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Phylogenetic analysis of some species of the Biarum genus in Turkey based on plastid *rps16* DNA sequences

Cahit ÇEÇEN[1], Arif PARMAKSIZ[2], Hasan AKAN[*2], Dilara ULUSAL SEVİMLİ[2], Mehmet Maruf BALOS[3]
ORCID: 0000-0001-6789-9397; 0000-0003-0321-8198; 0000-0002-3033-4349; 0000-0001-9090-5855; 0000-0002-9590-5237

[1] Elmalı village, Kızıltepe, Mardin, Turkiye
[2] Harran University, Arts and Science Faculty, Biology Department, Şanlıurfa, Türkiye
[3] Fatma Zehra Girls Anatolian Imam Hatip High School, Karaköprü, Şanlıurfa, Turkey

Abstract

Purpose: In this study, phylogenetic relationships of some species of *Biarum* genus naturally distributed in Turkey were determined by sequence analyses based on *rps16* region in chloroplast DNA.
Method: 8 *Biarum* species naturally distributed in different localities of Turkey were collected and DNA isolation was performed using a kit, and *rps16* region was amplified by Polymerase Chain Reaction (PCR). Genetic distances between species were calculated by analyzing DNA sequences and a phylogenetic tree was constructed using the Neighbor-Joining method.
Findings: As a result of the analyses, *Biarum bovei* and *B. crispulum* species were identified as the closest genetic relatives, while *Biarum davisii* subsp. *marmarisense* and *B. bovei* were determined as the most distant relatives. The obtained genetic analysis results confirm the classification based on morphological data and reveal that the phylogenetic structure of *Biarum* species should be supported by genetic analyses.
Conclusion: This study provides one of the first phylogenetic analyses based on the *rps16* chloroplast region in Turkish *Biarum* species and it is recommended that future phylogenetic studies be conducted using more comprehensive markers including geographical and ecological factors.

Keywords: Chloroplast DNA, *rps16* , phylogenetics, *Biarum*

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Türkiye'deki Biarum cinsine ait bazı türlerin plastid *rps16* DNA dizilerine dayalı filogenetik analizi

Özet

Amaç: Bu çalışmada, Türkiye'de doğal yayılış gösteren *Biarum* cinsine ait bazı türlerin filogenetik ilişkileri kloroplast DNA'sında bulunan *rps16* bölgesine dayalı dizi analizlerini tespit etmek amacıyla yapılmıştır.
Metod: Türkiye'nin farklı lokalitelerinde doğal yayılış gösteren 8 *Biarum* türü toplanarak DNA izolasyonu kit kullanılarak gerçekleştirilmiş ve Polimeraz Zincir Reaksiyonu (PCR) ile *rps16* bölgesi çoğaltılmıştır. DNA dizileri analiz edilerek türler arasındaki genetik mesafeler hesaplanmış ve Neighbor-Joining yöntemi kullanılarak filogenetik ağaç oluşturulmuştur.
Bulgular: Analizler sonucunda, *Biarum bovei* ve *B. crispulum* türlerinin en yakın genetik akraba olarak belirlenirken, *Biarum davisii* subsp. *marmarisense* ve *B. bovei* en uzak akraba türler olarak tespit edilmiştir. Elde edilen genetik analiz sonuçlarının, morfolojik verilere dayalı sınıflandırmayı doğrulamakta ve *Biarum* türlerinin filogenetik yapısının genetik analizlerle desteklenmesi gerektiğini ortaya koymaktadır.

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[*] *Corresponding author*: Tel.: +90 535 686 11 12; Fax.: +90 (0414) 318 3541; E-mail: hakan@harran.edu.tr

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Sonuç: İlk defa bu çalışma ile *Biarum* cinsine ait genetik veriler elde edilmiş olup gelecekte, coğrafi ve ekolojik faktörlerin de dahil olduğu daha kapsamlı markörlerin de kullanılarak filogenetik çalışmaların yapılması önerilmektedir.

Anahtar Kelimeler: Kloroplast DNA, *rps16* , filogenetik, *Biarum*

1. Introduction

The genus *Biarum* Schott, which is generally distributed in tropical areas, is monocotyledonous, rhizomatous or tuberous, perennial plants. It consists of 21 species of tuberous plants found in semiarid regions of South Europe, North Africa, Near and Middle East. The center of diversity is the Middle East, where 75% of the species are endemic [1] According to Flora of Turkey [2-3] the genus *Biarum* is represented by 8 taxa, while recent studies have shown that the number of species is 22 worldwide and 12 taxa in Turkey [1, 4, 5, 6,7, 8]. 5 of the taxa are endemic and the endemism rate is 41.6%. *Biarum* species are traditionally used in the treatment of some diseases by drying the underground parts and preparing them as a soup by turning them into powder [9] Therefore, the identification of species and knowing their characteristics are of both scientific and economic importance. In addition, the classification and identification of these plants are of great importance for the protection of biological diversity, the determination of medical and economic benefits, and scientific research. Correct classification will contribute to the conservation and sustainable use of these species. The earliest modern classification of the Araceae family was made by Schott [10] and based this classification mainly on flower morphology. In contrast, Engler [11] used a wider data set such as vegetative morphology and anatomy in addition to flower morphology in his classification system and added a distinct evolutionary perspective to his system [12-14].. Molecular phylogenetic studies conducted in recent years allow the evaluation of relationships between organisms within more independent and objective frameworks. Molecular markers are the preferred tools in the genetic analysis of plant groups [15-17]. With these markers, it is possible to detect specific DNA sequences and thus to elucidate evolutionary relationships. The chloroplast genome is considered an ideal marker for phylogenetic studies compared to mitochondrial and nuclear genomes due to its structural simplicity, nucleotide change rates and uniparental inheritance characteristics [18-20]. In addition, the rich mutation site information and high species resolution provided by the chloroplast genome allow for more accurate genetic characteristics of closely related species [21]. These genomes are widely used as a complementary genetic information source in the assessment of plant genetic diversity and development of conservation strategies, contributing to a better resolution of evolutionary relationships between species [22].The *rps16* intron has several features that make it attractive for comparative sequencing studies of plants; it can be easily amplified, multiple sequence alignment is simple and variable, and it is evenly distributed throughout the intron [23].

In this study, phylogenetic analyses were performed using chloroplast *rps16* DNA sequences to reveal the evolutionary relationships of some species of the *Biarum* genus collected from different localities in Turkey and to support species identification.

2. Materials and methods

2.1. Plant material and DNA isolation

The plant materials used in this study were collected from different localities during the 2020-2022 vegetation period and constitute the taxa of the *Biarum* genus with natural distribution in Turkey. Information about these samples is given in Table 1. After the identification of the plant samples, their leaves were preserved in silica gel. An average of 40 mg was weighed from the leaf samples that were dried and preserved in silica gel for DNA isolation and these samples were ground with the help of liquid nitrogen. After the grinding process, total DNA isolation was performed using the GeneMark Plant Genomic DNA Purification Kit.

Table 1. Information on plant samples

Takson	**Location**	**Altitude**	**Date**	**GenBank Accession no**
B. eximium	Adana	85 m	02.04.2021	PV324963
B. pyrami	Burdur	795 m	18.03.2021	PV324964
B. aleppicum	Şanlıurfa	352 m	04.03.2021	PV324968
B. davisii subsp. *marmarisense*	Muğla	20 m.	04.03.2021	PV324965
B. bovei	Şanlıurfa	522 m	03.04.2021	PV324967
B. crispulum	Hatay	743 m	23.03.2020	PV324966
B. rifatii	Antalya	1600 m	24.04.2021	PV324970
B. tenuifolium subsp. zelebori	Aydın	230 m	25.04.2021	PV324969

2.2. Chloroplast DNA *rps16* amplification and sequencing

In this study, the Polymerase Chain Reaction (PCR) process was performed using the BIO-RAD (T100™) device. Primer sequences for the chloroplast DNA *rps16* region are as follows. (5’□ 3’) F: GTG GTA GAA AGC AAC GTG CGA CTT; R: TCG GGA TCG AAC ATC AAT TGC AAC (Oxelman et al., 1997) PCR conditions were as follows: initial denaturation at 95 °C for 3 minutes, followed by denaturation at 95 °C for 1 minute for 35 cycles, annealing at 52.5 °C for 1 minute, extension at 72 °C for 1 minute, and finally, it was terminated by waiting at 72 °C for 5 minutes. In order to control the products formed after the PCR process, PCR products were run on 2% agarose gel under 100 V electric current for 30 minutes and visualized with an imaging device emitting UV light. Samples showing correct amplification were sequenced via commercial company (MEDSANTEK; Istanbul, Turkey) for sequence analysis.

2.3. Data Analysis

Raw data of chloroplast DNA *rps16* region sequences were evaluated using the FinchTV 1.4 program and the sequences of all species were aligned with BioEdit 7.2.5 software. Phylogenetic analyses were performed in the MEGA X program for the data of all species and The Neighbor-Joining phylogenetic tree was constructed using the Kimura 2-parameter model [24]. The reliability of tree branches (nodes) was evaluated with 1000 repeated bootstrap tests.

3. Results

For 8 species of the *Biarum* genus naturally distributed in Turkey, sequences belonging to the Chloroplast DNA *rps16* region were obtained by the Sanger method and visualized using the "Finch TV" program (Figure 1). These sequences belonging to all individuals were checked and reliable data were used. Genetic distances were calculated for sequences belonging to all species (Table 1) and a Neighbor-Joining phylogenetic tree was created in the MEGA X program (Figure 2).

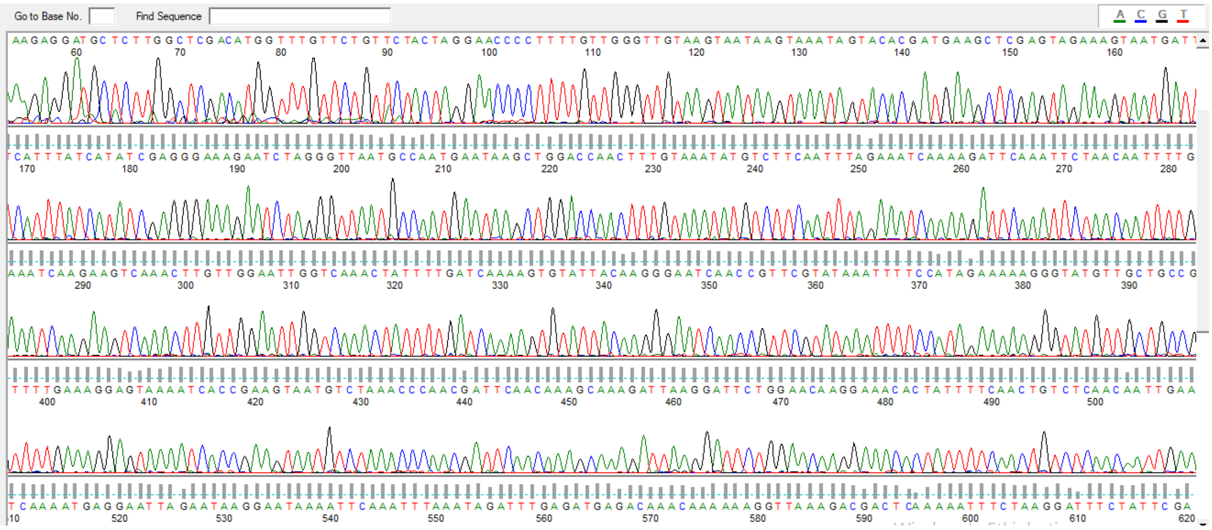

Figure 1. Chromatogram Image of Sequence Analysis of Chloroplast DNA *rps16* Region of *Biarum pyrami* species

This chromatogram was obtained by the Sanger method used for DNA sequencing analysis and was viewed using the "Finch TV" program. These sequences belonging to all individuals were checked and the sequences without base confusion or overlapping peaks were included in this study and reliable data were used.

Table 2. Genetic distance according to Chloroplast DNA *rps16* Region for 8 species of *Biarum* genus in Turkey.

	B. eximium	*B. pyrami*	*B. davisii* subsp. *marmarisense*	*B. crispulum*	*B. bovei*	*B. aleppicum*	*B. tennifolium*	*B. rifati*
B.eximium								
B.pyrami	0,01535							
B. davisii subsp. *marmarisense*	0,01534	0,00764						
B.crispulum	0,01340	0,01340	0,01728					

B.bovei	0,01534	0,01534	0,01922	0,00190				
B.aleppicum	0,00955	0,01341	0,01340	0,00381	0,00571			
B.tennifolium	0,01148	0,01148	0,01148	0,01340	0,01534	0,00955		
B.rifatii	0,00381	0,01534	0,01534	0,00955	0,01148	0,00572	0,01147	

According to Table 2, the lowest genetic distance (0.00190) was calculated between *B. bovei* and *B. crispulum*, indicating that these two species have the closest genetic relationship to each other. The highest genetic distance (0.01922) was calculated between *B. davisii* subsp. *marmarisense* and B. *bovei*, indicating that these two species are the most genetically different species. In addition, the genetic distance (0.00381) was determined between *B. eximium* and *B. rifatii*, indicating that these two species are very closely related and have recently diverged from a common ancestor.

In Figure 2, in addition to *Biarum* species in the Neighbor-Joining tree, Pinellia cordata species taken from NCBI gene bank was used as an outgroup. In this tree, the outgroup was effective in separating the branches and strengthened the assumption that species belonging to the *Biarum* genus form a monophyletic group. Two main branches formed by *Biarum* taxa are seen, *B. tenuifolium* subsp. *zelebori* taxon is positioned on a single branch and other species are positioned on the other branch. It is seen that *B. pyrami* and *B. davisii* subsp. *marmarisense*, *B. bovei* and *B. crispulum* and *B. eximium* and *B. rifatii* species are closer to each other (Figure 2). In addition, since the bootstrap values are above 70, it shows that the branching is quite reliable. These results support each other with morphological findings and it is seen that the closeness of the taxa to each other in the formed tree is compatible with morphological findings.

Figure 2. Neighbor-Joining tree based on Chloroplast DNA *rps16* region data of 8 species of the *Biarum* genus

4. Conclusions and discussion

Chloroplast DNA (cpDNA), due to its unique mode of inheritance and structural characteristics, is a widely utilized molecular marker in plant systematics and phylogenetic analyses. The gene regions within the chloroplast genome provide a broad range of applications in scientific studies, from species identification to elucidating evolutionary history. One of the most significant advantages of chloroplast gene regions is their generally conserved structure, which facilitates comparative analyses across different taxa. In this study, the *rps16* gene region located in the chloroplast DNA of *Biarum* species was sequenced, and a phylogenetic analysis of the species was conducted.

In the study conducted by Çeçen (25), the phylogenetic relationships of 13 species belonging to the genus *Biarum* were analyzed using sequences from the chloroplast DNA trnL-trnF intergenic region. Although the same species were also included in the study using chloroplast DNA *rps16* marker, PCR amplification was successful in only 8 species. No PCR products were obtained for *Biarum cinarense, Biarum carduchorum, Biarum angustatum, Biarum syriacum*, and *Biarum ditschianum* with the *rps16* marker.

Similarly, in a study conducted by Alqahtani and Jansen (26) using the same marker, amplification failure was also observed in certain species within the Euphorbiaceae family. This raises the possibility that either a mutation at the primer binding site or gene loss might be responsible for the lack of amplification in the *rps16* region.

In the study by Arabi et al. (27), a total of 103 species from 16 genera within the Caryophyllaceae family were used to construct a combined matrix based on available *rps16* data. Based on these data, a diagnostic key for the genera was provided, along with notes on their phylogenetic relationships, geographical distribution, and nomenclature.

When *Biarum* species are analyzed according to their positions, it is seen that some of the species show close genetic relationships and are located in close positions in the evolutionary process. It is thought that elements such as the genetic divergence levels of the species, population history, geographical distribution and environmental factors may have affected the phylogenetic structure. In the future, it is recommended that more comprehensive genetic analyses supported by geographical and ecological data be conducted to better understand the differentiation between these species.

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Conflicts of interest: No Conflict of Interest.

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Ethical statement: This study does not require ethical approval.

Author contributions: All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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