morphological Analysis and Herbarium Preparation

The study meticulously collected and preserved Campanula specimens for morphological characterization and herbarium preparation, adhering to strict botanical guidelines.

Specimen Collection:

1.Target Plant Parts: Careful attention was paid to collecting the essential plant parts: leaves, floral components, and fruits.

2.Cutting Technique: A sharp pruning shear was used to sever leafy, flowering, and fruiting branches from the parent plant.

Herbarium Preparation:

1.Pressing and Drying: The collected branches were arranged between newspaper sheets within press boards (29x41 cm) and pressed to flatten and dry the specimens. Cardboard sheets were inserted between the layers to ensure uniform pressure and prevent damage.

2.Drying Duration: The pressed specimens were left to dry for 48 hours at -20°C to eliminate any insect eggs or parasites that might be present.

Preparation for Identification:

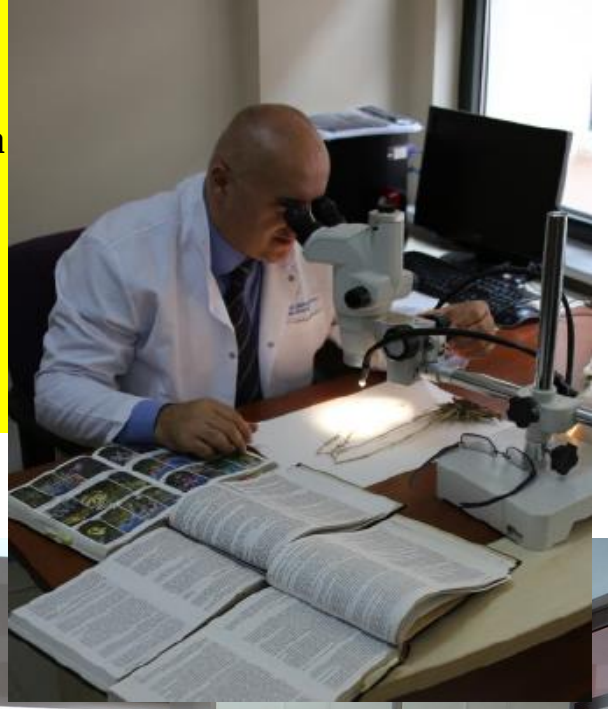
Once completely dry, the herbarium-quality specimens were ready for the identification process. The preserved plant parts would serve as crucial references for morphological characterization and taxonomic classification of the Campanula species.

Significance of Proper Preservation:

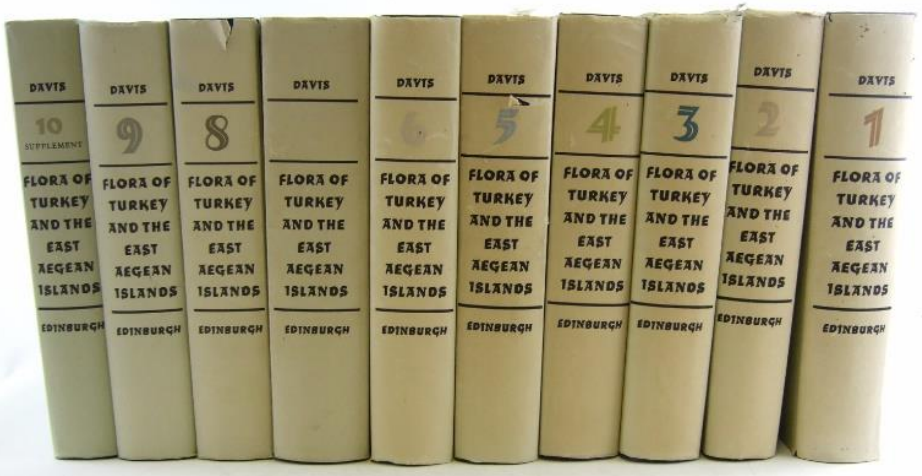
The meticulous collection and preservation techniques employed in this study ensure that the Campanula specimens retain their morphological integrity and provide reliable data for both herbarium preparation and detailed morphological analyses. This careful approach will facilitate accurate identification, characterization, and classification of these precious plant specimens.

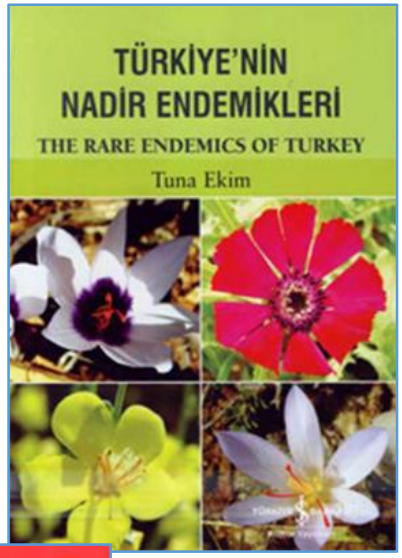
ARTVİN ÇORUH UNIVERSITY HERBARIUM (ARTH)

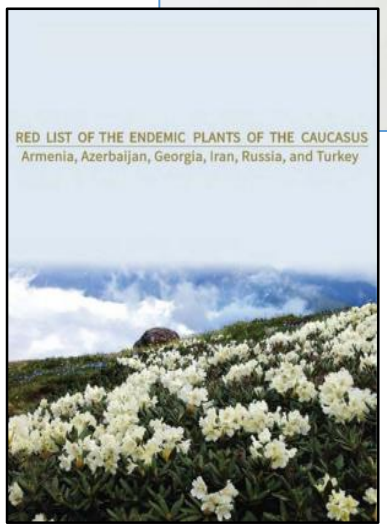
A Botanical Gem Among Turkey's Herbarium NetworkWith its impressive collection of over 40,000 plant specimens, ARTH stands as a botanical gem among Turkey's network of herbaria, ranking proudly as the fifth largest among approximately 70 herbaria nationwide. This vast repository of plant life not only reflects the rich botanical diversity of Turkey but also showcases ARTH's significant contributions to the field of botany.

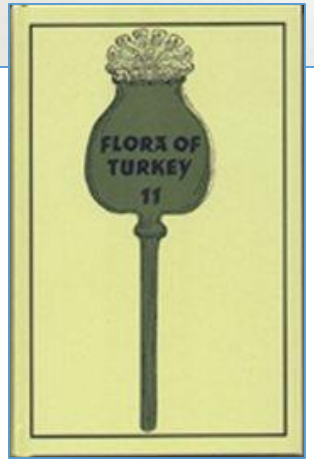


Unveiling Plant Diversity and Conservation Needs: Sources for Floristic Inventory and Risk Assessment



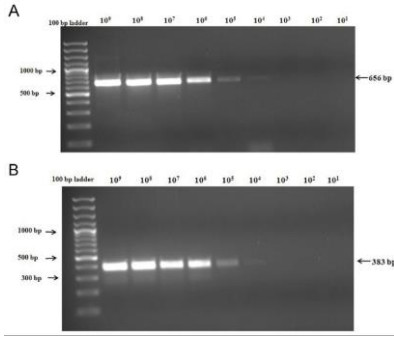




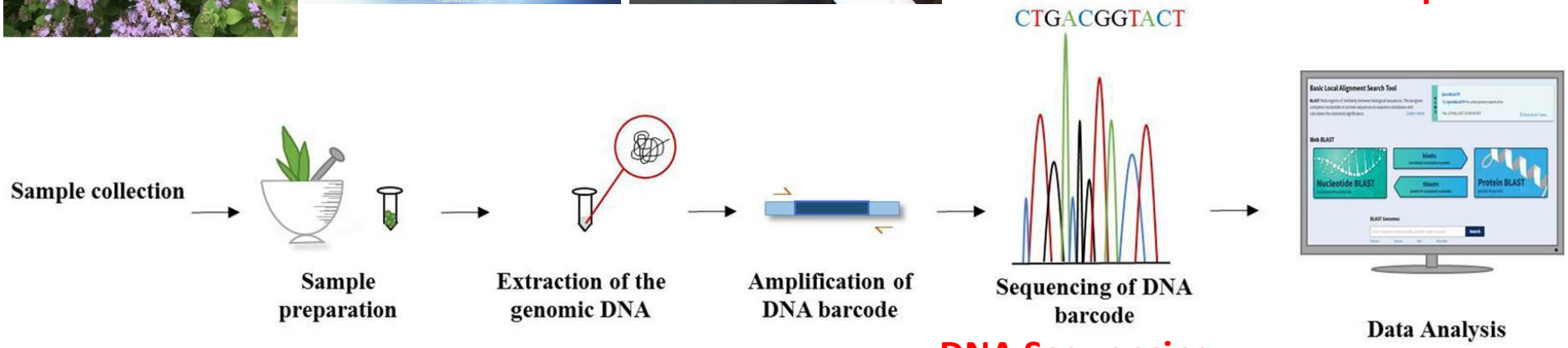


Molecular Methods

Polymerase Chain Reaction (PCR)



Gel Electrophoresis



DNA Sequencing

DNA Extraction

In the DNA isolation studies, a commercially available genomic DNA isolation kit (Qiagen Plant DNeasy Kit) was used. The protocol provided with the kit was followed as the methodology (Qiagen, 2016b).

DNA Quality and Concentration Control

Spectrophotometric measurements were performed using the Thermo Scientific Nanodrop 2000 (USA) device to control the quality, quantity, and purity.

Sample ID	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
Blank	27.09.2021 15:00:19	0	ng/µl	0,001	-0,017	-0,04	0,14	DNA
1	27.09.2021 15:01:14	31,5	ng/µl	0,63	0,356	1,77	1,06	DNA
2	27.09.2021 15:02:20	36,9	ng/µl	0,739	0,389	1,9	1,43	DNA
3	27.09.2021 15:03:15	54	ng/µl	1,08	0,6	1,8	1,34	DNA
4	27.09.2021 15:04:14	93,8	ng/µl	1,876	1,018	1,84	1,92	DNA
5	27.09.2021 15:05:41	36,4	ng/µl	0,729	0,355	2,05	1,03	DNA
6	27.09.2021 15:06:58	33,8	ng/µl	0,676	0,367	1,84	1,1	DNA
7	27.09.2021 15:07:59	24,7	ng/µl	0,495	0,262	1,89	1,07	DNA
8	27.09.2021 15:12:01	3,6	ng/µl	0,072	0,039	1,83	0,16	DNA
9	27.09.2021 15:14:31	22,9	ng/µl	0,457	0,247	1,85	0,81	DNA
10	27.09.2021 15:15:40	27,4	ng/µl	0,548	0,307	1,78	0,89	DNA

MATERIAL- METHODS

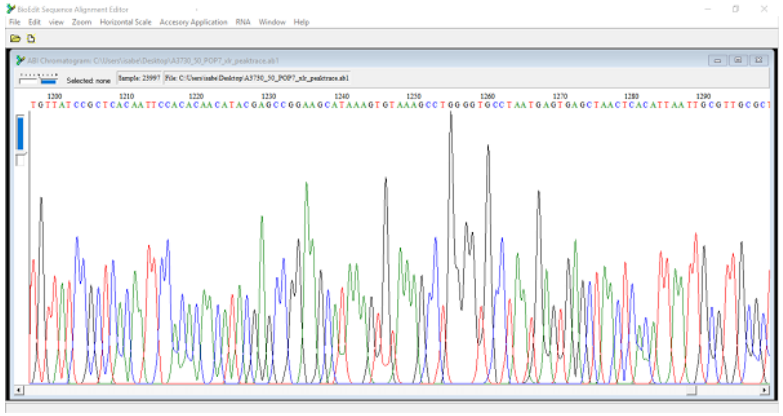
Molecular Analysis

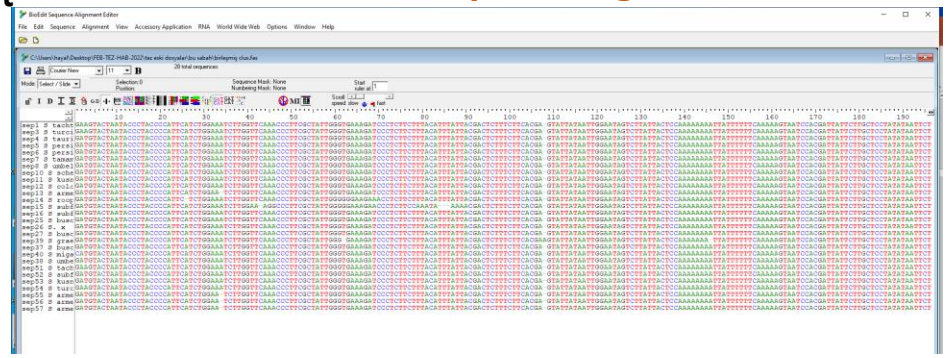
Acquiring Molecular Data

Targeted Plant Gene Regions for Contract Sequencing Services

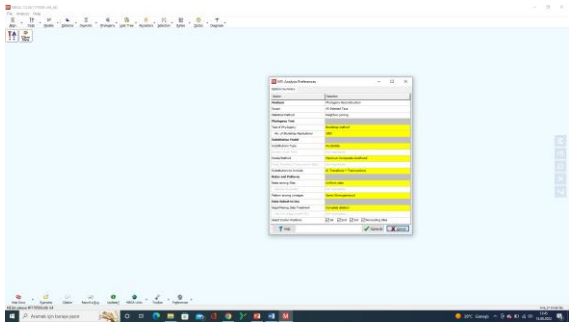
Sequences Analysis and Data Evaluation

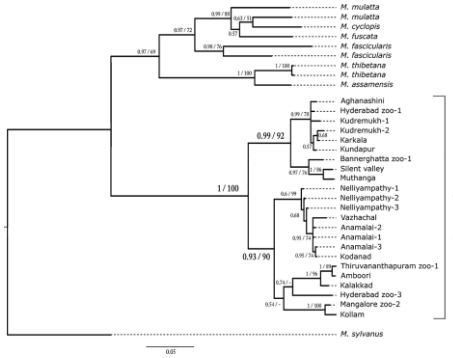
Bioedit





NJ, ML analizleri (MEGA 7.0)



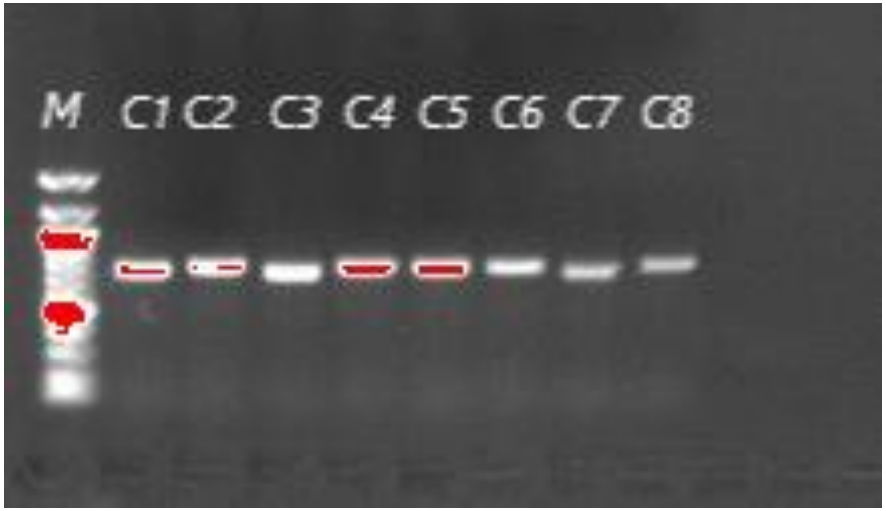


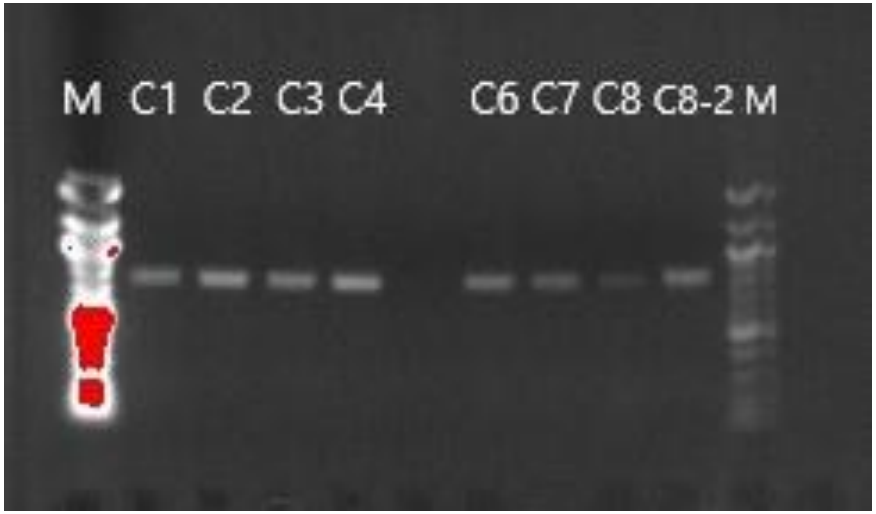
For phylogenetic analyses, DNA sequences from the gene regions obtained from the samples in Turkey were first compiled separately and together to create DNA data sets for all taxa. As a second data set, the DNA sequences identified in this study and those obtained from GenBank were compiled separately for each taxon to create a broader second group of DNA data sets for all taxa.

Results

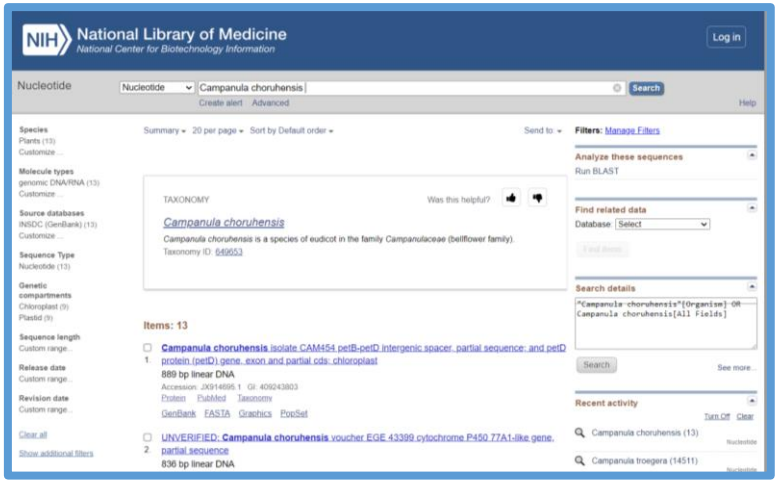
The DNA barcoding of the endemics *Campanula troegera, C. betulifolia* and *C. choruhensis* was investigated in this study. The plastid DNA (matK gene) sequences were successfully amplifed (650 bp).

Results





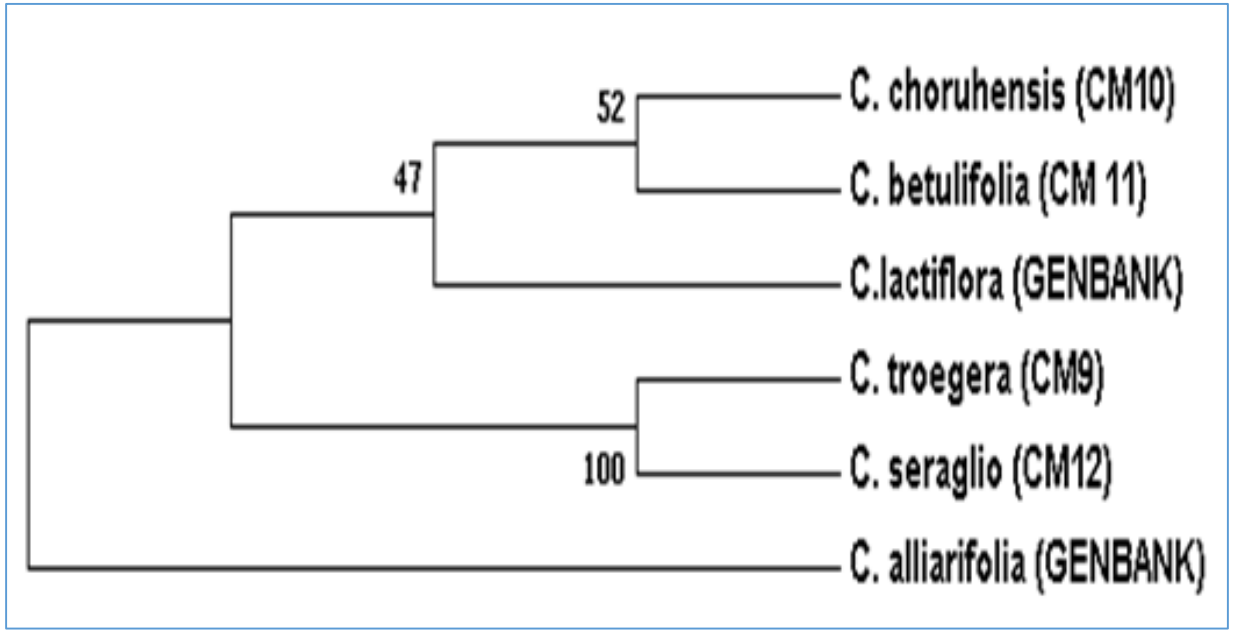
To generate a phylogenetic tree, these sequences were merged with GenBank sequences of other relative in genus *Campanula*. The BEAST program was used to generate the phylogenetic tree using the MEGA 7.0 (Fig. 1).



Results

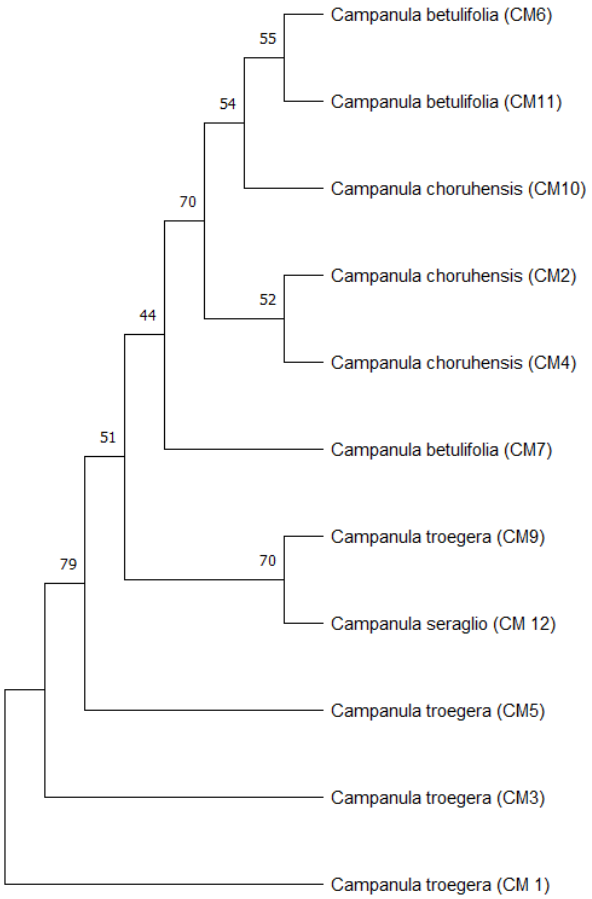
- As a result of phylogenetic analysis, *C. choruhensis* is close relative to *C. betulifolia*. Morphologically, these species were determined to be more similar to each other with flower and leaf characters.
- *C. troegera* formed a separate branch. *Campanula* species (*Campanula betulifolia* C. Koch, *C. choruhensis* Kit Tan & Sorger, *C. troegerae* Damboldt) native to the Artvin-Yusufeli region in Turkey.
- These ornamental plants will be subjects of in-depth cytogenetic and molecular analyses, unraveling their intricate genetic makeup and evolutionary relationships.

Results

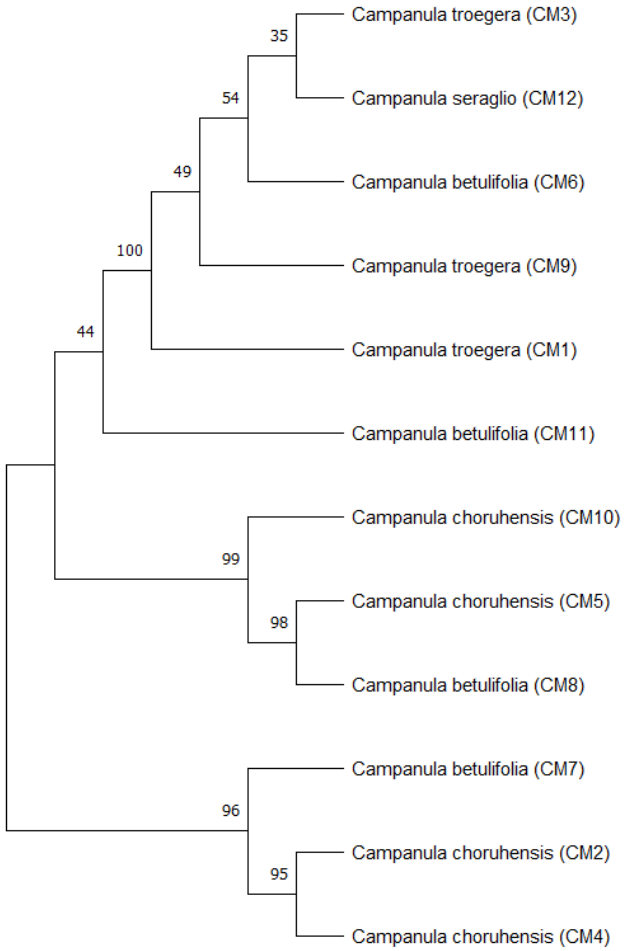


The phylogenetic tree was created by Maximum Likelihood approach using Tamura-Nei model based on chloroplast DNA. C. medium and C. alpina with a brightness value of 76%.

Results



trnK-rps 16 (cpDNA) gene region



trnL –rpL32 (cpDNA) gene region

The two barcoding loci specific primers resulted in robust amplification in the 3 *Campanula* species. Result of phylogenetic analysis, it was determined that *C. choruhensis* was relative to *C. betulifolia*. The phylogenetic tree constructed from trnK-rps16 region sequences was clustered into the two main groups. *C. choruhensis* were seperated from other *Campanula* species.

Identification Key for Endemic *Campanula* Species in Artvin, Turkey

1. Leaf dentate, bidentate, style included

2. Leaves narrowly elliptic, Corolla divided 1/3 lobes, triangular lobes, ciliate pilose……..…***C. choruhensis***

2. Leaves ovate, median largest, sometimes cordate base, Corolla divided 1/4 lobes, triangular lobes, ciliate pilose, ovate acute lobes, not ciliate……..…***C. betulifolia***

1. Leaf serrate, to biserrate, style long excerted

3. Leaves ovate, cordate, Corolla divided 2/3 lobes, obtuse, ciliate apex, densely pubescent outside……..…***C. troegera***

Identification Key for Endemic *Campanula* Species in Artvin, Turkey

Campanula choruhensis	***Campanula betulifolia***	***Campanula troegerae***
Saxatile, many stemmed, shortly and densely crispate-pubescent perennial.	Glabrous or finely pubescent,perennial	Densely pubescent perennial.
Rhizome thick, with persistent leaf bases. Stem simple, flexuous, ascending-erect, 7-10 cm.	Stem numerous ascending, flexuous or decumbent, rarely erect, 6-10-35 cm leafy, branched in upper part.	Stem ascending-erect, flexuous, 12-17 cm, simple, leafy.
Basal leaves narrowly elliptic, obovate,2,5-4*1,2-1,6 cm,irregularly, sharply dentate, pale green orgreyish green, broadly acute to obtuse, with 3-5 cm petioles. Cauline leaves smaller, 1-1.5x0,6 cm, shortly petiolate to subsessile.	Leaves thick, almost glabrous, rarely finely pubescent. Basal and cauline leaves ovate, to 4-6 cm, petiolate, median largest, ovate to broadly ovate with sometimes cordate base, 1-7x 0.7-4 cm, acutely dentate, bidentate or irregularly dentate obtuse or acute petiolate; upper most oblong- lanceolate, +-sessile	Leaves ovate, cordate, acute, serrate to biserrate, greenish pubescent on surface greyish silky below, basal having petiole to 6 cm; median largest, 1.5-3x1.5-2.5 cm, petiole 0.5-2 cm.
Flowers terminal solitary suberect, pedicellate, strongly protandrous. Bracts and bracteoles absent.	Flowers 1-4, erect, pedicellate, in almost corymb-like inflorescence.	Flowers 1-3 erect, terminal pedicels to 5 mm.
Calyx lobes broadly triangular, 7-10.5x4-5.5mm green, acute, exappendiculate. Corolla whitish, faintly tinged pink especially in bud campanulate, c. 3 cm, adpressed – pubescent or puberulent outside, divided to 1/3 into broadly triangular acute lobes, lobes 1-1.4x1.2 cm, ciliate pilose within.	Calyx lobes very variable in size and form, broadly triangular to lanceolate, acute, 6-15x4-6 mm, subentire, glabrous or pubescent, spreading erect, patent after flowering. Calx appendages lanceolate, acute, 2-5 mm or absent, shorter than obconical ovary. Corolla campanulate, 16-35x 10-30 mm,divided to ¼ into ovate, acute, lobes, White or pale pink, glabrous or slightly pubescent outside.	Calyx lobes broadly lanceolate or ovate, acute, spreading erect, 8-12x4-6mm silky. Appendages lanceolate, acute 2mm longer than shortly obconical tube. Corolla broadly infundibular, 2x3-4.5 cm, divided to 2/3 lobes, +- obtuse with ciliate apex, densely pubescent outside, pale pink in bud +- White in flower.
Style included, stigma 4-6.5 mm.recurved after anthesis.	Style included, Stigmas 3. Capsule erect, obconical 6-4 mm.	Style long exserted. Stigmas 3, long. Capsule and seeds unknown.

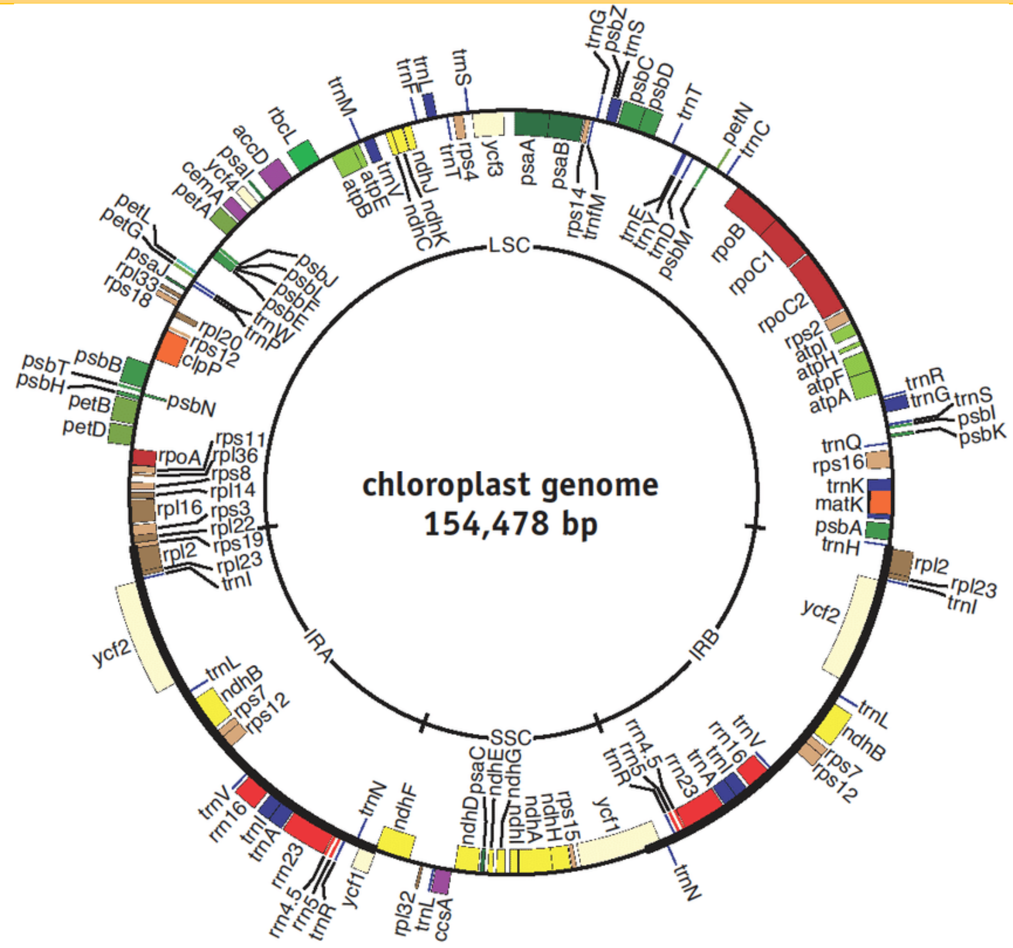
Conclusion

- A field survey was conducted to rediscover and document three endemic *Campanula* species (*C. troegera, C. choruhensis, and C. betulifolia*) distributed in Artvin province and its vicinity. The species were morphologically characterized and subjected to molecular analyses.
- All three species were successfully rediscovered and their field records were updated.
- Detailed morphological descriptions were provided for each species, including vegetative and reproductive characters.
- Molecular phylogenetic analyses using matK sequences confirmed the taxonomic status of the species and their relationships within the genus *Campanula.*
- This study provides valuable information for the conservation and management of these rare and endemic *Campanula* species. The updated field records and morphological characterizations will aid in their identification and monitoring. The molecular phylogenetic analyses contribute to our understanding of the evolutionary relationships within the genus *Campanula.*

Conclusion

- **matK Gene Region Effectively Distinguishes Endemic *Campanula* Species**
- The matK gene region proved to be an effective tool for distinguishing between the endemic *Campanula* species C. troegera and C. choruhensis.
- This is particularly significant as this gene region had not been previously studied for these species.
- Morphological characterization revealed that two specimens initially identified as *C. betulifolia* based on leaf characteristics were more closely related to *C. choruhensis* according to molecular analyses.
- The findings highlight the importance of integrating molecular data with morphological analyses for accurate species identification and classification.
- The matK gene region can serve as a valuable marker for distinguishing between *C. troegera* and *C. choruhensis,* especially in cases where morphological characters may overlap.
- The translation emphasizes the contribution of molecular analyses in resolving taxonomic uncertainties. The identification of *C. choruhensis* in the previously misidentified specimens underscores the need for comprehensive taxonomic assessments.The study demonstrates the value of combining multiple approaches for accurate species delimitation and understanding evolutionary relationships.

In summary, the matK gene region was equally suited for DNA barcoding of endemic *Campanula* taxa was generally enough to discriminate the species, but in identifcation-based DNA barcoding investigations, a combination of matK with a more variable region can be suggested.



Recommendations

Endemism and Habitat Destruction Threaten the Survival of *Campanula* Species

The endemism of *Campanula* species and the destruction of their habitats due to road construction activities are leading to their extinction. Although many conservation efforts have been undertaken in Artvin province, these species have not been able to survive in the relocated areas due to the different soil structures of their rocky habitats. It is necessary to create suitable environments for these species in specific areas, relocate them, and monitor them carefully.

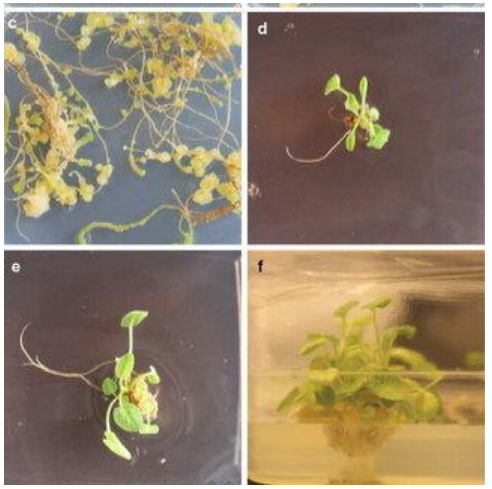
High Temperatures Hinder Seed Maturation and Reproduction

Endemic *Campanula* species prefer to grow in rock crevices and complete their vegetative growth when ambient temperatures are high, potentially preventing seed maturation. This can harm the next generation of the plant, which would develop from seeds. If mature seeds cannot be formed, the plant can use its basal perennial stem, which develops from the same base, to reproduce. To ensure the survival of these plant species, they need to be protected and cultivated in suitable environments.

Recommendations

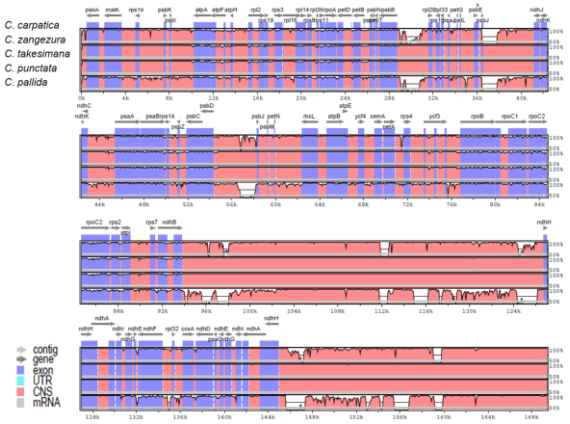
Challenges in Obtaining Root Explants

Obtaining root explants from small-seeded plants that prefer rock crevices is often difficult due to the inability of seeds to mature and dry conditions. Therefore, to conduct cytological studies, it is necessary to first cultivate the plants under controlled conditions to obtain mature seeds or use tissue culture and growth-promoting techniques such as media to facilitate root explant production.Additional Notes:The translation focuses on conveying the main ideas and concepts of the original text.Specific terminology and scientific details may require further translation and clarification.The translation is intended for informational purposes and should not be considered a substitute for professional scientific guidance.



Further Research Directions

Future studies could investigate the broader utility of the matK gene region for resolving phylogenetic relationships within the genus *Campanula.* A more detailed morphological examination of the *C. betulifolia* specimens could reveal subtle characters that distinguish them from *C. choruhensis.* Integrating molecular data with ecological and distributional information could provide a more comprehensive understanding of the evolutionary history and conservation status of these endemic *Campanula* species.



Thank you for your attention