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Astragalus L. Cinsinin Bazı Taksonlarındaki Genetik Çeşitliliğin RAPD Tekniği ile Araştırılması: Koruma ve Taksonomiye Yönelik Çıkarımlar

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ÖZ

Astragalus L., Avrasya ve Amerika'da bulunan, yaklaşık 3092 türden oluşan, dünya çapında en büyük damarlı bitki cinsi olarak kabul edilmektedir. Türkiye 63 farklı taksonomik seksiyona ayrılmış 493 türe ev sahipliği yapmaktadır. Bu çalışma, *Astragalus* cinsine ait seksiyonlar üzerinde yürütülen sınırlı sayıdaki moleküler çeşitlilik çalışmalarına odaklanmaktadır. Çalışmada öncelikli olarak Türkiye'de doğal yayılış gösteren 10 (on) *Astragalus* taksonu üzerinde çalışıldı. Genetik polimorfizmlerin varlığını değerlendirmek için Rastgele Çoğaltılmış Polimorfik DNA (RAPD) tekniği kullanıldı. Elde edilen filogenetik ilişki sonuçlarına göre *Anthylloidei* DC (*A. anthylloides*, *A. halicacabus* ve *A. ermineus*), *Macrophyllium* Bunge (*A. oleaefolius* ve *A. dipodurus*), *Hymenostegis* Bunge (*A. lagopoides*, *A. velenowskyi* ve *A. hirticalyx*) yakından ilişkili bulunurken, *Poterion* Bunge (*A. russelli*) ve *Hymenocoleus* Bunge (*A. vaginans*) seksiyonlarının farklı konumlar olduğu tespit edilmiştir. Çalışmamız, *Astragalus* taksonlarının moleküler karekterizasyonuna katkıda bulunmayı, moleküler biyoçeşitlilik envanterlerini oluşturmayı ve filogenetik ilişkileri belirlemeyi amaçlamaktadır. Genetik çeşitlilik ve filogenetik pozisyonlarına ilişkin bu bulgular, gelecekteki araştırmalar için önemli çıkarımlara sahiptir ve geleneksel tıp, tarım ve peyzaj dahil olmak üzere birçok alanda ekonomik önemi olan *Astragalus* türlerini vurgulamaktadır.

A RAPD-Based Exploration of Genetic Variation in the Some taxa of *Astragalus* L. Genus: Implications for Conservation and Taxonomy

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ABSTRACT

Astragalus L. is regarded as the most extensive genus of vascular plants globally, encompassing around 3092 species and distributed across Eurasia and the America. Türkiye hosts 493 species categorized into 63 distinct taxonomic groups. This study examines the restricted molecular diversity studies pertaining to the sections of the genus *Astragalus*. The study primarily investigated ten (10) *Astragalus* taxa native to Türkiye. The Randomly Amplified Polymorphic DNA (RAPD) method was utilized to evaluate the existence of genetic polymorphisms. The phylogenetic analysis revealed that the sections *Anthylloidei* DC (*A. anthylloides*, *A. halicacabus*, and *A. ermineus*), *Macrophyllium* Bunge (*A. oleaefolius* and *A. dipodurus)*, and *Hymenostegis* Bunge *(A. lagopoides*, *A. velenowskyi*, and *A. hirticalyx*) are closely related,

whereas the sections *Poterion* Bunge (*A. russelli*) and *Hymenocoleus* Bunge (*A. vaginans*) are situated in distinct locations. This study seeks to enhance the molecular characterization of *Astragalus* taxa, create molecular biodiversity inventories, and elucidate phylogenetic relationships. The results concerning genetic diversity and evolutionary relationships bear substantial implications for future research and underscore *Astragalus* species that possess economic significance in diverse domains, such as traditional medicine, agriculture, and landscaping.

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1.Introduction

The genus *Astragalus* L. is recognized as the largest genus of vascular plants, comprising an estimated 3092 species in the world (POWO, 2025). It has a dual distribution pattern, mostly concentrated in two major regions of the globe, namely Eurasia (Old World) and America (New World). The majority of the species are distributed in the Old World, comprising over 2400 species (Chaudhary et al. 2008; Zarre and Azani 2013). According to Chaudhary et al. (2008), Anand (2010) and Zarre and Azani (2013), there is a restriction of 500 species to the New World. The genus *Astragalus* has 493 taxa within 63 sections in Türkiye (Aytaç et al. 2012; Taeb and Uzunhisarcıklı 2012; Dinç et al. 2013; Karaman Erkul and Aytaç 2013; Çeçen et al. 2016; Karaman Erkul et al. 2016; İlçim and Behçet 2016; Dönmez and Uğurlu Aydin, 2018; Hamzaoğlu, 2020; Tunçkol et al. 2020; Aytaç et al. 2020; Duman et al. 2020; Uzun et al. 2021; Karaman et al., 2022; Karaman et al. 2023; Hamzaoğlu 2024; Fırat 2024a, 2024b).

In contemporary times, the task of comprehending taxonomic relationships through morphological characteristics has grown more challenging. Therefore, the importance of molecularly derived phylogenetic trees has increased, as they facilitate the comprehension of taxonomic relationships. Chloroplast DNA is a significant source of data for genetic and phylogeographic investigations in plant populations due to its characteristics of ancestral inheritance, haploidy, and widespread recombination (Perdereau et al., 2017). Previous research on the genus *Astragalus* has predominantly involved the integration of morphological studies to assess genetic distances among species, identify variations, and discover novel species (Zhang and Jiang, 2020; Martinez, 2024).

In recent times, molecular techniques have been increasingly employed in problems with taxonomic characteristics to ascertain the diversity of plant species, yielding significant outcomes. In particular, molecular characteristics are more dependable than morphological characteristics due to their immunity to environmental influences. By incorporating fingerprint techniques, particularly the RAPD method, among the species of the genus *Astragalus*, this study is expected to establish a new advancement in the scientific literature. Through the dissemination of acquired results, data derived from the existing body of literature will be revised and will serve as a foundation for subsequent investigations. In subsequent analyses, alternative methodologies will be employed to assess the biodiversity of species within the genus *Astragalus*, and the polymorphism rates of those species that have been quantified with respect to biodiversity will be ascertained.

The Random Amplified Polymorphic DNA approach (RAPD) is commonly employed in numerous molecular investigations across various plant species for the purpose of identifying genetic polymorphism (Vos et al., 1995; Karp et al., 1997). PCR-based molecular investigations play a crucial role in the identification and characterization of genetic variation, particularly at the inter-species and inter-genus levels. Polymorphism diagnosis benefits from their utilization, particularly in the accurate determination of primers employed, hence enhancing the overall quality of molecular investigations (Nebauer et al., 1999). The RAPD technique will be employed to ascertain the polymorphism rates of ten taxa within the genus *Astragalus* that are naturally distributed in Türkiye. Additionally, a molecular phylogenetic relation will be established between these taxa. Thus, molecular records will be annotated in the biodiversity records of naturally distributed *Astragalus* species found in the literature.

The primary objective of this study is to comprehensively evaluate both molecular and morphological data in the aspect of conserving plant diversity in Türkiye. Our research is dedicated to establishing and safeguarding molecular biodiversity inventories for naturally occurring species. Using the RAPD technique, we aim to document the diversity of the genus *Astragalus,* a members of economically significant Fabaceae family. Economic importance in *Astragalus* used as a traditional Chinese medicine (Huang et al., 2019), a valuable forage for livestock (Rao et al., 2018), a soil fertilitizer in agriculture (Jafari et al., 2018), a stabilizer in soil with their deep root systems (Eldridge et al., 2015), to an ornamental plant in horticulture and landscaping (Ranney et al., 2014). Consequently, these economically significant taxa will not only be classified based on morphological attributes but also on molecular characteristics. Furthermore, this study aims to determine the phylogenetic positions and relationships among *Astragalus* taxa, contributing to the molecular characterization of valuable genetic resources.

2.Materials and Methods

Samples belonging to 10 *Astragalus* taxa from different regions of Türkiye were collected mainly from their natural habitats as a part of project by S. Karaman (TÜBİTAK Project No: TBAG 110 T 911). The field works were carried out from early spring to mid-summer of 2014. In the field, the shoots with fresh leaves were collected and preserved in package filled with silica gel for molecular analyses, while a large branch with a leaf and fruit was taken and pressed for morphometric analyses. All samples were conserved at GAZI, AKSU and Tuna Ekim herbaria for collections. Information on the samples and their locations was presented in detail in Table 1.

Table 1. Information on *Astragalus* samples and their locations

No	Section	Taxon	Endemism	Turkish Name	Province
1	*Anthylloidei* DC.	*Astragalus anthylloides* Lam.	-	*Torbalı geven*	Ankara
2	*Anthylloidei* DC.	*A.halicacabus* Lam.	-	*Sepet geveni*	Erzurum
3	*Anthylloidei* DC.	*A. ermineus* V.A.Matthews	endemic	*Sansar geveni*	Van
4	*Poterion* Bunge	*A. russelii* Banks & Sol.	-	*Ballan*	Şanlıurfa
5	*Macrophyllium* Bunge	*A. oleaefolius* DC.	-	*Deli geven*	Aksaray
6	*Macrophyllium* Bunge	*A. dipodurus* Bunge	-	*Gürbüz geven*	Konya
7	*Hymenostegis* Bunge	*A. lagopoides* Lam.	-	*Som geven*	Van
8	*Hymenostegis Bunge*	*A. hirticalyx* Boiss. & Kotschy ex Boiss.	-	*Tüylüçanak*	Van
9	*Hymenostegis* Bunge	*A.velenovskyi* Nábělek	endemic	*Berçelan geveni*	Van
10	*Hymenocoleus* Bunge	*A. vaginans* DC.	endemic	-	Niğde

Table 2. Information about the primers used in the study (Anand et al., 2010)

No	Primer name	Sequence	Total band number	The number of polymorphic band	The percentage of polymorhism
1	OP-B08	GTCCACACAG	24	24	100
2	OP-B09	TGGGGGACTC	25	25	100
3	OP-C05	GATGACCGCC	21	21	100
4	OP-C15	GACGGATCAG	20	20	100
5	OP-N06	GAGACGCACA	26	26	100
6	OP-N08	ACCTCAGCTC	27	27	100
7	OP-N17	CATTGGGGAG	32	32	100
8	OP-N18	GGTGAGGTCA	24	24	100
9	OP-N19	GTCCGTACTG	27	27	100
10	OP-N20	GGTGCTCCGT	26	26	100

Total DNA was isolated from leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle, 1987). The presence and quality of the DNA were assessed using a spectrophotometer. The diluted DNA (10 ng/µl) was stored at 4ºC. The genetic diversity among individuals of the *Astragalus* genus belonging to 10 different taxa (Table 1) was tried to be determined using the RAPD technique. 10 primer markers amplified from different regions were studied according to their polymorphism rates (Table 2).

Initially, regional-specific optimization studies were conducted. To guarantee accurate amplification of the DNA region, it is critical in molecular research to optimize PCR mixture solutions and PCR cycling

conditions. Scanning of DNA followed the clarification and optimization of the temperature and PCR mixtures of the region. By manipulating their concentrations, primers for the region chosen for the PCR method were produced. By utilizing PCR mix, which comprises all enzymes and essential buffer solutions, the barcode region of DNA samples was amplified by PCR. It was determined whether the intended region had been amplified by loading the amplified PCR products onto an agorose gel using agorose gel electrophoresis. PCR products are inserted into wells that have been accessed utilizing combs, while the polymerized gel is immersed in the tank containing the buffer solution. A voltage range of 80-100V is utilized to operate agarose gel for a specified duration. Bands of the amplified DNA products are observed under ultraviolet light once electrophoresis is complete.

The aim of the RAPD technique is to determine the polymorphism rates in randomly cut regions. For this reason, the detection of polymorphic fragments begins with the counting of the bands in the PCR products carried out on agarose gel. If any type of band is present in the amplified profiles, it is coded as "1", otherwise it is coded as "0". The polymorphism rate of each primer is manually calculated after these bands are calculated and formulated as 'Polymorphism % = (Np/Nt) ×100. Here, Np gives the total number of polymorphic bands and Nt gives the total number of bands. Genetic association is calculated after the detection of polymorphic bands. Also, clear and visible amplified band numbers are sufficient for genetic relationship analysis. Manually, the GD (genetic diversity) ratio is made according to the following formula applied by Nei and Li (1979): G.D = 1-{2Nab/(Na + Nb)}. Where Nab: total number of bands seen in all individuals; Na: number of bands in individual a; Nb: is the number of bands in individual b. Genomic diversity rates are represented by the matrix resulting from these calculations. Today, clustering analyses are utilized to generate GD trees. The programs with the UPGMA method, which was developed by Sneath and Sokal in 1973, is particularly prevalent in these investigations. In the current study, The Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 2.1 application was utilized for all analyses (Rohlf, 2004). The genetic diversity among the resultant trees and 10 distinct species was assessed by employing polymorphic bands.

3.Result and Discussion

The Polymerase Chain Reaction (PCR) technology was employed to evaluate the efficacy of 10 distinct primers across a set of 10 distinct species. In the PCR protocol, the amplification process commenced with an initial denaturation step at a temperature of 94°C for a duration of 5 minutes. This was followed by a total of 45 cycles, each consisting of three steps: denaturation at 94°C for 1 minute, annealing at 35°C for 1 minute, and extension at 72°C for 1 minute. Finally, a final extension step was programmed at 72°C for 5 minutes. The cycle was repeated for each primer. The results indicate that band profiles were obtained using the utilization of the electrophoresis technique, specifically employing a 2% agarose gel. The bands were enumerated individually and subsequently organized for analysis within an excel spreadsheet (Figure 1, 2, 3, 4, 5).

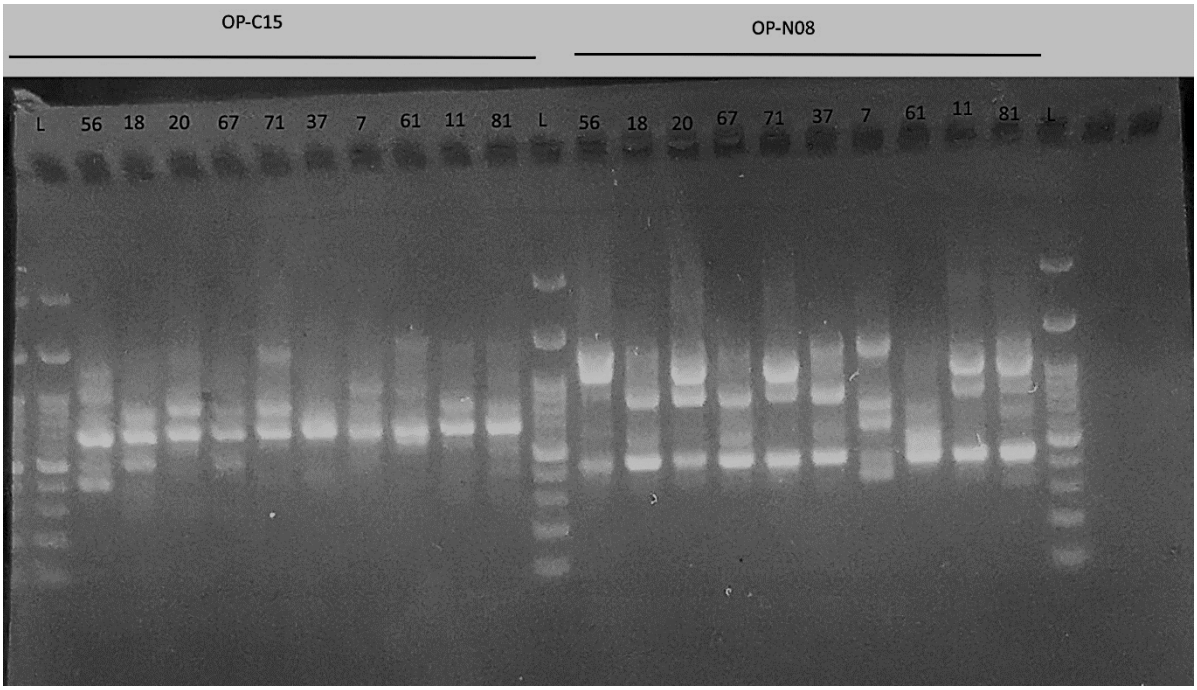

Figure 1. Gel image of primers OP-C15 & OP-N08

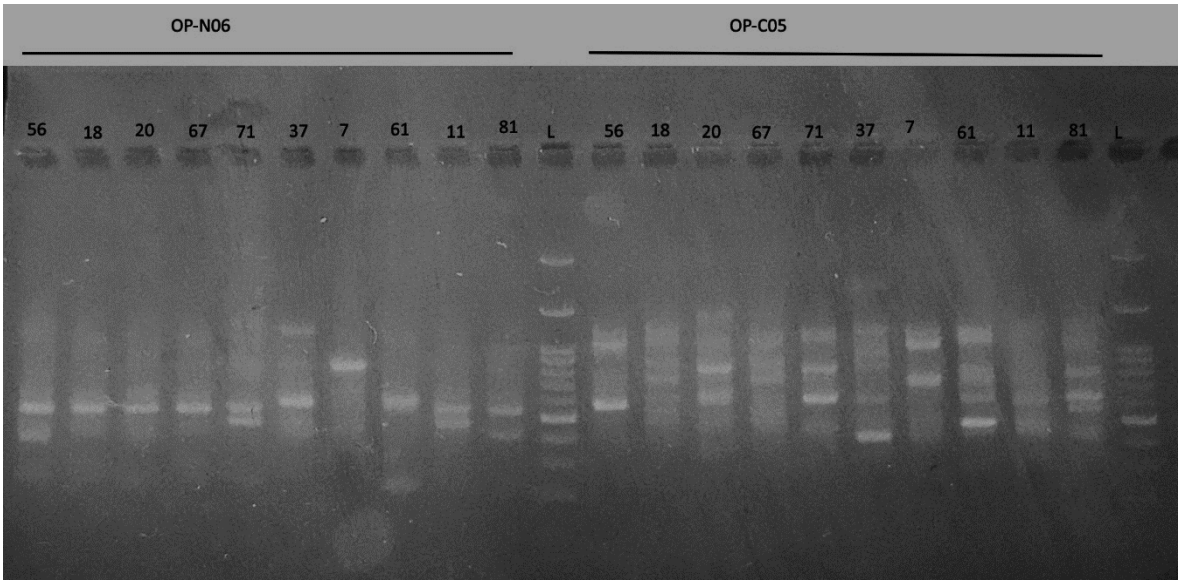

Figure 2. Gel image of primers OP-N06 & OP-C05

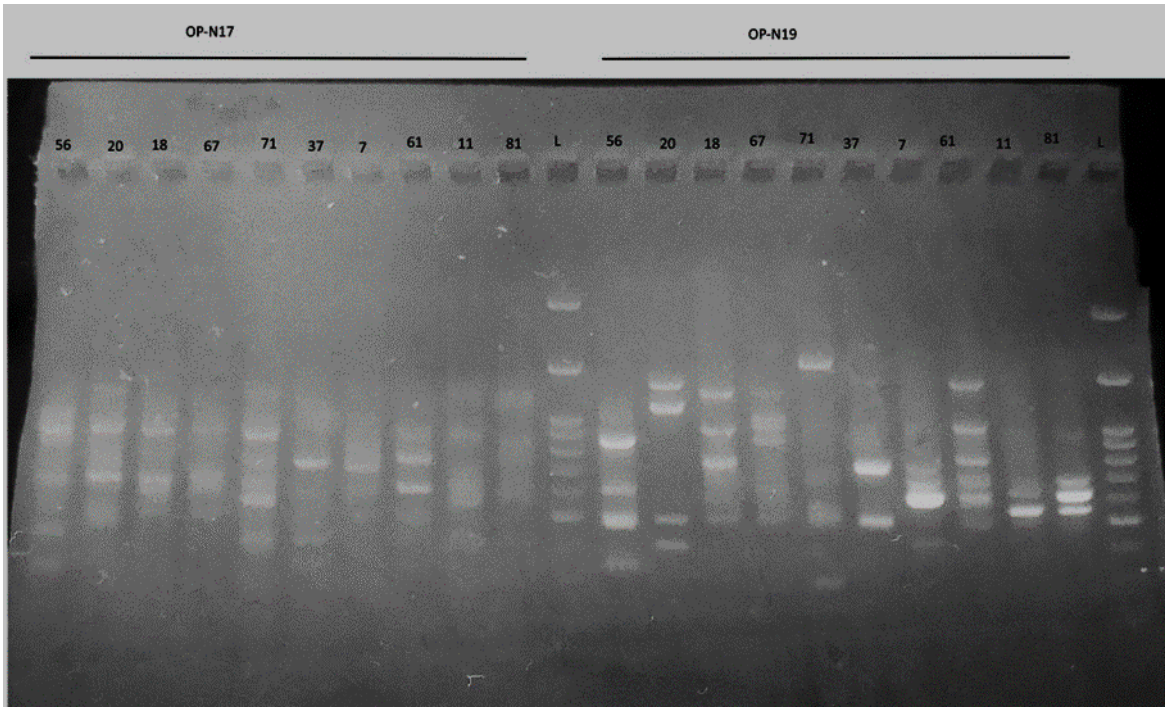

Figure 3. Gel image of primers OP-N17 & OP-C19

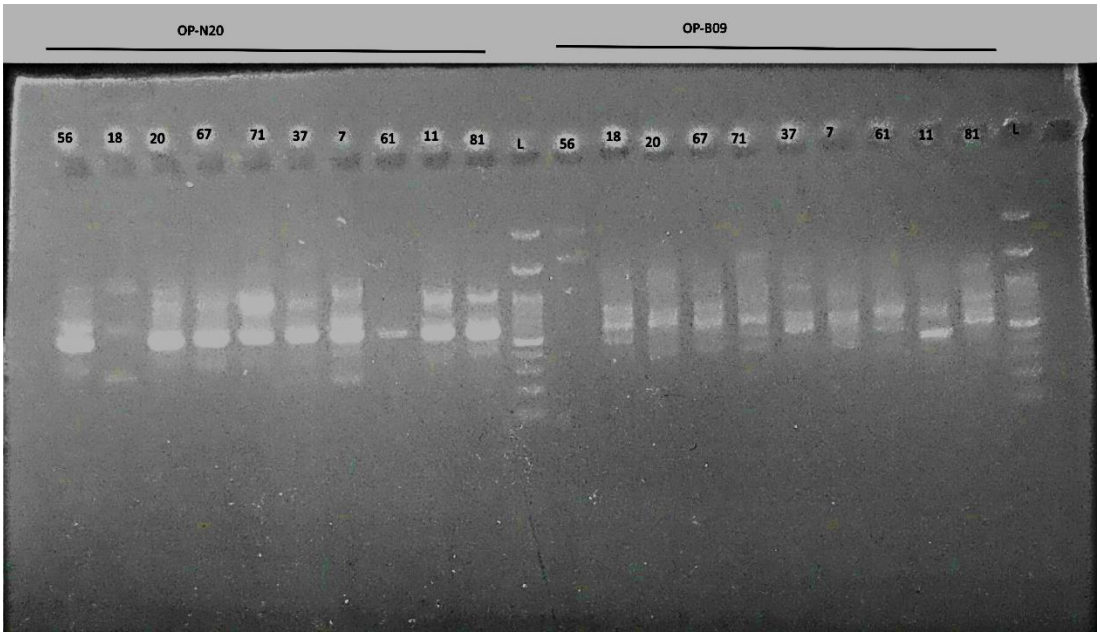

Figure 4. Gel image of primers OP-N20& OP-B09

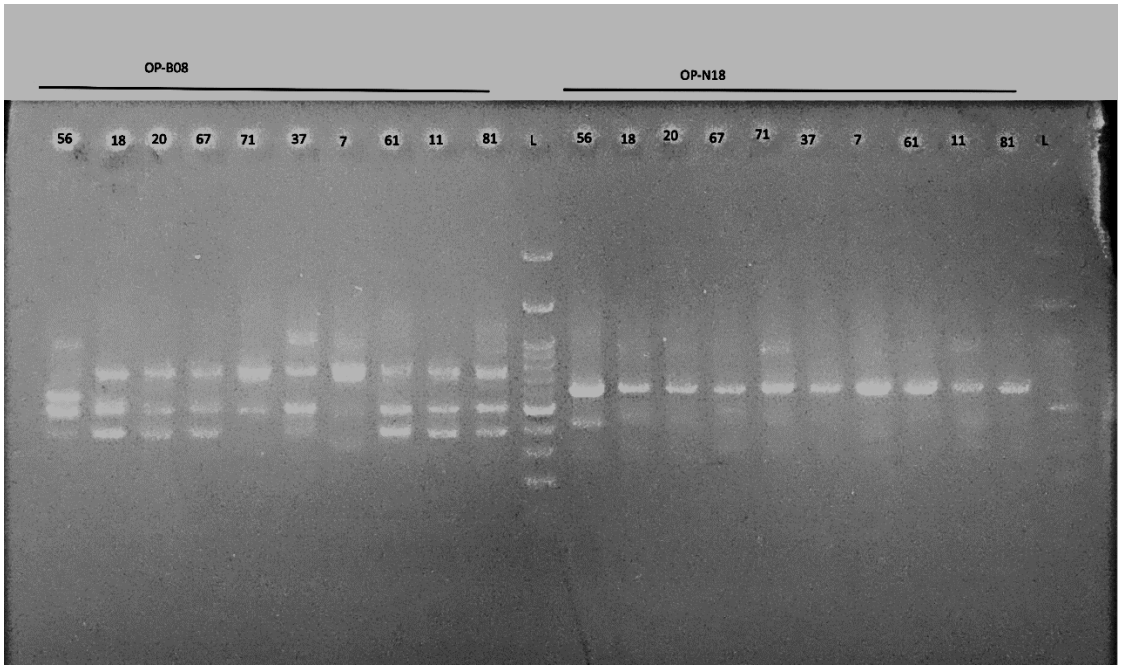

Figure 5. Gel image of primers OP-B08& OP-N18

Table 3 presents the quantification of polymorphic bands based on the calculations derived from the gel image of the band profiles. In line with this, it is worth noting that while the number of bands observed in the primers' bands is lower compared to those depicted in the reference figure, they exhibit a considerable level of polymorphism, as indicated by the data represented in Table 3.

Table 3. Number of amplified products from 10 different oligonucleotide primers. 56: *A. anthylloides,* 18: *A. oleaefolius,* 67*: A. dipodurus,* 20: *A. lagopoides,* 71: *A. velenovskyi,* 11: *A. hirticalyx,* 37: *A. halicacabus,* 7: *A. russelii,* 61: *A. vaginans,* 81: *A. ermineus.*

No	Primers	Total RAPD bands	Polymorphic band number	Amplified band number									
				56	***18***	***20***	***67***	***71***	***37***	***7***	***61***	***11***	***81***
1	**OP-C15**	6	5	5	3	3	3	3	1	3	3	3	4
2	**OP-N08**	7	6	3	3	4	2	4	3	5	2	4	5
3	**OP-N06**	5	5	2	1	1	1	2	2	1	1	2	2
4	**OP-C05**	5	5	2	0	2	1	2	2	2	3	1	1
5	**OP-N17**	3	3	1	2	2	2	1	1	1	2	0	0
6	**OP-N19**	7	6	3	2	3	2	2	1	1	3	1	3
7	**OP-N20**	2	2	2	0	2	2	2	2	2	1	2	2
8	**OP-B09**	2	2	0	2	2	2	2	2	2	1	2	1
9	**OP-B08**	4	4	3	3	3	3	3	4	1	3	3	3
10	**OP-N18**	3	2	2	2	2	1	2	1	1	1	2	1

The phylogenetic tree seen in Figure 6 was derived from the analyses conducted using the NTSYS program. Based on the analysis of the tree, the studied species can be classified into two distinct categories (Clade 1 and 2). *A. halicacabus* and *A. russeli* are classified as subgroups within the first main clade (Clade 1), whereas *A. anthylloides* is distinctively positioned as a separate branch among the same clade. The remaining *Astragalus* species comprised the first clade (Clade 1), whereas *A. vaginans* and *A. ermineus* collectively constituted the second clade (Clade 2). Species such as *A. oleaefolius* and *A. dipodurus*, as well as *A. lagapoides* and *A. velenoskyi*, exhibited notable genetic similarities. The observed distribution of species in the phylogenetic tree provides empirical evidence that aligns with the established morphological section classifications.

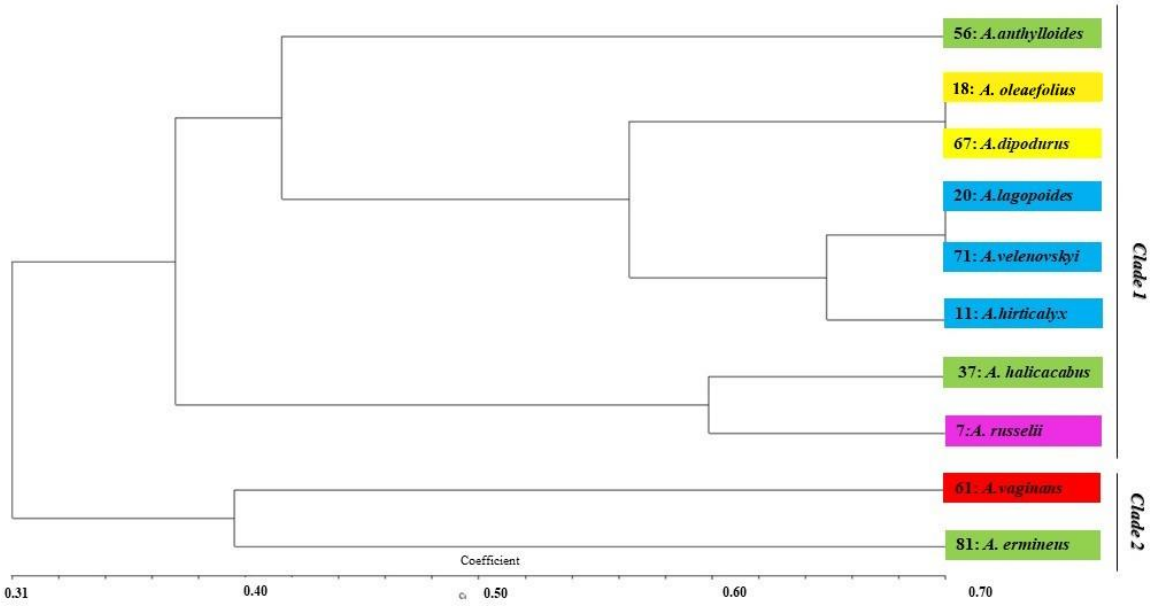

Figure 6. Phylogenetic tree constructed by the NJ method in the NTSYS 2.2 program. 56: *A. anthylloides*, 18:*A. oleaefolius*, 67*: A. dipodurus*, 20: *A. lagopoides*, 71: *A. velenovskyi*, 11: *A. hirticalyx*, 37: *A. halicacabus*, 7: *A. russelii*, 61: *A. vaginans*, 81: *A. ermineus.* (The members of *Anthylloidei* DC. are shown in green, *Macrophyllium* Bunge in yellow, *Hymenostegis* Bunge in blue, *Poterion* Bunge in pink and *Hymenocoleus* Bunge in red).

Upon examination of the phylogenetic tree, it can be observed that the *Anthylloidei* DC. in green (*A. anthylloides* and *A. halicacabus*), *Macrophyllium* Bunge in yellow (*A. oleaefolius* and *A. dipodurus*), *Hymenostegis* Bunge in blue *(A. lagopoides*, *A. velenowskyi*, and *A. hirticalyx*), and *Poterion* in pink sections are grouped together in the first clade. The *A. oleaefolius* and *A. dipodurus* species within the *Macrophyllium* Bunge section exhibit the highest degree of morphological similarity among all species in the section. Thus, they are allied together in sub clade of Clade 1. Also the section *Hymenostegis* Bunge clustered under the second sub clade of Clade 1. The species *A. lagopoides* and *A. velenovskyi* in Clade 1 exhibit morphological similarities and share the characteristic of having yellow blooms. These two species are in close proximity to each other on the sub clade of Clade 1. The purple-flowered

A. hirticalyx, situated in the sub clade, is in close proximity to the other two species but belongs to a distinct sublineage. The *Poterion* Bunge (*A. russelli*) is the section found in the third sub clade (Clade 1). About *Poterion* section had detailed studies that this section had many different morphological and molecular characters. The distinct position of the section in Clade 1 supported by morphological (short and white the on the calyx) and polymorphic sites on nrDNA and cpDNA gene regions (Ateş, 2017). The members of *Astragalus* located in Clade 1 exhibited more support for the morphological evidence. Additional molecular investigation is needed to done better understand the close proximity of *A. russelii* and *A. halicacabus* species sub clade of Clade 1, as their morphological characteristics do not support the molecular analysis.

Within Clade 2, *A. vaginans* (in red) from the *Hymenocoleus* Bunge section and *A. ermineus* from the *Anthylloidei* DC section are in close proximity. *A. ermineus* is the sole species within the *Anthylloidei* division possesses a spiny rachis which is found on Clade 1. *A. vaginans* is a species characterized by a rachis covered in spines. Those two species were found very close in the formerly constructed tree based on both nrDNA ITS and cpDNA *matK* gene regions (Ateş, 2017). Also *A. vaginans* from the *Hymenocoleus* section showed close relations to the *Anthylloidei* section and found no clear phylogenetic placement according to previous study based on ITS sequence data (Bagheri et al., 2017). Our findings supported proximate relationship of *A. vaginans* from the *Hymenocoleus* section to the *Anthylloidei* section. More sampling and detailed molecular analysis need to be done to determine the taxonomic position and conservation status of the *A. vaginans* which is an endemic species to Türkiye.

4. Conclusion

The application of the RAPD technique, a widely utilized method for discerning and analyzing genetic polymorphisms, has significantly contributed to scientific investigations. Despite the wealth of global research utilizing this fingerprinting technique on the genus *Astragalus*, studies conducted in Türkiye, especially those concentrating on endemic species, remain notably scarce. Understanding of the genetic diversity based on polymorphic sites, and phylogenetic positions of 5 sections and 10 distinct *Astragalus* species in newly constructed tree emphasize the need for conservations and more exclusive molecular research efforts, particularly endemic species in Türkiye. Our findings underscore the alignment between morphological data and the outcomes derived from the section examinations within the *Astragalus* genus. Especially, findings supported the proximate relationship of endemic species *A. vaginans* from the *Hymenocoleus* Bunge section to the *Anthylloidei* DC section. In the context of conservation biology, our study emphasizes for increased attention to the genetic intricacies of endemic *Astragalus* species, highlighting the potential vulnerability of these populations and the necessity for targeted conservation strategies to preserve the unique genetic heritage present in Türkiye's *Astragalu*s populations. The foundings are crucial for informed decision-making and sustainable management practices to ensure the long-term viability of these valuable plant resources.

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Conflict of Interest Declaration

The author's of the article declares that there is no conflict of interest.

Researchers' Contribution Statement

The authors declare that they have contributed equally to the article.

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