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MOLECULAR PHYLOGENY AND MORPHOLOGICAL EVALUATION OF THE RELICT-ENDEMIC *EPIGAEA GAULTHERIOIDES* (BOISS. & BALANSA) TAKHT

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Abstract

The genus *Epigaea* L., belonging to Ericaceae is represented worldwide by 3 species, *viz.*, *E. repens* L., *E. asiatica* Maxim. and *E. gaultherioides* (Boiss. & Balansa) Takht., exhibit limited distribution. Comprehensive study on phylogenetic differences of the relict *E. gaultherioides* is lacking and the phylogenetic relatedness of this species to two other species of *Epigaea* L. of the family has not yet been determined. In this study, the morphological and molecular differences of the *Epigaea* species and taxonomic description of relict *E. gaultherioides* were given in detail. nrDNA (*ITS 4-5*) and plastid (*matK*, *rbcL*) sequences were used for phylogenetic analysis. Morphologically, the main differences between *E. gaultherioides* and the other two species are in the color, shape, and size of corolla. Molecular results from ML analyses indicated that *Epigaea* L. is monophyletic and clearly separated from other genera within the Ericaceae in the phylogenetic trees and also *E. gaultherioides* is phylogenetically distinct from the other two species.

Introduction

The Ericaceae is the largest family in the order Ericales, with approximately 4,500 species (IPNI 2025). It is particularly abundant in mountainous and cold climate regions of the Northern Hemisphere (Nestby *et al.* 2019). Extant Ericaceae began to diversify around 90 million years ago from an ancestor in the Nearctic and/or Palearctic biogeographical realms, with many deeper nodes originating in the Nearctic and exhibits diverse floral structures with unique visual appeal (Rose *et al.* 2018).

Epigaea species are present in the alpine zones, although they are relatively few in number in these environments (Parolly 2020). The flowers are small, white or pink, and have a five-lobed tubular corolla, blooming in mid-spring. *E. repens* L. is widely distributed across the eastern United States, north of Mexico, *E. asiatica* Maxim. is native to Japan and nearby islands, and *E. gaultherioides* (Boiss.) Takht. is found in the Caucasus region of Eurasia, including Türkiye, at elevations of approximately 900-2300 meters in the border areas of Georgia and Türkiye (Gillespie and Kron 2013; Eminağaoğlu 2015; Ozturk *et al.* 2020). *Epigaea* was classified by Drude within the subfamily Andromedeae, but Watson noted its distinctiveness among Ericaceae due to the presence of cyclocytic stomata on its leaves (Kron *et al.* 2002). Boissier and Balansa (1875) described the genus *Orphanidesia* Boiss. & Balansa ex Boiss. and named the *Epigaea* species distributed in the Caucasus as *O. gaultherioides* Boiss. & Balansa. Later, Boissier indicated that *Orphanidesia* was a close relative of *Epigaea* (Watson 1965). The most significant morphological difference between these two genera is the mode of anther dehiscence: in *Orphanidesia*, anthers open by pores, whereas in *Epigaea*, they dehisce along their entire length. Observations of all *O. gaultherioides* specimens found in the Caucasus confirmed that their anthers open longitudinally. Consequently, the species has been renamed *Epigaea gaultherioides*

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(Boiss. et Bal.) Takht. (Stevens 2008). *E. gaultherioides* is an Euxine element with a tertiary origin, considered a relict species that is now at risk of extinction *Epigaea* species are cultivated as ornamental plants in rockeries, where they thrive in moist, acidic soil (Beridze and Dering 2021).

Epigaea asiatica was described by Maximowicz in 1867 but introduced to cultivation around 1929. It prefers shade and demands the same moist, peaty soil as *E. asiatica*, but seems to be just as difficult to cultivate (Clay and Ellstrand 1981). *E. repens*, also known as trailing *Arbutus*, is a native woodland sub-shrub in the Heath Family (Mcpherson 2013). It grows as an evergreen mat in sandy to peaty woods and clearings.

Although separate information has been provided for *E. repens* and *E. asiatica* in studies addressing the phylogeny of the Ericaceae family, there is no comprehensive study covering all *Epigaea* species (Gillespie and Kron 2013). The aim of this study is to identify the distinguishing features of *E. gaultherioides* (Boiss. & Balansa) Takht. from other species in the genus through morphological, molecular, and to construct a phylogenetic tree. To enable rapid and accurate identification, the gene sequence of *E. gaultherioides* was compared with those of two other *Epigaea* species obtained from GenBank.

Materials and Method

E. gaultherioides were collected from Murgul, Artvin during 2023-2024 (Table 1). Morphological features were analysed based on 6 specimens in ARTH and online images sourced from various herbaria and other platforms. Collections from the Artvin Coruh University Herbarium (ARTH), and digital images of *E. gaultherioides* from the web of University of Michigan Herbarium, Atlas of Florida Plants, Wisconsin State Herbarium (WIS), Royal Horticultural Society (RHS) were investigated (Table 2). Flora of Turkey and the East Aegean Islands (Davis 1965-85, Güner *et al.* 2012), illustrated plant atlases (Eminağaoğlu and Anşin 2003, Eminağaoğlu 2014, Eminağaoğlu *et al.* 2015) were used for identification. Morphological characters of *E. gaultherioides* which differ among *Epigaea* species, were measured. Measurements were obtained from both physical specimens and scaled images. Voucher specimens were preserved by drying and are stored at the ARTH herbarium (Tables 1and 2).

Table 1. İnformations regarding the collected specimens.

Species	Code	Collected Number	Herbarium Number	Locality	High (m)
E. gaultherioides	SHAB102	*H.Akyil* 201	ARTH 17303	Tiryal, Murgul, Artvin	1804 m
E. gaultherioides	SHAB104	*H.Akyil* 203	ARTH 17304	Şevvaltepe, Murgul, Artvin	2103 m
E. gaultherioides	SHAB111	*H.Akyil* 210	ARTH 17305	Tiryal, Murgul, Artvin	1961 m

Total genomic DNA was extracted from the fresh or silica-dried leaves by a commercially available genomic DNA isolation kit, DNeasy Plant Mini Kit (Qiagen 2016). The concentration of total DNA was determined using a Nanodrop (Thermo Microvolume UV-Vis) and visualized via 2% agarose gel electrophoresis. Obtained DNA was used in PCR amplification (Akyıldırım Beğen *et al.* 2024). Two chloroplast DNA (cpDNA) region (*matK* and *rbcL*) (Cuénoud *et al.* 2002, Shaw *et al.* 2007) and one nuclear DNA (nDNA) region (ITS 4-5) (White *et al.* 1990) were amplified by PCR and sequenced. ABI 3100 Genetic Analyzer was used for sequence analysis both directions by Macrogene Biotechnology.

All gene region of the *Epigaea* taxon was aligned using MAFFT (Multiple sequence alignment) in Geneious prime (2024.0) (Geneious 2024) and data entries were completed to the NCBI GenBank database (Genbank 2024). Plastid and nuclear DNA sequences of other *Epigaea* species were downloaded from the Genbank. In phylogenetic analyses, three gene regions were studied and phylogenetic trees were figured with related species. *Rhodothamnus chamaecistus* (EU U61321.1) and *Empetrum nigrum* (KC474702.1) species were selected as outgroups for matK region. To assess genetic distance among *Epigaea* species and obtain phylogenetic trees using Maximum Likelihood (Tamura Nei model) algorithm to evaluate the degree of support for given clades, a bootstrap analysis (10000 replicates) in MEGA 7 (Tamura *et al.* 2013).

Table 2. Details about the examined herbarium specimens.

Species	Collected Number	Locality	Herbarium	Accesession numbers
E. gaultherioides	*H.Akyil* 201	Tiryal, Murgul, Artvin	Artvin Coruh University Herbarium	ARTH 17303
E. gaultherioides	*H.Akyil* 203	Şevvaltepe, Murgul, Artvin	Artvin Coruh University Herbarium	ARTH 17304
E. gaultherioides	*H.Akyil* 210	Tiryal, Murgul, Artvin	Artvin Coruh University Herbarium	ARTH 17305
E. gaultherioides	*OEmin* 1234	Hatila Valley, Artvin	Artvin Coruh University Herbarium	ARTH 3205
E. gaultherioides	*H.Akyil* 221&Emin	Tiryal, Murgul, Artvin	Artvin Coruh University Herbarium	ARTH ..
E. gaultherioides	*H.Akyil* 222&Emin	Tiryal, Murgul, Artvin	Artvin Coruh University Herbarium	ARTH ..
E. repens	*Roberts, P.R. Pugh, B.*	Canada	Connell Memorial	17625
E. repens	A.E. Foote	Ontonagon	University of Michigan Herbarium	1258758
E. repens	S. L. Orzell 21470 with E.L. Bridges	Florida, Santa Rosa	Atlas of Florida Plants	226697
E. repens	John V. Freudenstein 846	North America		1258848
E. asiatica Maxim.	M. Togashi	Japan, Shiga, Mt. Hira, Shiga-gun.	Wisconsin State Herbarium (WIS)	8867
E. asiatica Maxim.	Marchant, W.J.	United Kingdom	Royal Horticultural Society (RHS)	RHS 1851

Results and Discussion

The morphological characteristics of *Epigaea gaultherioides* are described in detail, and its differences from other *Epigaea* species are revised (Figs 1and 2). The main differences between *E. gaultherioides* and the other two species are in the color, shape, and size of the corolla, as well as the inflorescence and leaf structure. *Epigaea gaultherioides* has a large campanulate corolla (4-7 cm) with lobes measuring 0.7-1.1 cm, 10 stamens, and an almost funnelform shape. Its inflorescence consists of 1-2, rarely 3 or 4, widely spaced flowers. In contrast, *E. asiatica* and *E.*

repens have much smaller corollas with long tubes (salverform), and their more numerous, closely spaced flowers form a very dense cluster (Fig. 2, Table 3). In this study, the morphological traits of *E. gaultherioides* specimens from Artvin were compared with *E. repens* and *E. asiatica*. Although all three share adaptations to harsh climates- such as leathery leaves and hairy stems- significant differences were observed, particularly in flower morphology and color. While *E. repens* and *E. asiatica* have more tubular flowers and form a closer group, *E. gaultherioides* displays distinct morphological traits, suggesting greater evolutionary divergence. Upon revision of the morphological characters of *E. gaultherioides,* it was determined that the petiole does not exceed 3.5 cm in length, the corolla is broader and campanulate in shape, and the ovary is glabrous (Table 3).

Fig. 1. *Epigaea* flowers. A: *Epigaea asiatica,* B: *E. repens,* C: *E. gaultherioides.*

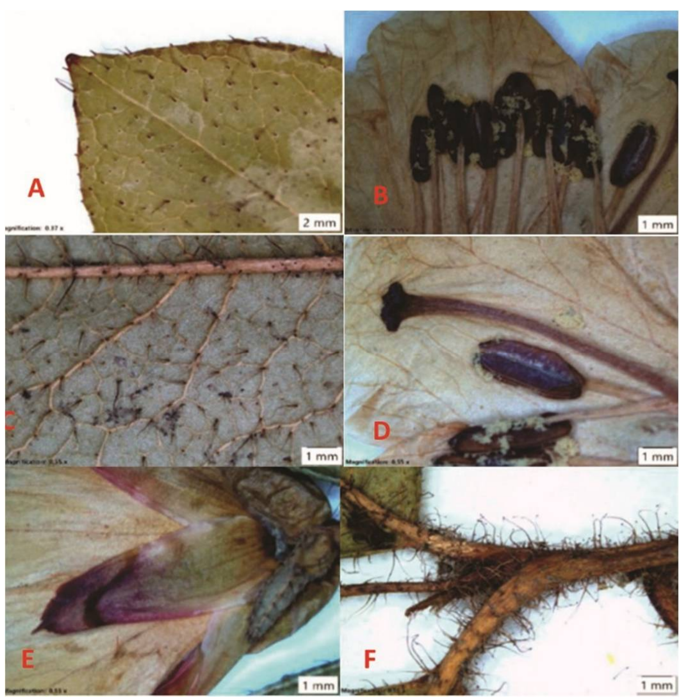

Fig. 2. Some morphological characters of *Epigaea gaultherioides*. A: leaf apex, B: stamen, C: leaf underside indumentum, D: ovary, E: calyx and F: hairs on the shoot.

Table 3. Morphological characters of three species of *Epigaea* spp.

Characters	*Epigaea gaultherioides* (Trailing Arbutus, Mountain Apple)	*E. repens* (Trailing Arbutus, Mayflower, Ground Laurel) (Langdon, 1894)	*E. asiatica* (Syn: *Parapyrola trichocarpa*) (Iwanashi or Trailing Arbutus) (Gillespie and Kron, 2013; Sugiura and Yamazaki, 2005)
Habit	Shrub, 0.2-0.5 m	shrub, 0.2-0.8m	Shrub 0.5-1m
Petiole	2.5-3.5 cm	1-5 cm, short rusty-hairy	1-5 cm
Leaves	Simple, entire, broad, oval, evergreen, elliptic, and short-acuminate, 6-11 X 3-5.5 cm.	Simple, 2-11 x 2-5 cm, wide, toothless, egg-shaped to oval - oblong.	Simple, dark green, 5-10 x 3 cm, elliptic, entire, oblong-ovatecordate, red flushed when young, lustrous and leathery
Hairs	Long ferruginous, rust-colored hairs, 1.2-1.3 cm	Brown hairs under laeves.	Gland-tipped hairs
Stem	Branched, reddish-brown-hairy, prostrate or creeping along the ground	Branched, prostrate or creeping, youngers covered in rusty hairs; older stems eventually become hairless, with flaking bark.	Prostrate or creeping, reddish-brown-hairy, twigs coarsely hirsute (especially new growth)
Corolla	Pale rose-colored, broadly funneliform, with 5 lobes, 20-60 mm, glabrous.	Waxy white or rose pink, tubular, 6–10(–15) mm, salverform, with a slender hairy tube spreading into five equal lobes, spotted brown and some even becoming transparent	Light to rose-pink with paler and a long tube (salverform) tubular to urn-shaped with five shallow, 10(–15) mm recurved, rounded lobes
Stamen /Style	Stamen 10, a five-lobed stigma, globose, anthers narrow-oblong 3·5 mm /3.8-4 x1.5 mm,	Stamen 5/10, a five-lobed stigma, a columnar style lender, with collar or ring of tissue partly adnate to stigma	Stamens short, hairy at the base, style stout, shorter than the stamens.
Flowers	Axillary, clusters, 1-5-flowered in the axils, campanulate shape, without hairs.	Axillary and terminal clusters, trumpet shaped, in tight clusters, 8 tubular flowers The inside of the tub dense white hairs.	Axillary or terminal, spikes or dense racemes, 2-6(-10)-flowered; perulae absent, tubular, softly pubescent with fine, short hairs
Ovary	Glabrous	Glandular-hairy	Hairy
Seed	0·3-0·4 mm, yellowish-brown	0.5 mm, brown The seeds are embedded in a sticky, white, fleshy pulp within the capsule	Ovoid to globose, not winged, not tailed; 0·1-0·2 mm, testa foveolate. flower-stalk hairy; seed-vessel orange-shaped, ca. 100,
Fruit	Globose, 0.9 mm long, loculicidal thin-walled.	Fleshy, 5-lobed, hairy, dehiscent capsule about 6 mm in diameter tiny, shiny, brown, hard seeds per apsule.	Capsular, depressed-globose, dehiscence septicidal
Flowering Time	5 - 7	4 -5	4 -5
Distribution	Türkiye, Transcaucasus	New England, Florida to Mississippi, New York, Pennsylvania, West Virginia, and Ohio	East Asia - Centre and North Japan
Altitude	920–2500 m	0-1500 m	100 - 1700 m
Habitat	Upper part of *Fagus orientalis* and *Picea orientalis* mix forest with *Vaccinium* spp. and *Rhododendron* spp.	Part shade, shade, moist to dry sandy or rocky acidic soil; pine forests, savannas, bogs Acidic, well-drained but moist, humus-rich.	The subshrubs prefer a half-shady to shady situation on moist soil. They tolerate temperatures down to -35°C. Open woods in the mountains.

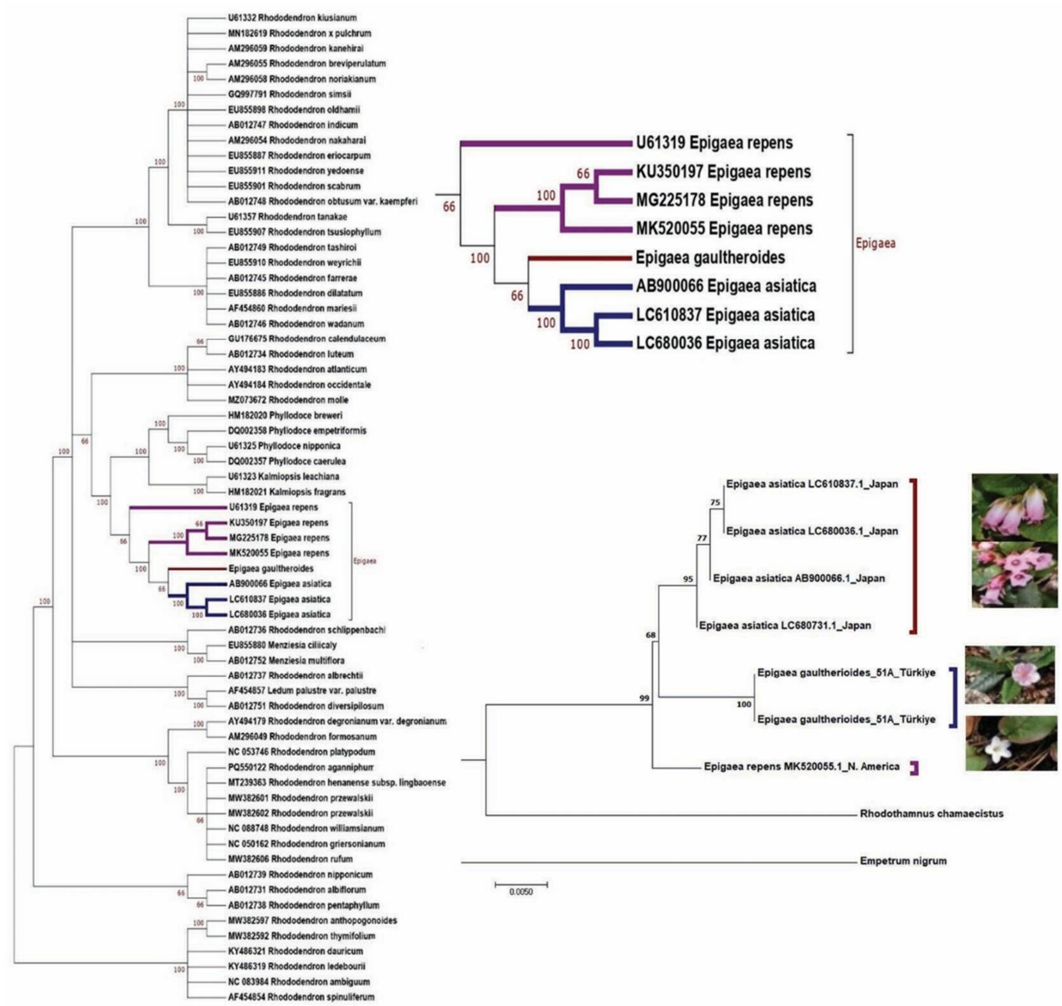

Fig. 3. Phylogenetic tree using Maximum Likelihood approach using Tamura-Nei model based on chloroplast DNA (*matK* region).

Molecular studies were conducted using *E. gaultherioides* samples collected from Türkiye, along with additional species obtained from GenBank. The results revealed that *E. gaultherioides* differs from the other two species both morphologically and molecularly. The placement of species from various genera within clades formed for the matK gene region of the Ericaceae family differs between the species tree and the concatenated tree constructed using Maximum Likelihood (ML). While most species are found within the *Rhododendron* clade (BS = 100), some species have resolved within a clade together with *Phyllodoce*, *Kalmiopsis*, and *Epigaea* (BS = 66). *Epigaea* is sister to the clade containing these genera (BS = 100). In the ML phylogenetic tree based on the matK gene region, *E. repens* is observed as the earliest diverging species within the tribe, while *E. gaultheroides* and *E. asiatica* appear to be more closely related (Fig. 3). The aligned rbcL dataset comprised 41 species representing nine Ericaceae genera. In the ML analyses of the chloroplast DNA *rbcL* gene region, *Phyllodoce, Rhodothamnus*, and *Epigaea* were placed

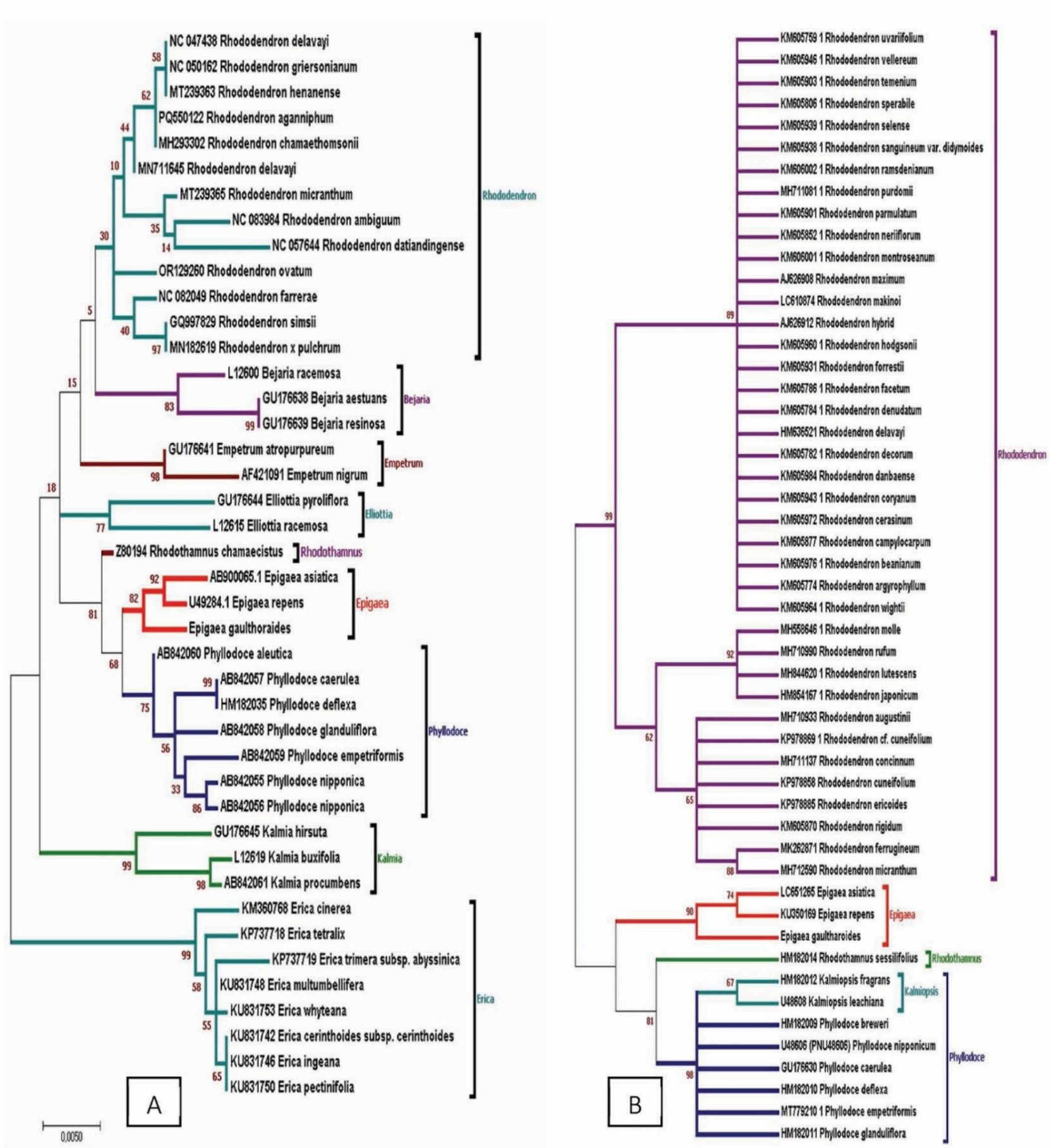

Fig. 4. Phylogenetic tree based on chloroplast DNA (**A**-*rbcL* region) and nuclear DNA (**B**-*ITS 4-5* region) using Maximum Likelihood approach using Tamura-Nei model.

within the same clade (BS = 81), while *Kalmia* and *Erica* formed distinct clades. *Epigaea asiatica* and *E. repens* resolved within the same clade (BS = 74), whereas *E. gaultheroides* formed a separate branch (BS = 82). The aligned ITS gene region dataset comprised 54 species representing five genera. In nuclear DNA analyses, *Rhododendron* species are placed in a completely separate clade, whereas in the chloroplast rbcL gene region analysis, *Bejaria, Empetrum, Rhodothamnus, Elliottia, Epigaea,* and *Phyllodoce* are grouped within the same clade (Fig. 4). In the ML phylogenetic tree based on the nuclear DNA ITS gene region, *Epigaea* formed a separate clade

(BS = 90), while *Phyllodoce, Rhodothamnus*, and *Kalmiopsis* formed a sister clade (BS = 81). *Epigaea asiatica* and *E. repens* resolved within the same clade (BS = 82), whereas *E. gaultheroides* formed a separate branch (BS = 90). In both nuclear and plastid DNA-based phylogenetic analyses, *Epigaea* species have been placed within the same clade. The closest related genus was identified as *Rhodothamnus.* When considering the distribution areas, these two genera are both found in alpine regions, and it has been observed that they naturally grow in the same areas in Türkiye.

This divergence is further supported by phylogenetic data. Gillespie *et al.* (2013) found *E. repens* and *E. asiatica* to be 66% similar based on 45 morphological traits, whereas *E. gaultherioides* showed 99% distinction. Phylogenetic analyses using both chloroplast (cDNA) and nuclear (nDNA) markers confirmed these separations, aligning with morphological differences and supporting the taxonomic distinctiveness of *E. gaultherioides.* Unlike Kron's (1997) earlier study, which lacked full resolution among *Epigaea* species, use of the matK region yielded clearer differentiation. Moreover, consistent with prior studies (Gillespie *et al.* 2013), analyses positioned *Epigaea* within the Phyllodoceae tribe, forming a sister relationship with *Rhodothamnus*, and closely related to *Phyllodoce* and *Kalmiopsis*. These findings highlight the significance of combining morphological and molecular data to understand the evolutionary history of the relict taxa like *E. gaultherioides*.

Epigaea gaultherioides is a rare relict species represented by a limited number of individuals in Türkiye. Its population decline is accelerating due to climate change and human activities. This study provides the first molecular analysis of the species, revealing clear distinctions from other *Epigaea* species alongside its unique morphological traits. Ex-situ conservation efforts have been initiated to preserve the species. The findings contribute to understanding its ecological requirements and offer a scientific basis for future conservation strategies.

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