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Determining Phylogenetic Relationships of Some Endemic and Rare *Astragalus* Taxa in the Van Lake Basin

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ABSTRACT
Astragalus, the most diverse genus in the world, contains about 3000 different taxa. This genus has 489 taxa and 63 sections in Turkey, giving it a 51% endemism rate. 82 *Astragalus* taxa were collected from the Van Lake Basin in 2019 for this research. These species' morphological diagnoses revealed that four of them were rare and 17 of them were endemic. In this research, the universal primers ITS4 and ITS5 were used to amplify the ITS1 and ITS2 sections, which included the 5.8S gene of rDNA. Different programs (SnapGene, CLC DNA Workbench) were used to analyze the genome information of species in the genus *Astragalus*. The DNA sequences of *Astragalus* species have been uploaded to the GenBank database of the National Center for Biotechnology Information (NCBI) (Maryland, USA), which is open to all researchers worldwide. The length of the ITS in the study, including the 5.8S sequence, varied from 669 to 687 bp. The results showed that the phylogenetic tree combined with the most stable secondary (2^0) structure obtained from the universal ITS4 and ITS5 primers is an effective tool for the identification of *Astragalus* taxa.

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Van Gölü Havzası'nda Bazı Endemik ve Nadir *Astragalus* Taksonlarının Filogenetik İlişkilerinin Belirlenmesi

ÖZET
Dünyanın en çeşitli cinsi olan *Astragalus*, yaklaşık 3000 farklı takson içermektedir. Bu cins Türkiye'de 489 takson ve 63 bölüme sahiptir ve bu da ona %51 endemizm oranı vermektedir. Bu araştırma için 2019 yılında Van Gölü Havzası'ndan 82 *Astragalus* taksonu toplanmıştır. Bu türlerin morfolojik tanıları, dördünün nadir, 17'sinin endemik olduğunu ortaya koymuştur. Bu araştırmada, rDNA'nın 5.8S genini içeren ITS1 ve ITS2 bölümlerini çoğaltmak için evrensel primerler olan ITS4 ve ITS5 kullanılmıştır. *Astragalus* cinsindeki türlerin genom bilgilerini analiz etmek için farklı programlar (SnapGene, CLC DNA Workbench) kullanılmıştır. *Astragalus* türlerinin DNA dizileri, Ulusal Biyoteknoloji Bilgi Merkezi'nin (NCBI) (Maryland, USA) dünya çapında tüm araştırmacılara açık olan GenBank veri tabanına yüklenmiştir. 5.8S dizisi de dahil olmak üzere çalışmadaki ITS uzunluğu 669 ila 687 bp arasında değişmiştir. Sonuçlar, evrensel ITS4 ve ITS5 primerlerinden elde edilen en kararlı ikincil (2^0) yapı ile birleştirilen filogenetik ağacın, *Astragalus* taksonlarının tanımlanması için etkili bir araç olduğunu göstermiştir.

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INTRODUCTION

After Orchidaceae and Asteraceae, the Fabaceae family has 19,400 species and 740 genera, making it the world's third-largest plant family. The largest genus in the Fabaceae family is *Astragalus* (Podlech & Zarre, 2013). The genus *Astragalus* has 469 taxa and 62 sections (groups) in the Turkish flora (Davis et al., 1988; Podlech, 1999; Aytaç & Ekici, 2012). Aytaç and Ekici (2012) report that 217 of Turkey's 469 *Astragalus* taxa are endemic, with an endemism rate of 46.2%. *Astragalus* is widely distributed in the steppe environment of low or high mountains in the Irano-Turanian phytogeographic region of Turkey (Chamberlain & Matthews, 1970; Podlech, 1999; Duran & Aytaç, 2005; Atasagun et al., 2021). The leaf rachis of this taxa is classified into two groups: those with spines and those without. In Turkey, locals refer to it as "Geven or Guni. The taxonomical problems of the genus *Astragalus* were mentioned in the Flora of Turkey (Davis et al., 1988). Due to their morphological similarities, some taxa of the *Astragalus* genus identified in Turkey might be difficult to distinguish. The Fabaceae family's most abundant member, *Astragalu*s, is considered a taxonomically difficult genus. (Podlech, 1986). To solve these taxonomical issues, the genus should determine the phylogenetic relationships.

The (ITS) Internal Transcribed Spacers region on ribosomal DNA is a popular choice today for PCR amplification used for phylogenetic analysis of closely related species and populations. The popularity of ITS regions has increased as universal primers that can bind to conserved rDNA regions have been developed. The information obtained from the analysis of the ITS base sequences makes important contributions to the solution of current problems in taxonomic categories. Usually, the kinship of the related taxa is attempted by examining the ITS variations that belong to the various taxa (Baldwin et al., 1995). In various systematic studies, ITS has been applied to a wide range of plants at the genus and species levels. The two interior spaces ITS-1 and ITS-2 are located between the genes encoding the 5.8S, 18S, and 26S nuclear ribosomal RNA (nrRNA) subunits. The ITS-1 and ITS-2 gaps as well as the 5.8S gene are referred to as the ITS region. While the 5.8S subunit in angiosperms has a constant length of 163–164 bp, ITS-1 and ITS-2 are about 300 bp long (Baldwin, 1992).

The ITS region can be amplified and sequenced with the help of all-purpose primers. Primers derived from yeast (Saccharomyces), insect (Drosophila), and plant (*Oryza sativa* and *Hordeum vulgaris*) lines were originally designed for fungal rRNA amplification (White et al., 1990). The ITS-1 or ITS-2 region sequence analysis results may produce phylogenetic trees with results that are not supported by other sequences. As a result, combining the data from the ITS-1 and ITS-2 regions produces results that produce trees that are more precise, reliable, and comprehensive (Baldwin, 1992; Baldwin et al., 1995).

Because the morphological features of many *Astragalus* species are complex, it is difficult to distinguish them differently. In that sense, standardized DNA marker-based DNA analysis is useful for identifying species (Zhang & Jiang, 2020). Recent years have seen a significant increase in the usage of these markers for endangered species conservation and species discrimination (Kress et al., 2005; Kress, 2017). Especially, the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (nrDNA) is now widely used for such purposes. Several studies, including some recent ones, have used ITS regions as molecular evidence to describe new *Astragalus* species (Pahlevani et al., 2020; Roofigar & Maassoumi, 2020; Abd El-Ghani et al., 2021).

In this study, some endemic and rare species of the *Astragalus* genus were collected in the Van Lake Basin. Phylogenetic analysis techniques were used to identify the degree of relationship between species. Molecular methods used in plant identification provide a great convenience in this sense and eliminate the mistakes made in systematic diagnosis in distinguishing species belonging to a genus from each other.

There are 211 endemic *Astragalus* taxa that have been identified in Turkey, and they are all perennial. According to our regions, the Central Anatolia Region (100 taxa; 29.1%) has the highest number of *Astragalus* taxa, followed by the Mediterranean (77 taxa; 22.4%), Eastern Anatolia (63 taxa; 18.3%), and the Black Sea Region (52 taxa; 15.1%) (Başbağ et al., 2018).

MATERIAL and METHOD

Taxon Sampling:

In July 2019, the study was carried out in the Van Lake Basin. Plant samples belonging to the genus *Astragalus* were collected from the Erciş, Muradiye, Çaldıran, Tuşba, Edremit, Özalp, Saray, Çatak, Bahçesaray, and Gürpınar districts of Van (Figure 1). The scientific identification of the plant samples was confirmed at Hakkari University Biodiversity Research Herbarium (VPH), in Turkey, and the voucher specimens were stored at VPH (Table 1).

DNA Isolation and PCR Amplification

Genomic DNA was extracted from fresh *Astragalus* spp.

The leaves of the samples selected during the field study and turned into herbarium samples were used for DNA isolation. A commercial DNA extraction kit

(Thermo Scientific, Lithuania) was used to isolate plant DNA, which is the main material of the present study. DNA isolation was applied to plant samples at the numbers which can represent each species. Molecular (sequence analysis and secondary structure pattern of nrDNA) and morphological (flower and leaf structure) analyses were primarily used to identify the species of the collected plant samples.

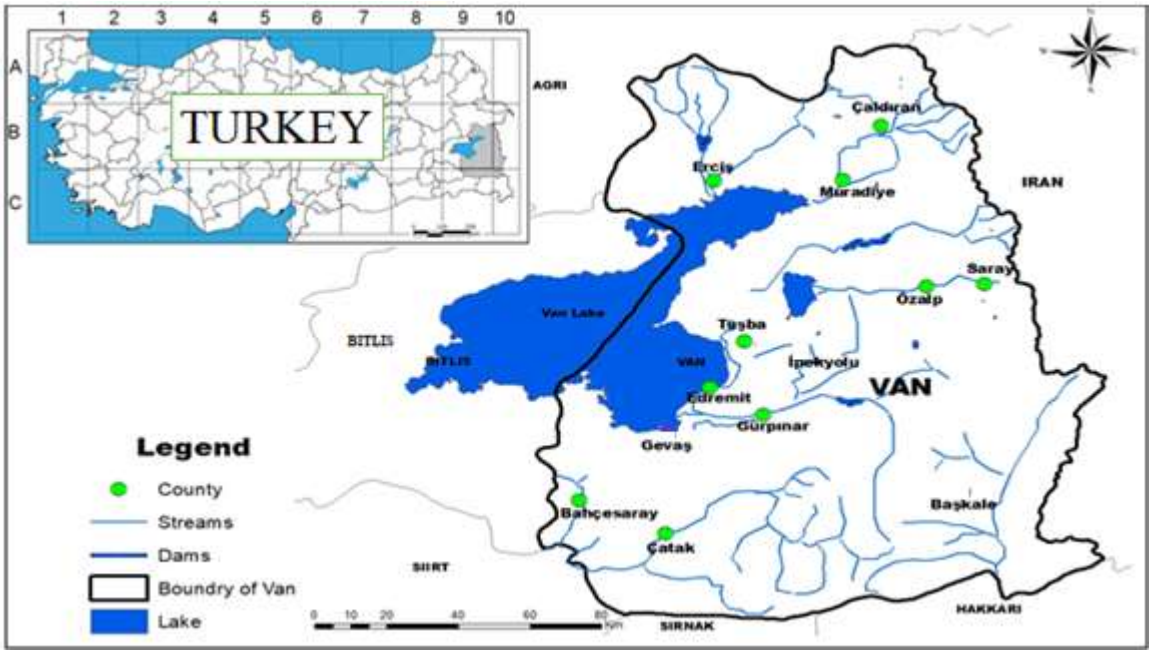

Figure 1. Locations where endemic and rare species belonging to the genus Astragalus are collected in the Van Lake Basin.

Şekil 1. Van Gölü Havzası'nda Astragalus cinsine ait endemik ve nadir türlerin toplandığı yerler.

Universal primers ITS4 –(5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAG G -3') were used to amplify the ITS region of the ribosomal DNA gene. With the help of these primers, ITS1, 5.8S, and ITS2 regions in rDNA were amplified by PCR. Amplification of these regions on double-stranded DNA was performed in 50 µl reaction volumes, which was a mixture containing 5 µl of 10X reaction buffer, 3 µl of $MgCl_2$, 1 µl of dNTP, 1 µl of each primer, 0.4 µl of Taq DNA polymerase enzyme (Fermantas) and 5 µl of genomic DNA. In the prepared PCR mixture 2 min DNA double chain separation at 94 °C, 1 min DNA double chain separation at 94 °C, 1 min primers binding at 55 °C, 2 min DNA synthesis at 72 °C, and finally 10 min final elongation at 72 °C were applied to the prepared PCR mixture in the form of 36 thermal cycles, respectively. PCR applications were performed on the Eppendorf MasterCycler device. The products PCR were run on a 1% agarose gel and the gel obtained at the end of electrophoresis was stained on a shaker platform for 20 minutes in 100 ml of sterile distilled water and 30 µl of EtBr (0.5 µg ml^{-1}) solution in a suitable container (Sambrook et al., 1989). To make the stained DNA visible, it was displayed in the gel imaging and analysis system. After the molecular studies, DNA next-generation sequencing (NGS) of the ITS regions of each taxon on the purified DNA was made by Sentebiolab A.Ş. The accession numbers of the studied sequences are given in Table 1 and registered in the GenBank database.

Predicting the Most Stable Secondary (2^0) Structure

All possible most stable secondary (2^0) structures of ITS1, 5.8S, and ITS2 regions for a total of 21 species sequences were modeled using the fold structure prediction package of CLC Main Workbench 6.7.1. The most stable rRNA molecule can be predicted by calculating the free energies of all possible secondary structures and maintaining one of the lowest energy (i.e., the most stable) ones (Chastain & Tinoco, 1991).

Phylogenetic Analysis

Nucleotide sequence similarity, multiple alignments, and phylogenetic tree were created using the CLC Mainwork bench 6.7.1 software (Qiagen, USA). The phylogenetic tree was created using the Neighbor-Joining algorithm with 1000 bootstrap replicates by the ITS (Internal Transcribed Spacer) region sequences of Astragalus species detected in the present study registered in the GeneBank. A plant species named *Phytolacca americana* L. with accession number JX6580 was used as an outgroup to provide better branching of the tree.

Table 1. NCBI accession number and the source of sequences used
Çizelge 1. NCBI erişim numarası ve kullanılan dizilerin kaynağı

Taxa	Herbarium/ Collector Code	Collected Coordinates	GenBank accession number	Base Length (bp)
**Astragalus aucheri* Boiss.	VPH: 358; GG-2	38º.8'.300"N42º.51'.3350" E 2341 m	MW208807	687
**Astragalus bashkalensis* D.F.Chamb.	VPH: 371; GG-15	38º.10'.642"N43º.54'.770" E 2742 m	MW177874	687
**Astragalus baytopianus* D.F.Chamb. & V.A. Matthews	VPH: 361; GG-5	38º.10'.996"N43º.12'.925" E 2188 m	MW207660	687
**Astragalus bicolor* Lam. subsp. *karputanus* (Boiss. & Noë) Ponert	VPH: 377; GG-21	38º.11'.511"N43º.54'.606" E 2700 m	MW205835	687
**Astragalus cinereus* Willd.	VPH: 357; GG-1	38º.15'.30"N43º.15'.115" E 1897 m	MW199105	686
**Astragalus comosoides* D.F.Chamb. & V.A. Matthews	VPH: 370; GG-14	37º.52'.422"N43º.01'.282" E 1676 m	MW177875	686
**Astragalus cryptocarpos* D.C.	VPH: 375; GG-19	38º.55'.311"N43º.47'.303" E 1949 m	MW208691	674
**Astragalus davisii* D.F.Chamb. & V.A.Matthews	VPH: 359; GG-3	38º.8'.300"N42º.51'.3350" E 2341 m	MW207663	686
**Astragalus delanensis* Sirj. & Rech. F.	VPH: 362; GG-6	38º.94'.87"N42º.58'.506" E 2400 m	MW207666	687
**Astragalus gevashensis* D.F.Chamb. & V.A. Matthews	VPH: 365; GG-9	38º.25'.811"N42º.53'.102" E 1808 m	MW207662	687
**Astragalus gymnalopecias* Rech. F.	VPH: 366; GG-10	38º.7'.350"N43º.3'.129' E 2250 m	MW208692	686
**Astragalus hareftae* (Nab.) Sirj.	VPH: 376; GG-20	38º.10'.996"N43º.12'.925" E 2188 m	MW208693	686
**Astragalus longifolius* Lam.	VPH: 363; GG-7	38º.8'.300"N42º.51'.3350" E 2341m	MW207665	686
**Astragalus lycius* Boiss.	VPH: 368; GG-12	38º.19'.421"N43º.48'.494" E 230 m	MW221495	686
***Astragalus pendulus* D.C.	VPH: 367; GG-11	38º.08'.463"N43º.02'.249" E 2291 m	MW208694	686
***Astragalus pinetorum* Boiss. subsp *declinatus* Podlech	VPH: 374; GG-18	38º.11'.511"N43º.54'.606" E 2700 m	MW289909	669
***Astragalus pulchellus* Boiss.	VPH: 372; GG-16	38º.11'.511"N43º.54'.606" E 2700 m	MW205827	686
**Astragalus sachanewii* Sirj.	VPH: 360; GG-4	38º.91'.92"N42º.54'.161" E 3047 m	MW207664	686
**Astragalus tauricolus* Boiss.	VPH: 364; GG-8	38º.19'.168"N43º.36'.565" E 2128 m	MW207659	686
**Astragalus tournefortii* Boiss.	VPH: 369; GG-13	37º.54'.542"N42º.52'.214" E 1790 m	MW208695	686
***Astragalus xylobasis* Freyn & Bornm.	VPH: 373; GG-17	38º.8'.300"N42º.51'.3350" E 2341 m	MW207661	687

*Endemic species, **Rare species

RESULTS

In the study, 82 *Astragalus* taxa were collected from the Van Lake Basin and morphological identifications of these taxa were performed. As a result of these diagnoses, it was identified that 17 of 82 taxa were endemic and 4 were rare. It has been studied with 21 taxa, which are most common in the Van Lake Basin. Due to the similarity of their morphological characteristics, the taxa of the genus *Astragalus* are difficult to identify. When the procedures outlined in this study and systematic diagnosis were used together, the identification of these species yielded

more precise and rapid results. These analyses showed that they were sufficient for identifying and categorizing *Astragalus* taxa. The ITS sequences used, including the 5.8S gene, were identified for 21 *Astragalus* taxa and accession numbers were submitted to the gene bank (Table 1).

Secondary (2^0) structures support phylogenetic studies and are very important in terms of providing certainty in the phylogenetic identification of species. Secondary (2^0) structures determined in this way can be easily used in diagnosis. Thanks to the phylogenetic tree and secondary (2^0) structures obtained, the morphological identification of taxa has been proven to be correct and it has been confirmed that they are endemic taxa.

When the phylogenetic tree in Figure 2 is examined; It has been revealed that endemic taxa named *A. tauricolus* and *A. sachanewii* are the closest taxa to each other. It was determined that the endemic species *A. aucheri* and *A. bicolor* subsp *karputanus*, both of which are in the same branch of the phylogenetic tree, are the closest taxa to each other. Likewise, taxa named *A. pulchellus* and *A. comosoides* were determined to be very close to each other. Located in the same branch of the phylogenetic tree, *A. pinetorum* subsp *declinatus* and *A. cryptocarpus* taxa were found to be close to each other. It has been determined that *A. tournefortii* and *A. longifolius* in the phylogenetic tree are very close to each other. It was determined that the taxa called *A. lycius*, *A. bashkalensis*, *A. xylobasis*, *A. gymnalopecias*, *A. cinerous, A. pendulus, A. hareftae, A. davisii, A. baytopianus, A. delanensis, A. gevashensis,* which are located in different branches of the phylogenetic tree, were not close to each other and were located in different branches of the phylogenetic tree.

Sequence Analysis and Determination of Secondary Structures

The nucleic acid base sequences of the ITS1-ITS2 region on the rDNA of the identified species were determined and secondary structures of the rRNA of the species were created using the CLC Main Workbench 6.7.1 software (Figure 3).

DISCUSSION and CONCLUSION

In the study, 82 *Astragalus* taxa were collected from the Van Lake Basin, and it was identified that 17 of these taxa were endemic and 4 of them were rare plants. A total of 21 taxa were studied. These taxa have been systematically identified and the taxa called *A. cinerous*, *A. aucheri*, *A. davisii*, *A. sachanewii*, *A. baytopianus*, *A. delanensis*, *A. longifolius*, *A. tauricolus*, *A. gevashensis*, *A. gymnalopecias*, *A. lycius*, *A. tournefortii*, *A. comosoides*, *A. bashkalensis*, *A. cryptocarpos, A. hareftae,* and *A. bicolor* subsp *karputanus* were found to be endemic. In cases where morphological characters are insufficient, sequence analysis is very useful for phylogenetic analysis (Yokoyama et al., 2000).

Species found to be rare were *A. pulchellus*, *A. xylobasis*, *A. pinetorum* subsp *declinatus,* and *A. pendulouss*. Recent advances in molecular biology help identify plant species by identifying species-species gene regions (Baldwin et al., 1995).

Based on the ITS region of the 21 taxa used to support the systematic diagnosis, a phylogenetic tree was constructed. DNA sequences of chloroplast DNA and nuclear genome regions are used to examine the phylogenetic relationships between plant species (Ateş, 2017). In such plants as *Astragalus* that are difficult to identify, phylogenetic relationships between subgenera and genera belonging to plants are distinguished by comparative sequencing of nrDNA with ITS and 5.8S (Osaloo et al., 2003).

In addition to the phylogenetic tree, secondary structures of 21 taxa studied for further supporting data, were created with the CLC Main Workbench 6.7.1 software, and accordingly, *A. cinerous*, *A. xylobasis*, *A. davisii, A. longifolius, A. gymnalopecias, A. comosoides, A. pinetorum* subsp *declinatus*, *A. cryptocarpus* taxa were found to have different secondary structures. RNA secondary (2°) structures, with symbolic representations of base pairs, double-helices, loops, bulges, and single-strands, represent a wide spectrum of information by forming branches to understand, organize, and decompose the three-dimensional (3D) structure, folding, and function of RNA (Petrov et al., 2014). When the groups formed in the phylogenetic tree of 21 *Astragalus* taxa identified in the study and the secondary structures of these species were compared, it was determined that the secondary structures of *A. tauricolus* and *A. sachanewii*, located in the same branch of the phylogenetic tree, were very close to each other. It has been determined that the secondary structures of *A. aucheri* and *A. bicolor* subsp *karputanus*, which are in the same branch of the phylogenetic tree, are different from each other. The secondary structure of *A. aucheri* with *A. tournefortii* and *A. bicolor* subsp *karputanus* with *A. lycius* was found to be similar. It was determined that the secondary structures of *A. pulchellus* and *A. comosoides*, which are on the same branch in the phylogenetic tree, are different from each other. The secondary structure of *A. pulchellus* and *A. bashkalensis* turned out similar. It has been determined that *A. comosoides* has a unique secondary structure. Similarly, it was determined that secondary structures of *A. pinetorum* subsp *declinatus* and *A. cryptocarpos*, located on the same branch in the phylogenetic tree, formed a unique structure. Considering the secondary structure of *A. tournefortii* and *A. longifolius*, which are in the same branch in the phylogenetic tree, it has been determined that the secondary structure of *A. tournefortii* is very close to

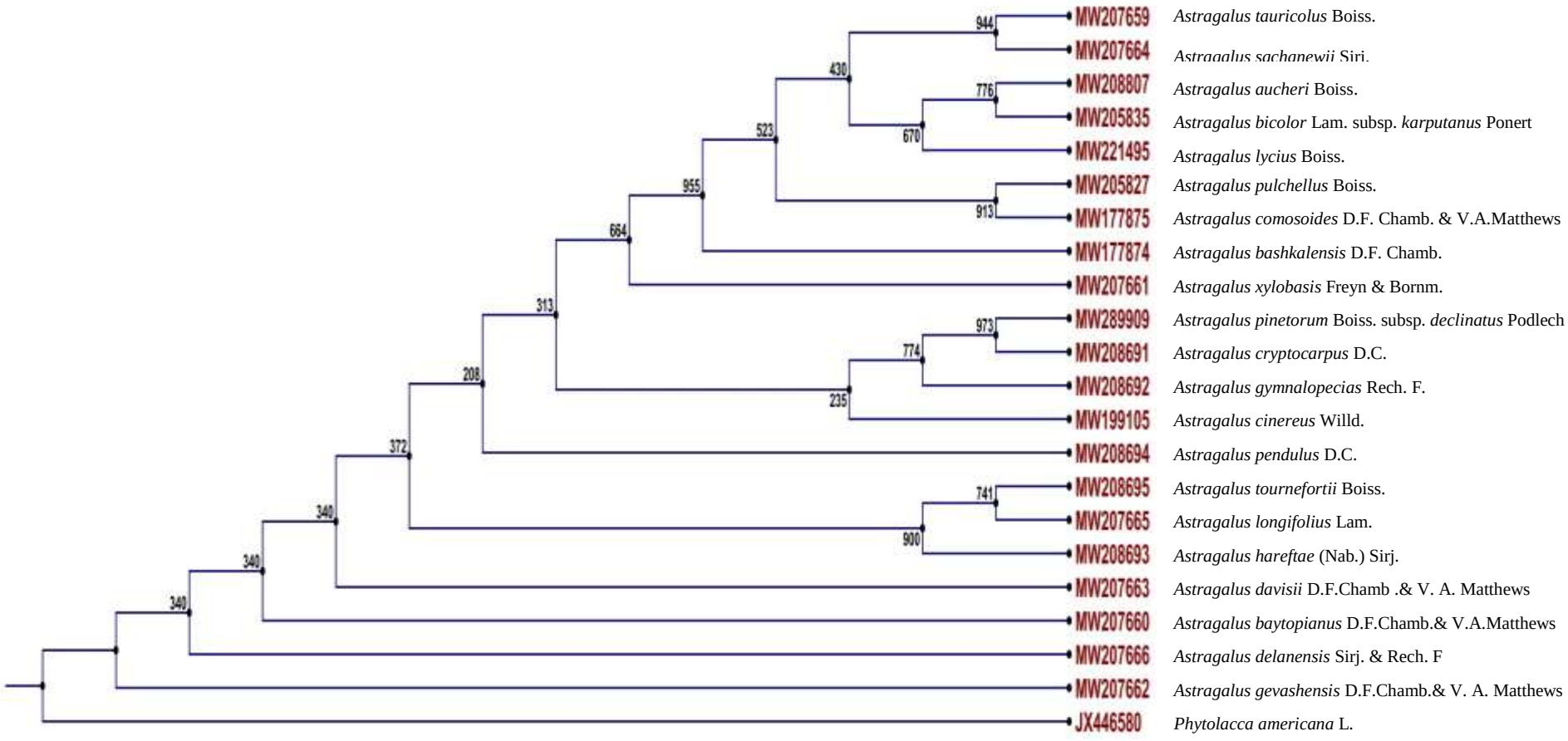

Figure 2. Phylogenetic tree of endemic taxa of the genus Astragalus obtained by CLC DNA Main Workbench 6.7.1 program
Şekil 2. CLC DNA Main Workbench 6.7.1 programı ile elde edilen Astragalus cinsinin endemik taksonlarının filogenetik ağacı.

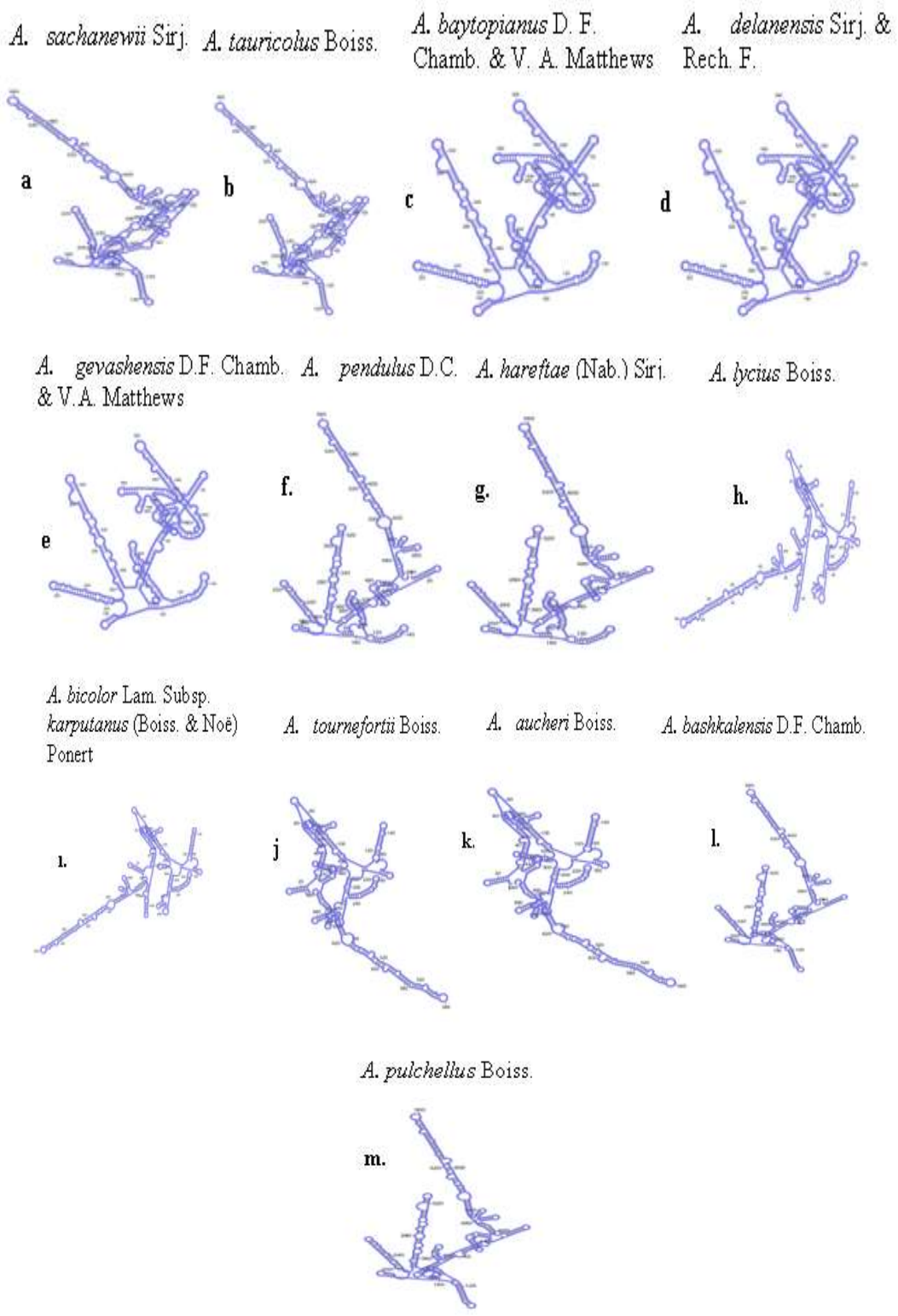

Figure 3. Secondary structures of ITS region sequences of Astragalus species: A. sachanewii (a) with A. tauricolus (b), A. baytopianus (c) with A. delanensis (d) and A. gevashensis (e), A. pendulus (f) with A. hareftae (g), A. lycius (h) with A. bicolor subsp. karputanus (i), A. tournefortii (j) and A. aucheri (k), A. bashkalensis (l) and A. pulchellus (m) secondary structures of the ITS sequences turned out to be similar.

Şekil 3. Astragalus türlerinin ITS bölgesi dizilerinin ikincil yapıları: A. sachanewii (a) ile A. tauricolus (b), A. baytopianus (c) ile A. delanensis (d) ve A. gevashensis (e), A. pendulus (f) ile A. hareftae (g), A. lycius (h) ile A. bicolor subsp. karputanus (i), A. tournefortii (j) ve A. aucheri (k), A. bashkalensis (l) ve A. pulchellus (m) ikincil yapılar birbirine benzer çıkmıştır.

that of *A. aucheri*. It was revealed that the secondary structure of *A. longifolius* formed a unique structure. Accurate and easily accessible secondary structures are essential for comprehending ribosomes, which are enormously large and highly complex three-dimensional objects (Petrov et al., 2014).

As a result, with this study, a total of 82 plant samples belonging to the genus *Astragalus*, were collected in the Van Lake Basin and their systematic identification was made. After a series of molecular processes, the degree of kinship between species was determined by phylogenetic analysis methods and their secondary (2°) structures have been researched. As a result, the relative positioning status and secondary structures of the species in the phylogenetic tree were evaluated, and it was found that the results were consistent with one another. At the same time, it was discovered that the nrDNA ITS region of plants ranged in length from 669 to 687 base pairs. This finding was lengthy enough to offer enough data for species identification.

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Contribution Rate Statement Summary of Researchers

The authors declare the contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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