Research Article

DNA barcoding and phylogenetic analysis of endemic *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* (Fabaceae): Evidence from nrDNA ITS and cpDNA *mat*K and *rbc*L sequences

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Abstract

In this study, we performed DNA barcoding and phylogenetic analysis using one nuclear (ITS) and two chloroplast DNA regions (*mat*K and *rbc*L) of endemic *Astragalus nezaketiae* A. Duran & Aytaçand *Vicia alpestris* Stev. subsp. *hypoleuca* (Boiss.) Davis taxa in Turkey. PCR reactions were performed using universal primers. Sequences of the PCR products were edited using BioEdit and FinchTV software and contigs were obtained. All contigs were Blasted at NCBI and similarities were analysed. Using the MEGA 6.0 program, maximum likelihood trees were constructed including some sequences retrieved from NCBI. For *Astragalus nezaketiae*; in the ITS analysis, *Astragalus nezaketiae* appeared separately from other species, and for matK, *Astragalus nezaketiae* appeared together with *Astragalus cicer* L. However, *rbc*L tree was polytomic. For *Vicia alpestris* subsp. *hypoleuca*; in ITS, *rbc*L and *mat*K results *Vicia alpestris* subsp. *hypoleuca* were found together with *Vicia cracca* L., *Vicia benghalensis* L. and *Vicia villosa* Roth species. Analysis of the combined data revealed similar results with all barcode regions for *Vicia alpestris* subsp. *hypoleuca* while different phylogenetic results were obtained for *Astragalus nezaketiae*.

Keywords: *Astragalus;* barcoding; ITS; *mat*K; *rbc*L; *Vicia*

Introduction

Fabaceae (Leguminosae), the third largest family within the Angiosperms, includes 946 genera and over 24,500 accepted species (Manzione *et al.*, 2022). The Fabaceae, one of the herbaceous, shrub, woody and climbing plants with a cosmopolitan distribution, is one of the greatest flowering plant families in Turkey after Asteraceae with respect to number of species, and features 1013 species belonging to 71 genera (Toksoy *et al.*, 2015; Ilcim and Behçet, 2016; Antonio-Domingues *et al.*, 2018). In addition, it consists of three subfamilies; Caesalpinioideae, Mimosoideae, and Papilionoideae (Han *et al.*, 2021a). Also known as the legume, pea or bean family, Fabaceae is an enormous and economically important member of flowering plants (Rahman and Parvin,

2014). Additionally, ethnobotanical studies have reported that some taxa belonging to the Fabaceae family are used in folk medicine, along with providing tree resources and dyes, resins, insecticides, fibers, feed, and the like, from a socioeconomic point of view (Molares and Ladio, 2012; Zengin *et al*., 2015). *Astragalus* L. is the largest genus of vascular plants with nearly 2900 species. In Turkey, it is represented by 483 taxa which are divided into 63 sections (Karaman Erkul *et al*., 2022). *Astragalus* is widely distributed in the steppe environment of low or high mountains in the Irano-Turanian phytogeographic region of Turkey (Duran and Aytaç, 2005; Atasagun *et al.,* 2021). Some species of *Astragalus* are used in farm and wild animal feed, foods, medicines, cosmetics, and as tea or as a source of herbal gum (Rios and Waterman, 1997). Furthermore, the medical use of the *Astragalus* species dates back ten centuries. In recent years, progress has been made in research on these species due to their anticancer compounds and medical uses (Ionkova *et al*., 2014). The genus *Vicia* L. belongs to the Fabaceae family and is the third largest family of flowering plants worldwide, containing about 210 species, with 22 divisions in two subgenera (*Vicia* and *Vicilla*), distributed throughout the temperate regions of Europe, Asia, and North and South America. In Turkey, 64 species, 22 subspecies, and 18 varieties of this genus have been recorded (Han *et al*., 2021b; Kaplan *et al*., 2021a, Kaplan *et al*., 2021b). The highest specific diversity of *Vicia* is found in Turkey and northwest Asia (Martin *et al*., 2018). The *Vicia* species, which are agriculturally and economically important, protein-rich, and valuable genetic resources, are used as high-quality green manure, medicine, food, cover, fodder, and ornamental and honey plants to reduce the occurrence of pests and diseases and increase soil fertility (Han *et al*., 2021b; Wu *et al*., 2021; Wu *et al*., 2020; Jo *et al*., 2022; Sun *et al*., 2022).

Plant molecular systematics has been developing rapidly, especially in the last 20 years. This development has started to contribute to molecular systematics with the use of DNA and amino acid sequence analysis and the discovery of new phylogenetic analysis methods (Inal and Karaca, 2019). The concept of "DNA barcode", which has been widely used in the literature, was first introduced by Hebert *et al*. (2003) and later managed to attract the attention of the science world (Filiz and Koç, 2012; Keskin and Atar, 2013). Thanks to this technique, it is easier to distinguish between species, and the identification process is quicker and more accurate (Koohdar *et al*., 2021). nrDNA ITS (internal transcribed spacer) and chloroplast DNA (*rbc*L, *atp*F-H, *rpo*B, *rpo*C1, *ndh*F, *mat*K, *trn*H-*psb*A, *rps*16-*trn*Q, *rpl*32-*trn*L and *trn*L-F) sequences are used frequently in molecular phylogenetic analyses of plants and barcoding (Yang *et al*., 2010; Filiz and Koç, 2012; Sun *et al*., 2012; Dong *et al*., 2012; Wang *et al*., 2013; Techen *et al*., 2014; Lin *et al*., 2015; Dastpak *et al*., 2018; Kim *et al*., 2020; Inal and Karaca, 2019). In this study, we conducted DNA barcoding and phylogenetic analysis using one nuclear (ITS) and two chloroplast DNA regions (*mat*K and *rbc*L) of endemic *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* taxa distributed in Turkey.

Materials and Methods

Study area and genomic DNA isolation

The study materials included the specimens of *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* taxa. Taxa and the locations they are collected from are presented in Table 1. For genomic DNA isolation, a commercial kit (GeneMark Catalog No: DP022) was used.

Table 1. Location of endemic *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* in Turkey

Taxa	Location and herbarium number
Astragalus nezaketiae	Tunceli province, Pülümür district, Bağırpaşa Mountain, calcareous rocky slopes, 2600 m a.s.l, 20 August 2019, *Başköse and Paksoy 3004*
Vicia alpestris subsp. *hypoleuca*	Tunceli province, Pülümür district, Bağırpaşa Mountain, calcareous rocky slopes, 2600 m a.s.l, 20 August 2019, *Başköse and Paksoy 3005*

PCR amplifications, sequencing and phylogenetic analysis

Primer sequences, PCR components and PCR protocols for amplifying the nrDNA ITS, cpDNA *mat*K and *rbc*L regions via PCR are given in Table 2.

Table 2. Primers, PCR components and PCR protocols

Primer name, 5'-3' sequences and references	PCR components	PCR Protocols
ITS5AF: 5'-CCTTATCATTTAGAGGAAGGAG-3' (White *et al.,* 1990) ITS4R: 5'-TCCTCCGCTTATTGATATGC-3' White *et al.,* 1990)	1 µL genomic DNA 1 µL primer (forward), 1 µL primer (reverse), 5 µL master mix (PCR buffer, 2 Mm MgCl$_2$, dNTP, 0.75 U Taq DNA polymerase) and 17 µL dH$_2$O	94°C, 5 min; 35× (94°C, 45 s., 50°C, 45 s., 72°C, 1 m.), 72°C, 10 min.
*mat*K-472f: 5'CCCRTYCATCTGGAAATCTTGGTTC-3' (Yu *et al.,*2011) *mat*K-1248r: 5'-GCTRTRATAATGAGAAAGATTTCTGC-3' Yu *et al.,*2011)	1 µL genomic DNA 1 µL primer (forward), 1 µL primer (reverse), 5 µL master mix (PCR buffer, 2 Mm MgCl$_2$, dNTP, 0.75 U Taq DNA polymerase) and 17 µL dH$_2$O	94°C, 4 min; 35× (94°C, 1 min; 48°C, 30 s; 72°C, 1 min); 72°C, 7 min.
*rbc*La-F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' (Levin, 2013) *rbc*La-R: 5'-GTAAAATCAAGTCCACCRCG-3' (Kress *et al.,* 2009)	1 µL genomic DNA 1 µL primer (forward), 1 µL primer (reverse), 5 µL master mix (PCR buffer, 2 Mm MgCl$_2$, dNTP, 0.75 U Taq DNA polymerase) and 17 µL dH$_2$O	95°C, 1 min; 35× (95°C, 30 s; 51°C, 30 s; 68°C, 1 min); 68°C, 5 min.

PCR products were sent to the TRIOGEN (Istanbul/Turkey) biotechnology company for service procurement of their distillation and cycle sequencing reactions. The BioEdit 7.2.3 (Hall, 1999) and Finch TV version 1.4.0 programs were used in processing the DNA sequences which came as files in the *ABI* prism format. A contig was created using forward and reverse sequences related to each species. Specific bases misread by a device performing the sequencing reactions were visually corrected with the help of the Bioedit 7.2.3 and Finch TV version 1.4.0 programs based on the strength and cleanness of the signals (peaks) in the chromatogram. By this way, the contig sequences were obtained. The MEGA 6.0 program was used for aligning the sequences (Tamura *et al.*, 2013). To evaluate the degree of support for clades, a bootstrap analysis (1,000 replicates) was applied (Felsenstein, 1985). In order to find out the phylogenetic relationships of the species whose sequences were obtained, MEGA 6.0 which is commonly used in biotechnology, molecular biology, genetic and bioinformatic research, was used. Data such as phylogenetic trees, genetic distance and nucleotide distance were acquired using the parameters and species in these programs.

Results and Discussion

The nrDNA ITS sequence of flowering plants is recommended as a core DNA barcode (Zhu *et al.*, 2021) as it has high discriminatory power to distinguish closely related species. In plant phylogenetic studies of

closely related species and populations, this region is preferred by molecular taxonomists (Dizkirici and Koroglu, 2018). The ITS sequence length of the endemic *Astragalus nezaketiae* species was 671 bases, and the nucleotide composition was determined as 22.70% adenine, 25.50% cytosine, 27.90% guanine and 24.00% thymine. The maximum likelihood phylogenetic tree was constructed including ITS sequences of some *Astragalus* species obtained from NCBI. *Astragalus nezaketiae* species was separated from other species (Figure 1). The ITS sequence length of the endemic *Vicia alpestris* subsp. *hypoleuca* species was 680 bases, and the nucleotide composition was determined as 22.10% adenine, 24.10% cytosine, 27.40% guanine and 26.50% thymine. The maximum likelihood phylogenetic tree constructed including ITS sequences of some *Vicia* species from NCBI, consisted of two clades (Figure 2). *Vicia alpestris* subsp. *hypoleuca* species coexisted with *Vicia cracca*, *Vicia benghalensis* and *Vicia villosa* in subclade A, and this branch received a bootstrap value of 94%.
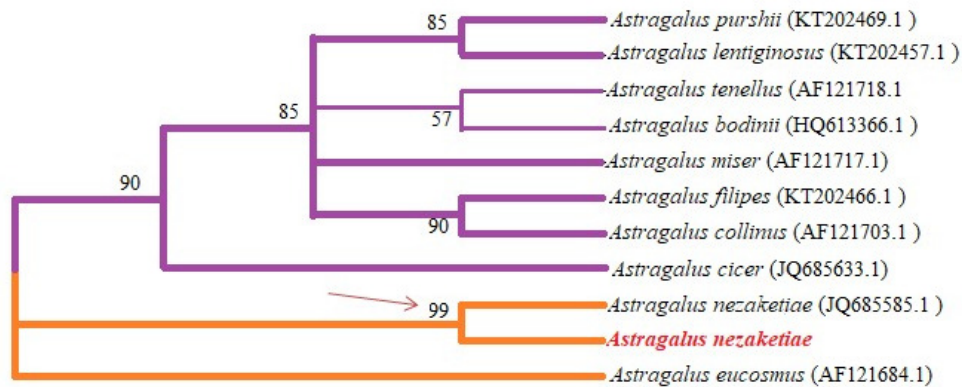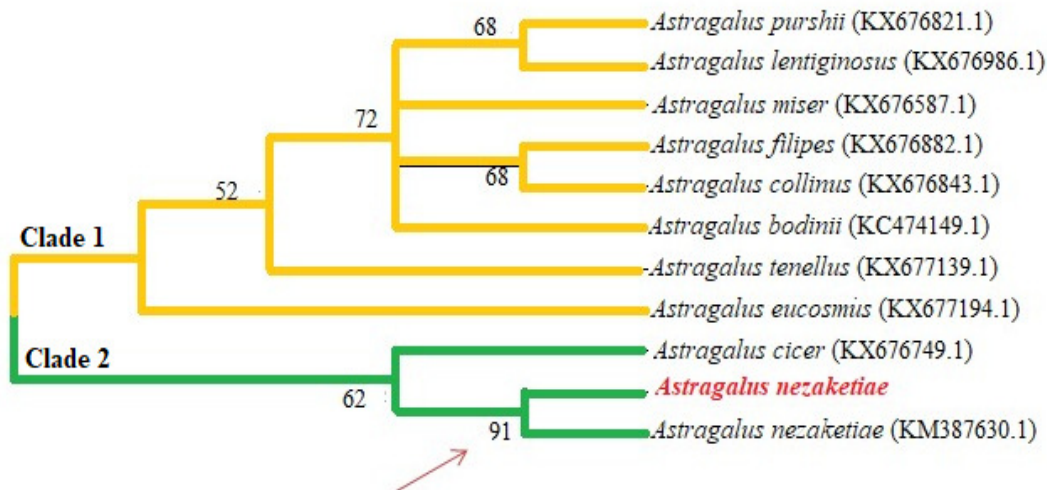

Figure 1. The maximum likelihood tree generated using nrDNA ITS sequences of *Astragalus nezaketiae* and other *Astragalus* sequences retrieved from NCBI
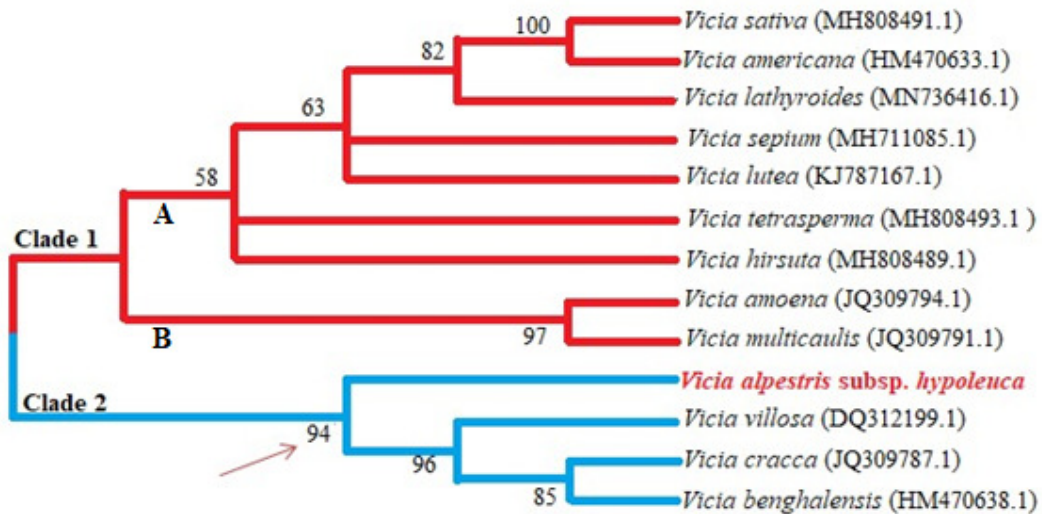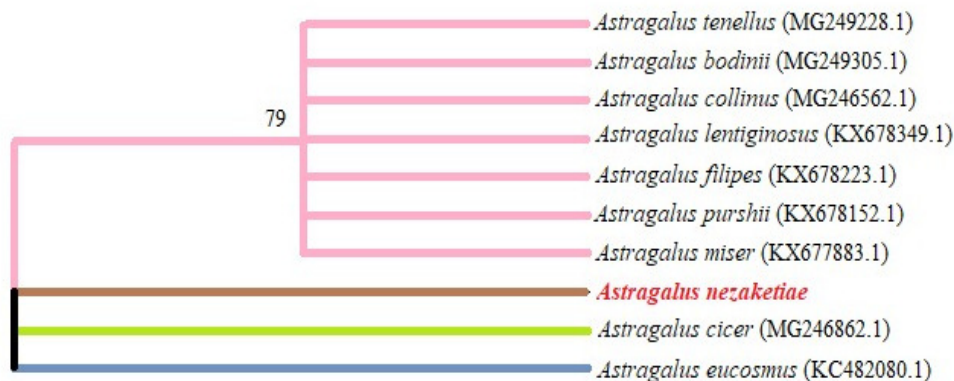

Figure 2. The maximum likelihood tree generated using nrDNA ITS sequences of *Vicia alpestris* subsp. *hypoleuca* and other *Vicia* sequences retrieved from NCBI

Advances in DNA sequencing techniques have allowed the studies of phylogenetic relationships with the widespread use of short DNA fragments especially of the chloroplast genome (Penjor *et al.,* 2010). DNA barcoding relies on information encoded in the nucleotide sequences of a genome's standard region as a tool for species identification. The CBOL working group proposed Rubisco large subunit (*rbc*L) and maturase K (*mat*K) as the standard plant barcode based on sequence quality and species separation levels (CBOL Plant Working Group, 2009; Bafeel *et al.*, 2011). The *mat*K sequence length of the *Astragalus nezaketiae* was 738 bases, and the nucleotide composition was determined as adenine 30.10%, cytosine 15.90%, guanine 15.40% and thymine 38.60%. Including *mat*K sequences of some *Astragalus* species from NCBI, a maximum likelihood phylogenetic tree composed of two clades was constructed (Figure 3). *Astragalus nezaketiae* and *Astragalus cicer* were placed in clade 2 which was supported with a 62% bootstrap value. The *rbc*L sequence length of *Astragalus nezaketiae* was 546 bases, and the nucleotide composition was determined as adenine 26.70%, cytosine 20.10%, guanine 23.30% and thymine 29.90%. In a maximum likelihood phylogenetic tree constructed including *rbc*L sequences of some *Astragalus* species from NCBI, *Astragalus nezaketiae* was found to be polytomic (Figure 4).
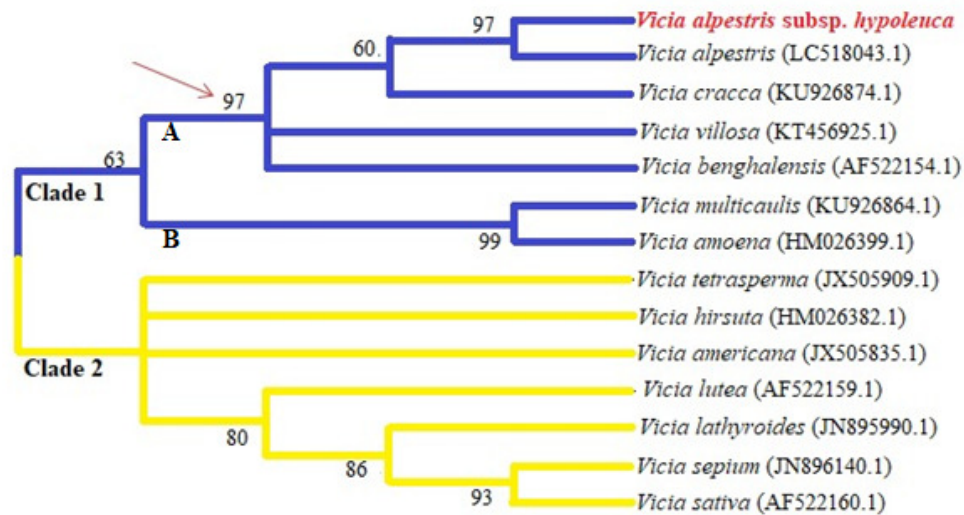

Figure 3. The maximum likelihood tree generated using cpDNA *mat*K sequences of *Astragalus nezaketiae* and other *Astragalus* sequences retrieved from NCBI
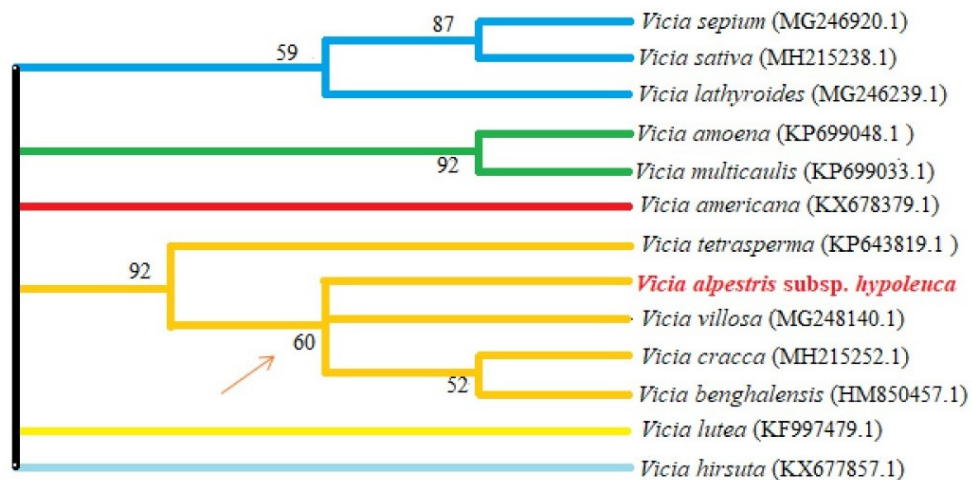

Figure 4. The maximum likelihood tree generated using cpDNA *rbc*L sequences of *Astragalus nezaketiae* and other *Astragalus* sequences retrieved from NCBI

The *mat*K sequence length of the *Vicia alpestris* subsp. *hypoleuca* species was 739 bases, and the nucleotide composition was determined as 38.84% adenine, 15.43% cytosine, 16.24% guanine and 29.50% thymine. The maximum likelihood phylogenetic tree constructed including *mat*K sequences of some *Vicia* species from NCBI consisted of two clades (Figure 5). *Vicia alpestris* subsp. *hypoleuca* species was placed with *Vicia alpestris, Vicia cracca, Vicia benghalensis* and *Vicia villosa* species in group A, and this group received a bootstrap value of 97%. The *rbc*L sequence length of *Vicia alpestris* subsp. *hypoleuca* was 546 bases, and the nucleotide composition included Adenine 28.2%, cytosine 20.1%, guanine 22% and thymine 29.7%. In the maximum likelihood phylogenetic tree constructed including the *rbc*L sequences of some *Vicia* species from NCBI, *Vicia alpestris* subsp. *hypoleuca* found in the same group with *Vicia cracca, Vicia benghalensis* and *Vicia villosa*, and this group received a 60% bootstrap value (Figure 6).

Figure 5. The maximum likelihood tree generated using cpDNA *mat*K sequences of *Vicia alpestris* subsp. *hypoleuca* and other *Vicia* sequences retrieved from NCBI

Figure 6. The maximum likelihood tree generated using cpDNA *rbc*L sequences of *Vicia alpestris* subsp. *hypoleuca* and other *Vicia* sequences retrieved from NCBI

Dizkirici *et al.* (2014) in ITS analysis study have determined *Astragalus nezaketiae* together with *Astragalus humillimus* Barneby*, Astragalus sanguinolentus* M. Bieb.*, Astragalus glaucophyllus* Bunge*, Astragalus achundovii* Grossh. and *Astragalus schizopterus* Boiss. species. A cpDNA *mat*K analysis study of Tekpinar *et al.* (2016) reported *Astragalus nezaketiae* together with *Astragalus czorochensis* Kharadze*, Astragalus humillimus, Astragalus sanguinolentus* and *Astragalus schizopterus* species. Kaplan *et al.* (2021a) in their cpDNA *trn*L intron UPGMA analysis determined *Vicia sativa* subsp. *sativa, Vicia sativa* subsp. *nigra* var. *nigra, Vicia sativa* subsp. *amphicarpa, Vicia lathyroides* L. in a group (bootstrap 100%), while *Vicia lutea* var, *hirta, Vicia villosa* subsp. *varia, Vicia villosa* subsp. *eriocarpa* and *Vicia cracca* subsp. *stenophylla* within a group identified In our ITS, *rbc*L and *mat*K analysis results, the *Vicia alpestris* subsp. *hypoleuca, Vicia cracca, Vicia benghalensis* and *Vicia villosa* were together determined. Kaplan *et al.* (2021b) in their ITS analysis found that the *Vicia sativa* subsp. *sativa, Vicia sativa* subsp. *amphicarpa, Vicia sativa* subsp. *nigra* and *Vicia lathyroides* were together, whereas the *Vicia villosa* subsp. *varia, Vicia villosa* subsp. *eriocarpa* and *Vicia cracca* subsp. *stenophylla* and *Vicia lutea* var. *hirta* were in a different group. In the NJ phylogenetic tree generated using ITS2 + *mat*K + *psb*A-*trn*H + *rbc*L sequences, Raveendar *et al.* (2017) determined the *Vicia sativa* and the *Vicia hirsuta* (L.) Grey within a group (bootstrap 100%), *Vicia tetrasperma* and *Vicia articulate* within a group (bootstrap 100%), the *Vicia lutea* L. and *Vicia peregrina* L. within another group (bootstrap 99%) and *Vicia cracca, Vicia benghalensis,* and *Vicia villosa* in another group (bootstrap 94%). Bozkurt *et al.* (2013) in their ISSR analysis determined *V. sativa* subsp. *sativa, V. sativa* subsp. *nigra* var. *nigra, V. sativa* subsp *incisa* (M. Bieb.) Arcang. var. *cordata* and *V. peregrina* and *V. hybrida* species within a group and the *V. cracca* subspecies together with the *V. palaestina* Boiss. Endo *et al.* (2010), in an ITS analysis performed with the Bayesian tree method determined the *Vicia cracca* and *Vicia benghalensis* species (bootstrap 100%) within a group and the *Vicia sativa* var. *angustifolia, Vicia sativa* and *Vicia americana* Muhl. ex Willd within another group (bootstrap 99%). In the cpDNA *mat*K analysis (Bayesian tree), they determined the *Vicia hirsuta* and *Vicia articulata* species within a group (bootstrap 100%); *Vicia lutea, Vicia sativa* and *Vicia americana* together, and the *Vicia villosa* and *Vicia benghalensis* species within a separate group (bootstrap 100%).

Conclusions

In this report, we studied endemic *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* species distributed in Turkey to run their phylogenetic analysis using ITS, *ma*tK and *rbc*L sequences as barcode regions. Of these barcode regions, only the *rbc*L region did not yield efficient results for *Astragalus nezaketiae*. For *Vicia alpestris* subsp. *hypoleuca,* the phylogenetic analysis of the ITS*, mat*K *and rbc*L barcode regions were congruent. We believe the result of this study will guide the further phylogenetic analyses of *Astragalus nezaketiae* and *Vicia alpestris* subsp. *hypoleuca* species.

Authors' Contributions

M.Y.P and İ.B collected plants samples. The experiments were performed and analyzed by E.S. E.S wrote the paper. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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