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Phylogenetic analysis of Kars' endemic plant species through amplification of the 26S rDNA gene region

Asiye ULUĞ [*1], Funda Özdemir DEĞIRMENCI[2]
ORCID: 0000-0001-5524-8431; 0000-0002-8875-0273

[1] Department of Biology, Faculty of Science and Literature, Kafkas University, Kars, Türkiye
[2] Department of Crop Science, Faculty of Agriculture, Ahi Evran University, Kırşehir, Türkiye

Abstract

The province of Kars, hosting 16% of Türkiye's plant cover, exhibits a high degree of floral diversity, including 71 endemic species. These species are located at the intersection of Türkiye's Caucasian lands, the Iran-Turan, Euro-Siberian, and Mediterranean flora regions. In this study, the 26S rDNA region of 14 endemic plant species sampled from Kars was amplified, and sequence data were obtained. NCBI GenBank database searches confirmed the first-time sequencing of the 26S rDNA gene region for these endemic plant species. The molecular characteristics of the 26S rDNA region of the 14 endemic plant species were examined, providing significant genetic data on the diversity and evolutionary relationships of endemic plants. Comparative analysis of the 26S rDNA sequences of the studied endemic species revealed notable genetic relationships within these plant groups, uncovering considerable variations among the species. The observed high polymorphism in the 26S rDNA region suggests its potential for accurate species identification. The genetic data obtained in this study have the potential to contribute to genetic research for the conservation of endemic species and biodiversity, emphasizing the importance of exploring and documenting the genetic uniqueness of endemic species. Furthermore, the genetic data obtained not only contribute to understanding the phylogenetic relationships among endemic species but also have implications for preserving and sustaining biological diversity by providing molecular identity to endemic plants in international databases.

Keywords: Kars, endemic, 26S rDNA, diversity, conservation

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Kars'ın endemik bitki türlerinin 26S rDNA gen bölgesi amplifikasyonu ile filogenetik analizi

Özet

Türkiye'nin bitki örtüsünün %16'sine ev sahipliği yapan Kars ili, 71'i endemik olmak üzere yüksek oranda floral çeşitliliğe sahiptir. Bu türler, Türkiye'nin Kafkas toprakları, İran-Turan, Euro-Sibirya ve Akdeniz bitki coğrafyasının kesişim noktasında bulunmaktadır. Bu çalışmada, Kars ilinden örneklenen 14 endemik bitki türünün 26S rDNA bölgesi amplifiye edilerek sekans verileri elde edilmiştir. NCBI GenBank veri tabanı eşleştirmeleri bu endemik bitki türlerinin 26S rDNA gen bölgesinin ilk defa sekanslandığını göstermiştir. 14 endemik bitki türünün 26S rDNA bölgesinin moleküler özellikleri incelenerek, endemik bitkilerin genetik çeşitlilikleri ve evrimsel ilişkileriyle ilgili önemli genetik veriler elde edilmiştir. Çalışılan endemik türlerin 26S rDNA dizilerinin karşılaştırılması, bu bitki grupları arasında önemli genetik ilişkileri ortaya koyarak, türler arasında dikkate değer varyasyonları açığa çıkarmıştır. Yüksek polimorfizm 26S rDNA bölgesinin doğru tür tanımlamasındaki potansiyelini göstermiştir. Bu çalışmada elde edilen genetik veriler, endemik türlerin ve biyoçeşitliliğin korunmasına yönelik genetik araştırmalara katkıda bulunma potansiyeline sahiptir ve endemik türlerin genetik benzersizliğinin keşfedilmesinin ve belgelenmesinin önemini açığa çıkarmaktadır. Ayrıca elde edilen genetik veriler, endemik türler arasındaki filogenetik ilişkilerin anlaşılmasına katkı sağlamasının yanı sıra, uluslararası veri tabanlarında endemik bitkilere moleküler kimlik vermede kullanılabileceğinden biyolojik çeşitliliğin korunması ve sürdürülebilir olması açısından önem arz etmektedir.

Anahtar kelimeler: Kars, endemik, 26S rDNA, çeşitlilik, koruma

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +9xxxxxxxxxxxx; Fax.: +9xxxxxxxxxxxx; E-mail: xxxxxxx
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1. Introduction

Endemic species, which are only found in specific geographic areas and not anywhere else in the world, are unique and often vulnerable components of biodiversity. These species make a significant contribution to overall biodiversity, and it is essential to conserve them in order to maintain ecosystem resilience and functionality [1]. Endemic plants have unique ecological roles and their presence contributes to the stability and functioning of ecosystems [2]. However, endemic plant species are often threatened by habitat loss, climate change, and other environmental pressures. Therefore, the molecular identification of endemic plant species is a crucial pursuit. Molecular identification techniques, such as DNA barcoding, are invaluable tools for studying and preserving these distinctive plants [3]. By using molecular identification, researchers can carefully monitor and identify these species, which empowers conservationists to develop targeted strategies for their preservation and sustainable management. Molecular methods also contribute to comprehensive biodiversity assessments by systematically identifying and cataloguing endemic plant species in specific regions [4]. Additionally, molecular identification allows researchers to study the biogeography of endemic plant species, providing insights into their historical distribution, migration patterns, and evolutionary relationships. This knowledge enhances our understanding of the complex processes that shape regional biodiversity [5, 6]. Molecular data are also crucial for reconstructing the evolutionary histories and phylogenetic relationships of endemic plant species. By identifying these species at the molecular level, we can effectively manage and utilize them as valuable genetic resources [7, 8].

The 26SrDNA gene region, which is part of the nuclear genome in plants, encodes a crucial segment of the 26S ribosomal RNA (rRNA), which is an essential component of the large ribosomal subunit. This gene region plays a fundamental role in protein synthesis and has become a focal point of scientific investigation [9]. Researchers use information from the 26SrDNA gene region, along with other molecular markers, to study plant systematics, taxonomy, and evolutionary relationships. The 26SrDNA gene region is highly conserved across a wide range of plant species, highlighting the fundamental role of ribosomal RNA in cellular translation. Phylogenetic studies, based on the 26S rDNA gene region, are key in unraveling the evolutionary history of plant species [10]. Due to its moderate evolutionary rate and versatility as a molecular marker, the 26S rDNA gene region is used to determine relationships at various taxonomic levels, from genera to families. Its universality and conservation make it an indispensable tool for comparing evolutionary relationships across diverse organisms. The gene region's evolutionary stability, combined with its ability to detect subtle variations, allows researchers to construct robust phylogenetic trees. These trees help us understand the relationships between different plant taxa and contribute to our knowledge of plant evolutionary history. The unique sequence variations in this region can be used as molecular fingerprints to distinguish between species. Phylogenetic studies using the 26S rDNA gene region also contribute to our understanding of biogeography and the historical distribution of species [11].

The Kars province in Türkiye has an exceptionally rich floral diversity, with 16% of Türkiye's flora consisting of 1615 species, 71 of which are endemic. This region represents Türkiye's Caucasian lands and serves as a convergence point for the Iran-Turan, Euro-Siberian, and Mediterranean flora regions [12]. Unfortunately, the region faces challenges such as excessive and uncontrolled grazing, as well as clearing activities, which pose a threat to the survival of various species, especially endemic ones [13]. To protect biodiversity, it is crucial to uncover and preserve the genetic diversity that allows species to adapt to changing environmental conditions. Among the 71 endemic species, 12 are specifically found around Lake Çıldır, Allahuekber Mountains, and Sarıkamış forests, which are designated as Important Plant Areas in Türkiye [12, 14]. By assigning molecular identities to endemic plants, we can make a significant contribution to the conservation and sustainable development of biological diversity in our country. In this study, our aim was to use the 26SrDNA gene region to perform phylogenetic identification of 14 endemic plant species in the Kars province. This will help us understand the phylogenetic relationships within and between species. The genetic data obtained in this study have the potential to contribute to genetic research for the conservation of endemic species and biodiversity. The use of molecular identification of endemic plant species could play an important role in understanding, conserving, and managing these species in the Kars province.

2. Materials and methods

DNA extraction and PCR cycling

Leaf samples were systematically collected from diverse locations within Kars province, encompassing 14 distinct endemic plant species (Table 1). Nuclear DNA extraction from leaf tissues was conducted using the modified CTAB DNA isolation method devised by Kistler (2012) [15]. To assess DNA concentrations and quality, readings at 230 nm, 260 nm, and 280 nm were obtained using the Biodrop l Lite 7141 V.1.0.4 spectrophotometer. The 26S rDNA forward and reverse primer sequences, specifically 5’-TTCCCAAACAACCCGACTC-3’ and 5’-GCCGTCCGAATTGTAGTCTG-3’ [16], were employed for the PCR reaction. The reaction mixture, total volume 20 µl, comprised 4 µl HOT FIREPol Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.5 µl of 200 nM forward and reverse primers, 5 µl of template DNA (diluted to 10 ng), and 10 µl of water. The PCR protocol included an initial cycle

at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 58 °C (Ta) for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. After PCR, electrophoresis was performed on 3% agarose gels at 90 V for 30 min.

Table 1. Information on 14 endemic plant species from Kars province

Endemic plant species	**Distrubution**	**Endemism**	**Family**
Onosma nigricaulis Riedl	North East Anatolia, Kars	Local Endemic	Boraginacea e
Onosma isaurica Boiss. & Heldr.	Anatolia, Sarıkamış	Endemic	Boraginacea e
Tragopogon aureus Boiss.	North Anatolia, Kars	Endemic	Asteraceae
Corydalis oppositifolia subsp. oppositifolia DC.	North, South, East Anatolia, Sarıkamış	Endemic	Papaveracea e
Rosa pisiformis (Christ) Sosn.	North East Anatolia, Kars	Endemic	Rosaceae
Lathyrus karsianus P.H. Davis	North East Anatolia, Sarıkamış	Local Endemic	Fabaceaea
Astragalus globosus Vahl	North Anatolia, Kısır Mountain	Endemic	Fabaceae
Lamium galactophyllum Boiss. & Reuter	North East Anatolia, Seli	Endemic	Lamiaceae
Salvia rosifolia SM	Anatolia, Kağızman	Endemic	Lamiaceae
Allium czelghauricum Bordz	North East Anatolia, Göl	Local Endemic	Amaryllidac eae
Papaver triniifolium Boiss	North East and South Anatolia, Çıldır	Endemic	Papaveracea e
Pastinaca armena subsp. dentata (Freyn et Sint.) Chamberlain	North East Anatolia, Arpaça	Endemic	Apiaceae
Vincetoxicum coskuncelebiana S.Makbul& S.Güven	North East Anatolia, Çıldır, Tşbaşı village	Local Endemic	Apocynacea e
Fritillaria michailovskyi Fomin	North East Anatolia, Sarıkamış	Endemic	Liliaceae

Data analysis

PCR products demonstrating the desired amplification were purified and sequenced at BM Labosis in Cankaya, Ankara. Chromatogram data visualization was performed using the TraceEditor tool included in MEGA 11 software. BLAST search [17] and CLUSTAL alignment [18] were conducted using MEGA 11 software [19]. MEGA 11 software was used to compute essential phylogenetic parameters and identify DNA polymorphism among the endemic species. BLAST analysis compared the 26SrDNA sequences of the 14 endemic species with sequences of closely related species from the NCBI database [20]. These analyses evaluated the correspondence between the acquired sequences and previously studied sequences of the same species or closely related species. To determine the evolutionary relationships among the endemic species, the Neighbor-Joining Method [21] was employed to construct a phylogenetic tree based on evolutionary distances [22]. The number of bootstrap replications was set at 500.

3. Results

A large amount of high-quality genomic DNA was obtained for many of the 14 endemic plant species studied using the modified CTAB method from Kistler and Shapiro (2011) [15] (Table 2). For the first time, the 26S rDNA region of the 14 endemic plant species from Kars province was successfully amplified and sequenced.

The length of the 26S rDNA region was found to be approximately 149 base pairs for all endemic species. However, due to the absence of previously published sequences of 26S rDNA for these species in the NCBI GenBank database [20], alignment with the same species could not be determined. Instead, the obtained sequences for these endemic plants were aligned with their relatives in the same genus or family (Table 3). Specifically, *Onosma nigricaulis* and *Onosma isaurica* showed 100% identity with *Echium plantagineum* (OL580770.1). Similarly, *Lamium galactophyllum* had the highest alignment with *Ballata nigra* (ON685391.1), while *Pastinaca armena* had the highest alignment with *Zizia*

aurea (MT610976.1), both with 100% identity. *Fritillaria michailovskyi* aligned with *Lilium michauxii* (AF205126.1) with a 99.31% identity value.

Table 2. DNA concentration of the studied endemic plants

Species	DNA concentration (μg/ml)	230/260 Ratio	OD	260/280 Ratio	OD
Onosma nigricaulis	837	0.65		1.55	
Onosma isaurica	750	1.85		2.02	
Tragopogon aureus	1057	1.25		1.88	
Corydalis oppositifolia subsp. oppositifolia	1677	1.28		2.04	
Rosa pisiformis	608	0.67		1.19	
Lathyrus karsianus	1006	1.55		1.99	
Astragalus globosus	2794	1.64		1.68	
Lamium galactophyllum	655	0.40		2.5	
Salvia rosifolia	316	0.99		1.75	
Allium czelghauricum	27	2.83		1.84	
Papaver triniifolium	65	2.81		1.80	
Pastinaca armena	26	2.19		1.84	
Vincetoxicum coskuncelebianus	178	2.53		2.11	
Fritillaria michailovskyi	1171	0.98		1.91	

The sequence of the 26S rDNA of *Tragopogon aureus, Corydalis oppositifolia, Rosa pisiformis, Lathyrus karsianus, Salvia rosifolia, Astragalus globosus, Allium czelghauricum*, and *Papaver triniifolium* corresponded with the sequence of members of the same genus. *Allium czelghauricum* exhibited the lowest sequence similarity with *Allium altaicum* (MK049255.1), with a 96.48% identity (Table 3).

Table 3. GenBank Alignment results of 26S rDNA gene region for the studied 14 endemic plant species

Species	Amplified product length (bp)	Aligned species	Accession number	Query cover	e value	Percentage of identity
26S rDNA						
Onosma nigricaule	149	*Echium plantagineum*	OL580770.1	98	1e-68	100
Onosma isaurica	149	*Echium plantagineum*	OL580770.1	98	1e-68	100
Tragopogon aureus	149	*Tragopogon dubius*	KT179725.1	97	9e-78	100
Corydalis oppositifolia subsp. oppositifolia	149	*Corydalis wilsonii*	LN610850.1	100	2e-74	100
Rosa pisiformis	149	*Rosa chinensis*	XR_002934681	98	6e-72	100
Lathyrus karsianus	149	*Lathyrus decaphyllus*	KT459234.1	98	2e-72	99.32
Astragalus globosus	149	*Astragalus canadensis*	MT610924.1	98	2e-70	99.32
Lamium galactophyllum	149	*Ballata nigra*	ON685391.1	100	1e-69	100
Salvia rosifolia	149	*Salvia carduaceae*	MK257800.1	100	3e-73	100
Allium czelghauricum	149	*Allium altaicum*	MK049255.1	94	7e-57	96.48
Papaver triniifolium	149	*Papaver somniferum*	XR_003342571.1	100	5e-68	99.33
Pastinaca armena	149	*Zizia aurea*	MT610976.1	100	1e-69	100
Vincetoxicum coskuncelebianus	149	*Asclepias tuberosa*	KY860923.1	100	2e-66	98.66
Fritillaria michailovskyi	149	*Lilium michauxii*	AF205126.1	96	3e-65	99.31

When comparing the 26S rDNA sequences of 14 endemic plant species, we observed 38 variable sites (Figure 1). There was no variation between *Onosma isaurica* and *Onosma nigricaulis*. However, we did observe one variable site at the 52 bp location between *Lathyrus karsianus* and *Astragalus globosus*, which are both members of the Fabaceae family. In the Asteraceae family, there were four nucleotide substitutions between *Corydalis oppositifolia subsp. oppositifolia* and *Papaver triniifolium*, occurring at the 52, 77, 86, and 112 bp locations. Despite both belonging to the Lamiaceae family, *Lamium galactophyllum* and *Salvia rosifolia differed* at nine base positions in the 26S rDNA sequence (Figure 1). We have uploaded the sequence data for each endemic plant species to the GenBank database.

Figure 1. Multiple alignments of 26S rDNA gene region for the studied 14 endemic plant species

A phylogenetic tree was generated using the obtained sequences of 26S rDNA for 14 endemic plant species in order to detect the evolutionary relationships among them. The studied species were found to belong to different branches of the phylogenetic tree, with a low level of genetic differentiation among closely related taxa. Although members of each family were clustered together in separate genetic clusters, *Corydalis oppositifolia subsp. oppositifolia* and *Papaver triniifolium* were grouped with *Tragopogon aureus* and *Vincetoxicum coskuncelebianus*, respectively. When the 14 endemic species were analyzed, *Allium czelghauricum* and *Pastinaca armena* were found to be significantly different from the other endemic species (Figure 2).

4. Conclusions and discussion

The 26S rDNA region was successfully amplified and sequenced for the first time in 14 endemic plant species of Kars province, providing valuable insights into their genetic characteristics. The alignment results revealed intriguing relationships between the studied endemic species and their relatives in the same genus or family, emphasizing the genetic relationships within these plant groups. The comparison of 26S rDNA sequences among the 14 endemic plant species has revealed a notable level of genetic variation, as evidenced by the identification of 38 variable sites. These findings offer valuable insights into the molecular diversity within this group of plants and contribute to our understanding of their evolutionary relationships. The absence of variation between *Onosma isaurica* and *Onosma nigricaulis* suggests a high degree of sequence conservation within these two closely related species. Within the Fabaceae family, a single variable site at 52 bp distinguishes *Lathyrus karsianus* and *Astragalus. globosus*. This limited nucleotide substitution between members of the same family underscores the importance of exploring intraspecific variation, as even closely related species can exhibit subtle genomic differences that may have functional implications [25]. In contrast, a higher level of divergence within the Asteraceae family was observed in *Corydalis oppositifolia* subsp. *oppositifolia* and *Papaver triniifolium*, with four nucleotide substitutions at positions 52, 77, 86, and 112 bp, suggesting a greater evolutionary distance or potential ecological factors influencing genetic variation between these two species. Similarly, despite their membership in the same Lamiaceae family, *Lamium galactophyllum* and *Salvia rosifolia* exhibit variation at nine base positions in the 26S rDNA sequence. This highlights the complexity of genetic divergence even within taxonomically related groups, emphasizing the need for a nuanced understanding of evolutionary relationships at the species level [23].

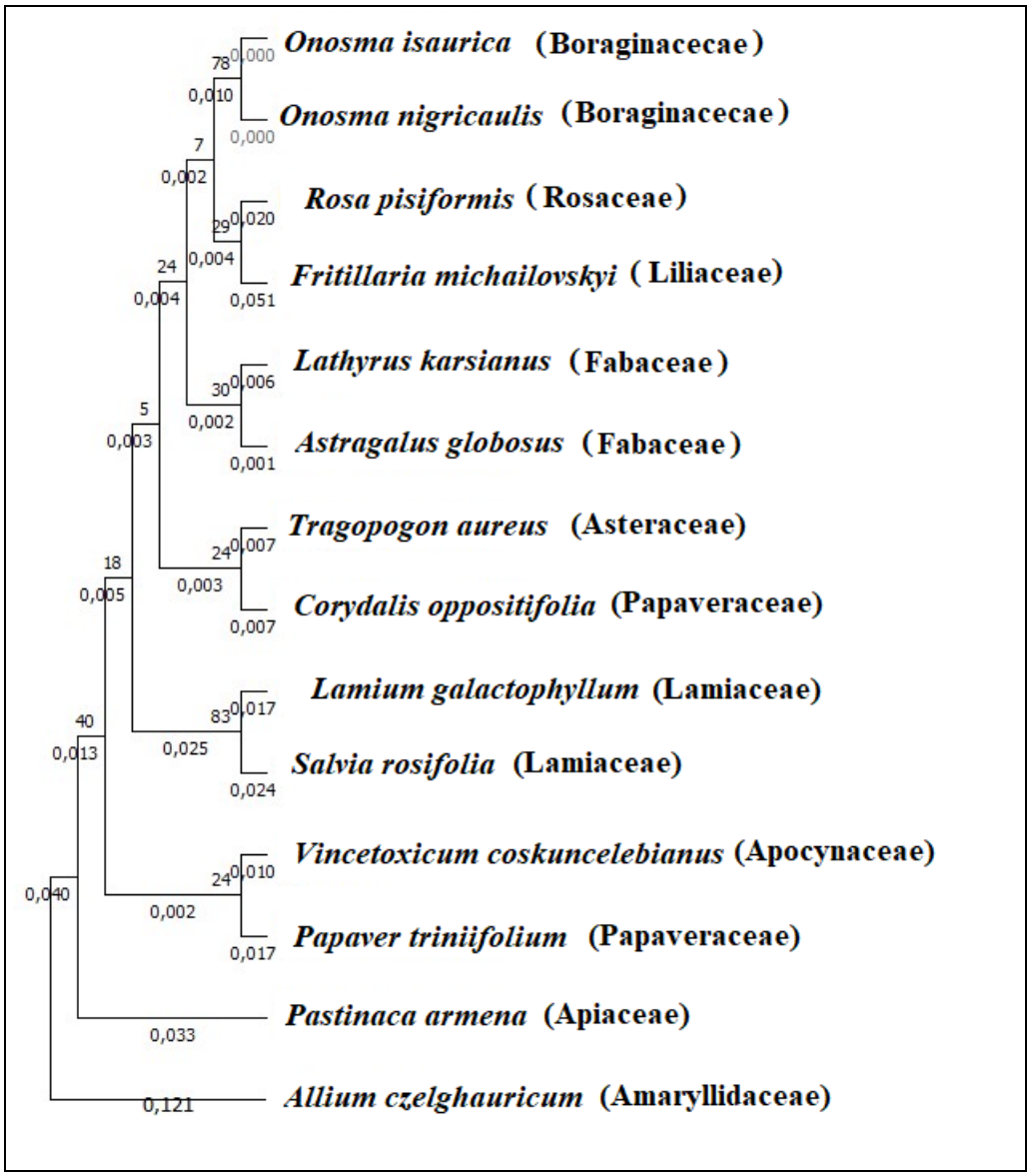

Figure 2. Phylogenetic tree based on the sequence of 26S rDNA gene region for 14 endemic plant species

The observed variable sites in the 26S rDNA sequences provide valuable markers for future studies aiming to explore the population genetics, phylogeography, and adaptive evolution of these endemic plant species [10, 11]. Investigating the functional significance of these variable sites could offer insights into their potential roles in adaptive processes or responses to environmental factors.

The construction of a phylogenetic tree using the 26S rDNA sequences of 14 endemic plant species has provided valuable insights into their evolutionary relationships. The phylogenetic analysis generally reveals a clustering of members within the same family. The observed clustering of members of each family into distinct genetic clusters aligns with expectations, suggesting a shared evolutionary history and genetic similarity among closely related species. However, the unexpected grouping of *Corydalis oppositifolia subsp. oppositifolia* and *Papaver triniifolium* with unrelated species challenge conventional taxonomic expectations. This discrepancy may be indicative of convergent evolution, or complex evolutionary processes that transcend traditional taxonomic boundaries [24]. A notable finding in the phylogenetic analysis is the significant genetic differentiation observed in *Allium czelghauricum* and *Pastinaca armena* compared to other endemic species. The distinct placement of these species in the phylogenetic tree suggests unique evolutionary trajectories or ecological adaptations that set them apart from their counterparts. The high polymorphism detected in the 26S rDNA region for these species indicates the dynamic nature of their genomes, reflecting ongoing evolutionary processes, potential gene flow, or adaptive responses to environmental factors [25]. It is seen that there is a paraphyletic relationship between the endemic species in terms of the 26S rDNA gene region. Although it is a very successful gene region in species identification, it is insufficient alone to explain the evolutionary relationship between these endemic species. The phylogenetic relationship between the studied endemic species can be revealed with different evolutionary approaches by studying different gene regions in addition to the 26S rDNA.

Genetic studies on endemic plant species have only recently begun to be carried out in Türkiye [26, 27]. It is clear that more detailed molecular identification and phylogenetic studies should be performed to protect the biodiversity of Türkiye.

Our study presents the first insights into the molecular characteristics of the 26S rDNA in 14 endemic plant species. The phylogenetic analysis of 26S rDNA sequences has revealed both expected and unexpected patterns in the evolutionary relationships among these 14 endemic plant species. This underscores the significance of our study in providing valuable genetic information for these endemic species. The establishment of genomic resources for these endemics may contribute to a broader understanding of plant diversity and evolution, emphasizing the ongoing need for efforts to explore and document the genetic uniqueness of endemic species. The preliminary results obtained from this study lay the groundwork for future genetic investigations aimed at conserving these endemic species and broader biodiversity. The addition of genetic data to national and international databases will contribute to a growing repository of information, facilitating collaborative research efforts and enhancing our understanding of plant evolution and diversity. The genetic data generated in this study serve as a valuable resource for future research initiatives focused on the conservation of Türkiye's rich biodiversity and contribute to the global discourse on biodiversity conservation.

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