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Original article (Orijinal araştırma)

Comparative analyses of *COI* and *ITS2* in the molecular identification of *Chrysolina* Motschulsky, 1860 (Coleoptera: Chrysomelidae) in Türkiye[1]

Türkiye'de dağılış gösteren *Chrysolina* Motschulsky, 1860 (Coleoptera: Chrysomelidae) cinsine dahil türlerin moleküler tanımlamasında *COI* ve *ITS2* belirteçlerinin karşılaştırmalı analizleri

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Abstract

This study investigated the effectiveness of two molecular markers—the cytochrome c oxidase subunit I (*COI*) gene and the internal transcribed spacer II (*ITS2*) region—in delineating species boundaries in insects. While *COI* is widely used in insect taxonomy to define species boundaries, *ITS2* has been less frequently tested. A total of 25 individuals from the genus *Chrysolina* (Coleoptera: Chrysomelidae), distributed across Türkiye, were analysed. Molecular analyses were conducted in 2017 at the Molecular Systematics Laboratory of the Department of Zoology, Faculty of Science, Ege University, and data analyses were completed in 2025. Using a Bayesian approach, they examined phylogenetic tree topology and species monophyly based on each marker and combined datasets. This marks the first comparative study of *COI* and *ITS2* within *Chrysolina* species in Türkiye. Results showed that both markers supported morphological distinctions at the molecular level. All species formed monophyletic groups in trees constructed from *COI, ITS2,* and combined datasets. These findings suggest that the *ITS2* may be as effective as the *COI* in evaluating genetic diversity and identifying species. The study highlights the potential of *ITS2* as a reliable molecular marker in insect taxonomy and enhances understanding of species boundaries within the genus *Chrysolina*.

Keywords: Chrysolina, COI, DNA barcoding, ITS2

Öz

Bu çalışma, böceklerde tür sınırlarını belirlemede iki moleküler belirtecin—sitokrom c oksidaz alt birimi I (*COI*) geni ve iç transkribe edilmiş aralık II (*ITS2*) bölgesinin—etkinliğini araştırmıştır. *COI* geni, böcek taksonomisinde tür sınırlarını belirlemede yaygın olarak kullanılmasına rağmen, *ITS2* bölgesi bu amaçla daha az test edilmiştir. Türkiye genelinden toplanan *Chrysolina* cinsine (Coleoptera: Chrysomelidae) dahil 25 birey analiz edilmiştir. Moleküler analizler 2017 yılında Ege Üniversitesi Fen Fakültesi Zooloji Bölümü Moleküler Sistematik Laboratuvarı'nda gerçekleştirilmiş, veri analizleri ise 2025 yılında tamamlanmıştır. Her bir belirteç ve birleşik veri seti temelinde filogenetik ağaçların topolojisi ve türlerin monofiletikliği Bayesian yaklaşımıyla değerlendirilmiştir. Bu çalışma, Türkiye'de *Chrysolina* türleri için *COI* ve *ITS2* belirteçlerinin ilk karşılaştırmalı analizini sunmaktadır. Elde edilen sonuçlar, her iki belirtecin de morfolojik ayrımları moleküler düzeyde desteklediğini ortaya koymuştur. *COI, ITS2* ve birleşik veri setleriyle oluşturulan filogenetik ağaçlarda tüm türler monofiletik gruplar oluşturmuştur. Bulgular, *ITS2* bölgesinin genetik çeşitliliğin değerlendirilmesi ve tür tanımlanmasında *COI* kadar etkili olabileceğini göstermektedir. Bu çalışma, *ITS2*'nin böcek taksonomisinde güvenilir bir moleküler belirteç olarak potansiyelini vurgulamakta ve *Chrysolina* cinsi içindeki tür sınırlarının anlaşılmasına katkı sağlamaktadır.

Anahtar sözcükler: Chrysolina, COI, DNA barkodlama, ITS2

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Introduction

DNA barcoding is the identification of species using a specific gene region (Hebert et al., 2003a, b). An effective barcoding region must display adequate sequence divergence to enable reliable species-level discrimination, while maintaining sufficient conservation to ensure that intraspecific variation remains lower than interspecific divergence (Yao et al., 2010). To identify a gene region as an appropriate DNA barcode, three criteria are necessary: (1) pronounced genetic heterogeneity and interspecific divergence at the molecular level; (2) short sequence length to enhance DNA extraction and amplification efficiency; and (3) amplification with universal primers (Kress & Erickson, 2008). Research has shown that the mitochondrial cytochrome c oxidase subunit I (*COI*) gene provides the foundation for a universal identification system in animals, with a standardized region functioning as a species-level taxonomic barcode (Hebert et al., 2003a, b; Kress & Erickson, 2008). The *COI* gene has demonstrated high resolution in distinguishing cryptic species (Hebert et al., 2004), and its effectiveness has been validated across multiple insect orders, such as Coleoptera (Löbl & Leschen, 2005), Diptera (Scheffer et al., 2006), Ephemeroptera (Ball et al., 2005), Hemiptera (Lee et al., 2011), Hymenoptera (Smith et al., 2008), and Lepidoptera (Hajibabaei et al., 2006).

The internal transcribed spacer 2 (*ITS2*) region of ribosomal DNA (rDNA) is also used as a molecular marker (Arnheim, 1983). Among its components, the *ITS2* region—situated between the 5.8S and 28S rRNA genes—represents a rapidly evolving, non-coding segment of nuclear rDNA (Arnheim, 1983; Miller et al., 1996). The *ITS2* marker is utilized for species comparison and the determination of cryptic species (Campbell et al., 1993; Edel, 1998; Alvarez & Hoy, 2002; Prinsloo et al., 2002; Rokas et al., 2002; Ashfaq et al., 2005).

In insects, the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (*COI*) gene serves as the standard DNA barcoding region and is extensively utilized as a molecular marker for delineating species boundaries (Garin et al., 1999; Hebert et al., 2003a; Becerra, 2004; Lopes et al., 2015). On the other hand, the usage of *ITS2* as a molecular identifier in insect taxonomy is rare. Probably due to the challenges associated with secondary structure prediction and alignment issues. Recent advancements have enabled the assessment of more distantly related taxa, thereby promoting the increasingly frequent use of *ITS2* in insect taxonomy (Budak et al., 2016).

Leaf beetles, belonging to the Chrysomelidae family, represent one of the most diverse and expansive groups within the order Coleoptera (Lopatin, 1977). The genus *Chrysolina* Motschulsky, 1860 (Coleoptera: Chrysomelidae), belongs to the subfamily Chrysomelinae, the tribe Chrysomelini, and the subtribe Doryphorina. Within the subfamily Chrysomelinae, *Chrysolina* is considered to be the most taxonomically controversial genus. Today, *Chrysolina* comprises nearly 500 species worldwide, with approximately 70 subgenera and several species groups. The members of *Chrysolina* often exhibit clear morphological differences. Forty-nine of these species are distributed across Türkiye (Bezděk & Sekerka, 2024).

Comparisons of *COI* and *ITS2* genes have been conducted in various groups within the Chrysomelidae family (Becerra, 2004; Swigonova & Kjer, 2004; Germain et al., 2013). The study of Park et al. (2011) conducted with *Chrysolina aurichalcea* (Mannerheim, 1825) (Coleoptera: Chrysomelidae) from Korea can be given as an example. Furthermore, mitochondrial and nuclear DNA comparisons of the genus *Chrysolina* have been conducted across gene regions other than *COI* and *ITS2* (Garin et al., 1999; Jurado-Rivera & Petitpierre, 2015). In this study, the comparison of the mtDNA *COI* gene region and rDNA *ITS2* region of the genus *Chrysolina*, distributed in Türkiye, has been presented for the first time.

We aimed to determine if the *ITS2* region is as effective as the *COI* marker in determining the genetic diversity among the species. In order to achieve this, we compared the mitochondrial *COI* gene and ribosomal *ITS2* region of 25 *Chrysolina* species from Türkiye.

Materials and Methods

Sampling

The study material consists of 25 specimens from six different species of *Chrysolina*, collected from Türkiye. *Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae) was used as the outgroup (Table 1). To

ensure a clear taxonomic differentiation, it is essential to select a taxon from a different subtribe as an outgroup. The genera *Chrysolina* and *Gonioctena* Chevrolat, 1836 included in this study belong to the tribe Chrysomelini. Taxonomically, *Chrysolina* is assigned to the subtribe Doryphorina (Motschulsky, 1860), whereas *Gonioctena* is categorized under the subtribe Gonioctenina (Motschulsky, 1860) (Bezděk & Sekerka, 2024).

A total of 50 *Chrysolina COI* and *ITS2* sequences were transferred to the Barcode of Life Database (BOLD) (Ratnasingham & Hebert, 2007) under the project name '*Chrysolina* in Türkiye [CHREU]'. Moreover, total of 8 *Gonioctena fornicata COI* and *ITS2* sequences were transferred to the BOLD under the project name '*Gonioctena* in Türkiye [GONEU]' (www.barcodinglife.org).

Table 1. Morphologically identified species, collection regions and dates

Species names	Subgenus	Region of collection	Date of collection	Number of individuals
Chrysolina orientalis (Olivier, 1807)	Ovasoma Motschulsky, 1860	Konya - Akşehir	11.06.2015	8
Chrysolina vernalis (Brullé, 1832)	Ovasoma Motschulsky, 1860	İzmir - Urla	05.04.2015	2
Chrysolina gypsophilae (Küster, 1845)	Stichoptera Motschulsky, 1860	Karaman - Karadağ	18.04.2015	1
Chrysolina didymata (Scriba, 1791)	Hypericia Bedel, 1899	Isparta - Aşağıgökdere	09.05.2015	8
Chrysolina herbacea (Duftschmid, 1825)	Synerga Weise, 1900	İzmir - Urla	05.04.2015	1
Chrysolina coerulans (Scriba, 1791)	Synerga Weise, 1900	Isparta - Aşağıgökdere	09.05.2015	5
Gonioctena fornicata (Bruggemann, 1873)	Outgroup genus	Yozgat - Sorgun	19.04.2015	4

*Morphological identifications of all species were performed by Prof. Dr. İsmail Şen.

Molecular analyses

Molecular analyses were performed in 2017 at the Molecular Systematics Laboratory of the Department of Zoology, Faculty of Science, Ege University. Genomic DNA was isolated using the Promega A1120 Wizard Genomic DNA Purification Kit, following the manufacturer's protocol. Amplification of *COI* and *ITS2* sequences was performed using specific primer sets (Table 2).

Table 2. Primers used for Polymerase Chain Reaction (PCR) analysis

Molecular Marker	Primer Name	Primer Sequence (5′–3′)	Reference
COI	Forward (s1859)	GGA ACI GGA TGA ACW GTT TAY CCI CC	Simon et al., 1994
	Reverse (a2590)	GCT CCT ATT GAT ARW ACA TAR TGR AAA TG	
ITS2	Forward (cas5.8s)	ATG AAC ATC GAC ATT TCG AAC GCA TAT	Ji et al., 2003
	Reverse (cas28s)	TTC TTT TTC TTC GCT TAG TAA TAT GCT TAA	

Polymerase Chain Reaction (PCR) was carried out using a master mix with a final volume of 25 µl, comprising 12 µl Trehalose, 2.5 µl NH_4 Buffer, 1.25 µl $MgCl_2$, 0.5 µl of each forward and reverse primer, 0.5 µl dNTPs, 0.1 µl Taq polymerase, and nuclease-free water to reach the final volume. PCR amplification conditions for the *COI* gene included an initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 51ºC for 45 seconds, and extension at 72ºC for 1 minute, concluding with a final extension at 72ºC for 10 minutes. For the *ITS2* region, the PCR protocol consisted of an initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 51.3ºC for 1 minute, and extension at 72ºC for 1 minute, with a final extension step at 72ºC for 10 minutes. Amplified products were visualized via 1% agarose gel electrophoresis and subsequently submitted for sequencing.

The sequences of the *COI* gene and *ITS2* regions were edited using Geneious 10.2.6 (Geneious, 2025) and Sequencher 4.10.1 (Gene Codes). *COI* sequences in FASTA format were aligned using the ClustalW (Thompson et al., 1994). Codon positions and the genetic code for the *COI* marker were determined using Mesquite (Maddison & Maddison, 2011), and the alignments of both markers were also checked in the same program. A total of 655 base pairs were generated for the matrix using the mtDNA *COI* gene region marker. The MAFFT platform (Katoh et al., 2019) was used to align *ITS2* sequences, providing accurate multiple sequence alignments for non-coding regions. For the *ITS2* marker, 469 base pair datasets were obtained, including the insertion/deletion (indel) regions present.

Data analyses

Data analyses were completed in 2025. The optimal substitution models for the mitochondrial *COI* gene and nuclear *ITS2* regions were determined using JModelTest v0.1.1 (Posada, 2008). Haplotypes, polymorphic regions, and conserved regions for both markers; synonymous and non-synonymous regions for the *COI* marker; and indel regions for the *ITS2* marker were analysed using the DnaSP v.6.12.03 (Rozas et al., 2017). Bayesian inference with Markov Chain Monte Carlo (MCMC) analysis was conducted using BEAST v2.6.7 (Bouckaert et al., 2019). This method estimates the final probability value for species by evaluating the topology of the species tree and testing species monophyly. In the BEAST2 analyses, four specimens of *Gonioctena fornicata* Bruggemann, 1873 (Coleoptera: Chrysomelidae) were designated as outgroup taxa for both *COI* and *ITS2* markers. A total of 29 samples, including the outgroup, were analysed. For both markers, the strict clock model was used under the GTR substitution model. The Yule process was selected as the prior for the tree. The age range was not specified. The analyses were run for 20 million generations. The Effective Sample Size (ESS) values of the trees obtained for both markers were assessed using Tracer v1.6.0 (Rambaut et al., 2014). A maximum clade credibility (MCC) tree was constructed using TreeAnnotator v2.6.0, based on the posterior distribution of trees, with the initial 20% discarded as burn-in. The gene trees obtained from the analyses were visualized using FigTree v1.4.2 (Rambaut, 2009).

The coding mtDNA *COI* gene region and the non-coding rDNA *ITS2* region were concatenated with the datasets using Sequence Matrix 1.8 (Vaidya et al., 2011). The combined evaluability of these two markers was tested using the Incongruence Length Difference (ILD) test in PAUP 4.0a 159 (Swofford, 2002). A P-value greater than 0.01 obtained from the analysis indicates that the two regions can be evaluated together (Farris et al., 1994).

Finally, an evolutionary operational criterion for species delimitation was investigated using the tree-based General Mixed Yule Coalescent (GMYC) model (Pons et al., 2006). When species delimitation relies solely on DNA sequences, identifying a ‘species boundary’ based on observed genetic variation constitutes a fundamental step in the process. This method was applied to ultrametric trees obtained using BEAST v2.6.7 (Bouckaert et al., 2019), with analyses performed in R Gui 4.4.2 (R Core Team, 2022) using the APE (Paradis & Schliep, 2019), PARAN (Dinno, 2018), RNCL (Michonneau et al., 2023), and SPLITS (Ezard et al., 2021) packages. Single-gene trees and a combined tree were estimated to infer GMYC species under both the single-threshold (Pons et al., 2006) and multiple-threshold (Monaghan et al., 2009) models.

Results

In mtDNA *COI* gene region analyses, 655 base pairs of sequence data were used. In *ITS2* region analyses, 469 base pairs of sequence data, including indel regions, were used.

The parameters obtained using DnaSP v6.12.03 (Rozas et al., 2017) are summarized in Supplementary Figure 1. Seventeen haplotypes were observed with the *COI* marker, while eighteen haplotypes were observed with the *ITS2* marker. Haplotype diversity was 0.9310 for the *COI* marker and 0.9557 for the *ITS2* marker. Two conserved regions were found in the *COI* sequences, while six conserved regions were found in the *ITS2* sequences. Sequence conservation was 0.689 for the *COI* gene region and 0.660 for the *ITS2* region. The number of polymorphic sites was 204 for the *COI* region and 146 for the *ITS2* region. Additionally, the *ITS2* region contained 59 indel sites.

Transition/transversion rates were determined for the mtDNA *COI* gene region according to the TIM1+I model, based on BIC parameters, using JModelTest 0.1.1 (Posada, 2008) [R (AG= 15.4445), (AC= 1. 0000), (AT= 8.1402), (CG= 8.1402), (CT= 40.2487), (GT= 1.0000); equilibrium base frequencies A= 0.3112, C= 0.1418, G= 0.1380, T= 0.4090; p-inv value 0.6250 (Supplementary Figure 2). According to the TPM2+G model, selected based on BIC parameters for the rDNA *ITS2* region in JModelTest 0.1.1, the transition/transversion rates [R (AG= 3.3426), (AC= 2.3401), (AT= 2.3401), (CG= 1.0000), (CT= 3.3426), (GT= 1.0000); gamma shape value was 0.3400 (Supplementary Figure 3).

The accuracy of the BEAST2 analysis for the mtDNA *COI* and rDNA *ITS2* datasets was evaluated using Tracer, confirming that all analyses fell within the confidence interval and that posterior distributions for both markers were consistent. The *COI* marker had an ESS value of 6386.7164, a mean of -2341.2605, a median of -2340.9082, a variance of 32.3371, and a standard deviation of 5.6866 (Supplementary Figure 4). The *ITS2* marker

had an ESS value of 3625.3569, a mean of -1635.4064, a median of -1635.0427, a variance of 31.7037, and a standard deviation of 5.6306 (Supplementary Figure 5).

The trees obtained from BEAST v2.6.7 (Bouckaert et al., 2019) analyses conducted with the mtDNA *COI* gene region and rDNA *ITS2* region dataset were reciprocal (Figure 1). In the *COI* and *ITS2* trees, *Gonioctena fornicata* was an outgroup. The closely related species exhibited branching among themselves. The *COI* and *ITS2* markers identified species as monophyletic groups. Support for the inferred tree topology is reflected in the posterior probabilities assigned to each branch.

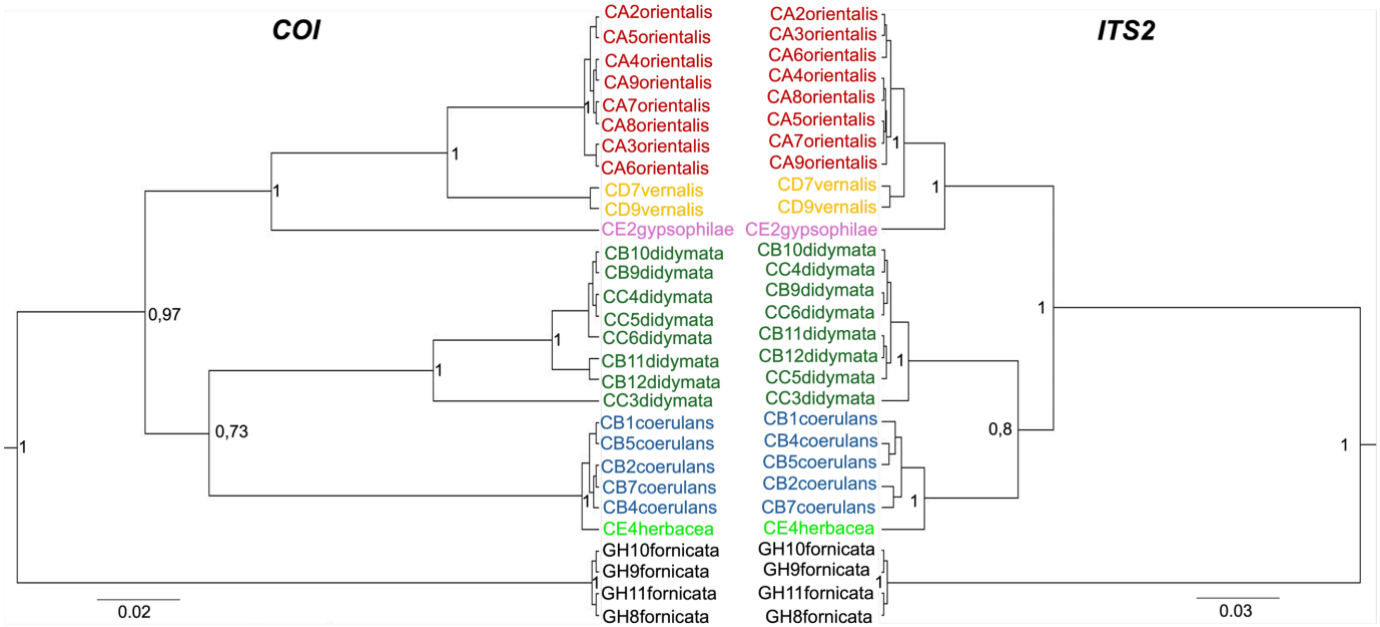

Figure 1. BEAST2-inferred trees based on *COI* (left) and *ITS2* (right) markers. Interspecific branching in gene trees showed similar patterns for *COI* and *ITS2* markers.

Phylogenetic analyses to determine species boundaries were performed separately for the both markers and gene trees were obtained. As a result of further analysis using integrated data sets, species trees were obtained. GMYC analysis of the mtDNA *COI* gene identified 6 clusters and 8 entities using the single-threshold method, whereas the multiple-threshold method detected 7 clusters and 9 entities. Similarly, analysis of the rDNA *ITS2* region yielded 6 clusters and 9 entities under the single-threshold method, while the multiple-threshold method recognized 6 clusters and 13 entities. Additionally, GMYC analysis based on concatenated sequence data (*COI* and *ITS2*) revealed 3 clusters and 3 entities using the single-threshold method and 4 clusters and 5 entities using the multiple-threshold method (Supplementary Figure 6-11).

To evaluate the combinability of the *COI* and *ITS2* markers, the Incongruence Length Difference (ILD) test was performed using PAUP version 4.0a 159 (Swofford, 2002). The result from Sequence Matrix 1.8 (Vaidya et al., 2011) generated a concatenated dataset, and the ILD test was performed with 1000 replicates by specifying the locations of the *ITS2* and *COI* regions in the PAUP (Swofford, 2002) program. The ILD test yielded a P-value of 0.30 (p> 0.01), indicating no significant phylogenetic incongruence between the two datasets; therefore, their concatenation is justified.

Phylogenetic analyses were conducted using BEAST v2.6.7 (Bouckaert et al., 2019) on a concatenated dataset of 1124 base pairs, comprising *COI* (positions 1–655) and *ITS2* (positions 656–1124), assembled in Sequence Matrix v1.8. For the concatenated dataset, the BEAST 2 parameters defined in the data analysis were applied. The combined tree derived from the merged dataset is presented in Figure 2. In the combined tree, constructed with the joint evaluation of both markers, the species were distinctly separated into monophyletic groups. *Gonioctena fornicata* individuals formed the outgroup. The combined tree of *Chrysolina* species is structured into two major clusters. One clade comprises *Chrysolina orientalis* (Olivier, 1807), *Chrysolina vernalis* (Brullé, 1833), and *Chrysolina gypsophilae* (Küster, 1845), while the other includes *Chrysolina didymata* (Scriba, 1791),

Chrysolina coerulans (Scriba, 1791), and *Chrysolina herbacea* (Duftschmid, 1825). Photographs of voucher specimens of *Chrysolina* species, with adapted scales, are presented. Voucher specimen photographs presented in Figure 2 are also provided as Supplementary Figure 12-18. Images of the specimens were captured using an Olympus SZX7 stereo microscope's camera. Moreover, in the tree obtained from the analysis, the subgenera to which the species belong are indicated. The phylogenetic analysis revealed that the subgeneric classification of the studied *Chrysolina* taxa is highly congruent with molecular data. The subgenus *Ovasoma* (Motschulsky, 1860) (*C. orientalis* and *C. vernalis*) formed a monophyletic group with a posterior probability of 1.00, where *C. vernalis* appeared as the sister group to the *C. orientalis* cluster. The subgenus *Stichoptera* (Motschulsky, 1860) (*C. gypsophilae*) was recovered as the sister group to the *Ovasoma* clade, suggesting a closer evolutionary relationship and a more recent common ancestor between these two subgenera. Furthermore, the subgenus *Hypericia* (Bedel, 1899) (*C. didymata*) constituted a distinct and robust clade. Within the subgenus Synerga (Weise, 1900), *C. coerulans* and *C. herbacea* constituted a clade, with the sole specimen as *C. herbacea* located basally to the *C. coerulans* group.The posterior probability values support the branching patterns observed in the tree.

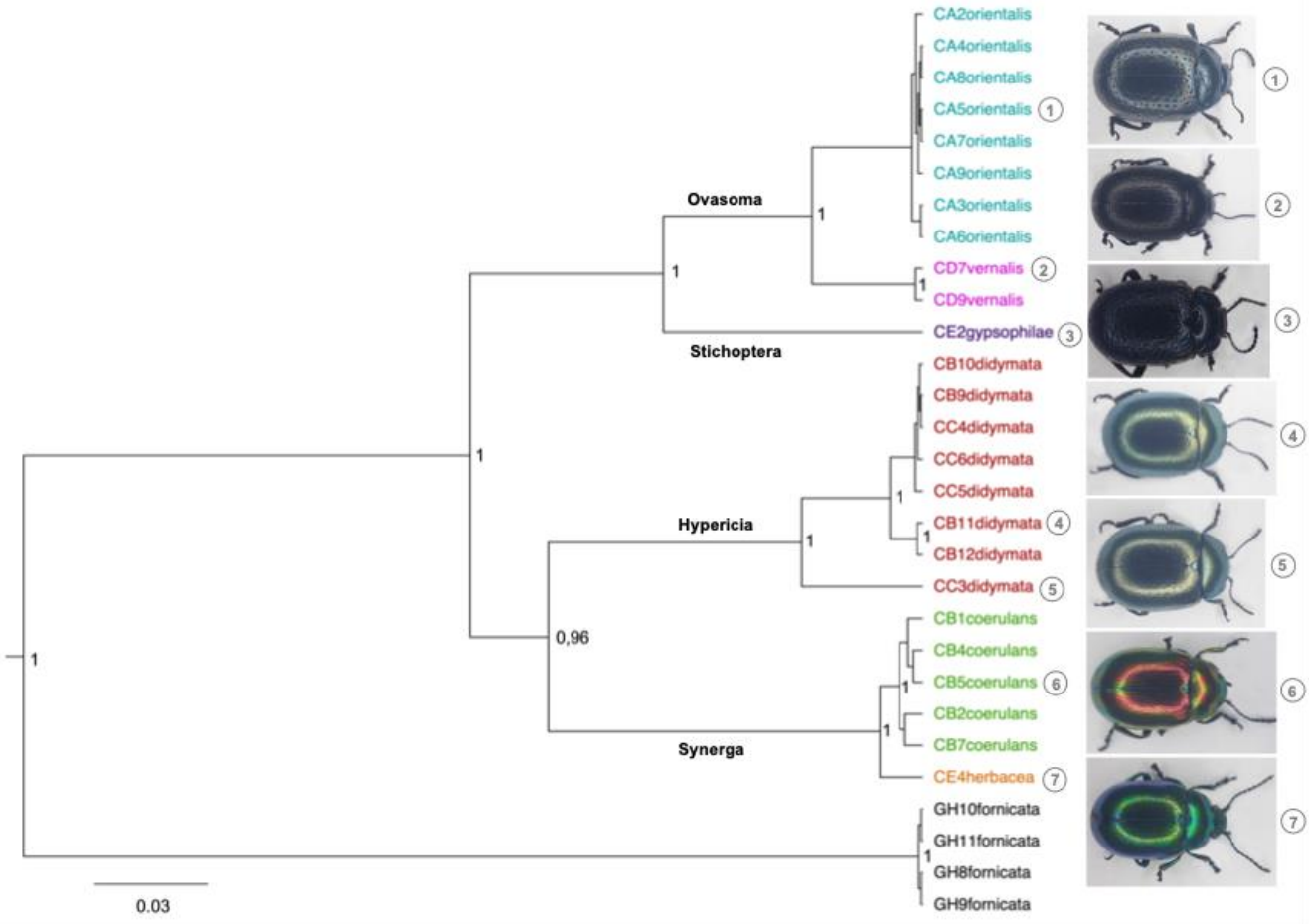

Figure 2. The combined tree derived from BEAST2 analyses of *COI* and *ITS2* markers. The bootstrap values on branches delineating species confirm species boundaries. Photographs of voucher specimens of *Chrysolina* species, with adapted scales, are shown.

Discussion

Traditional entomological studies rely heavily on morphological characters for species identification. However, in groups such as the genus *Chrysolina*, characterized by high species diversity and complex morphological variations, identification processes based solely on external morphology can be insufficient due to the presence of cryptic species. At this juncture, molecular markers have become indispensable tools for clarifying taxonomic boundaries and validating phylogenetic relationships. Contrary to single-locus analyses, a multilocus approach—in which cytochrome c oxidase subunit I (*COI*) and internal transcribed spacer II (*ITS2*) regions are evaluated in tandem—maximizes taxonomic accuracy.

While the mitochondrial *COI* gene is widely accepted as the "barcode region" in animal systematics and serves as a powerful tool for species-level differentiation, it may fail to provide adequate resolution in certain instances. *COI* sequences can exhibit overlapping variations between species, potentially leading to erroneous phylogenetic inferences. This study aims to test the taxonomic utility of the *ITS2* region, a nuclear ribosomal marker, across specific *Chrysolina* species represented in the fauna of Türkiye. Due to its location within the nuclear genome and its rapid evolutionary rate, *ITS2* serves as a complementary asset to the data provided by mitochondrial DNA.

The *COI* gene is widely recognized as a standard DNA barcode for animal taxa (Garin et al., 1999; Hebert et al., 2003a; Becerra, 2004; Lopes et al., 2015), whereas the *ITS2* region has proven to be a reliable marker for species identification across a broad range of taxa (Ben-David et al., 2007; Prasad et al., 2009; Li et al., 2010; Calzolari et al., 2024).

In plants, by comparing seven candidate DNA barcodes (*psbA-trnH, matK, rbcL, rpoC1, ycf5, ITS2,* and ITS), it was presented that the *ITS2* marker could potentially be used as a standard DNA barcode to identify medicinal plants (Chen et al., 2010). Despite its high mutation rate, the *ITS2* region maintains a conserved secondary structure due to its essential role in ribosome biogenesis (Budak et al., 2016). This structural stability, along with its amplification using universal primers, presence of conserved regions, ease of reproduction, and sufficient genetic variation, makes *ITS2* a promising DNA barcode candidate for distinguishing closely related animal species (Yao et al., 2010).

Research has consistently demonstrated that combining *COI* and *ITS2* markers can significantly enhance the resolution of phylogenetic relationships. For example, Jurado-Rivera & Petitpierre (2015) performed a phylogenetic analysis of the genus *Chrysolina* using mitochondrial (*COI* and *rrnL*) and nuclear (*H3*) DNA sequences, reporting findings that align with our results. Similarly, Germain et al. (2013) analysed *COI* and *ITS2* markers in *Epitrix* Foudras, 1859 (Coleoptera: Chrysomelidae) species, demonstrating that *ITS2* is an effective complement to *COI* for species identification. Moreover, Wang et al. (2023), in their comparative study of *ITS2* and *COI* markers in *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae), emphasized the complementary nature of these markers in molecular identification and their combined effectiveness in enhancing phylogenetic resolution for species delimitation. Additionally, Calzolari et al. (2024) utilized *COI* and *ITS2* markers for DNA-based species delimitation of the *Anopheles maculipennis* Meigen, 1818 (Diptera: Culicidae) incorporating morphometric data into their analysis. Their results indicated that while the *COI* tree exhibited a more complex branching pattern, the intraspecific groups identified through *COI* lacked morphometric support, whereas *ITS2* demonstrated greater reliability in species differentiation within this framework. In summary, while previous studies have applied *COI* and *ITS2* markers to various Chrysomelidae taxa, this research represents the first comparative analysis of *COI* and *ITS2* sequences within the genus *Chrysolina* distributed in Türkiye. Moreover, a significant lack of information remains on the Chrysomelidae family in Western Anatolia due to the limited number of studies.

In this study, the monophyly of species were assessed using a Bayesian approach, incorporating *COI* and *ITS2* markers in selected *Chrysolina* species. As a result, species that were morphologically distinct were also found to be significantly differentiated at the molecular level. For both markers, *Chrysolina* species exhibited monophyletic segregation. All trees were structured into two primary clades. *C. orientalis, C. vernalis,* and *C. gypsophilae* cluster within one clade, while *C. didymata, C. coerulans,* and *C. herbacea* form the other (Figure 1 and Figure 2).

The combined tree closely resembled the trees constructed using *COI* and *ITS2* markers, indicating strong concordance between the individual marker analyses. In the combined tree, where both markers were analysed together, the species were distinctly monophyletic, demonstrating clear genetic separation. Additionally, the posterior probability values assigned to each branch in the gene trees obtained from the analyses further support the phylogenetic structure. Species within the same subgenus clustered together on the gene tree, reflecting their phylogenetic relationships (Figure 2).

To explain in terms of subgenus and morphological characters; *C. vernalis* has a dorsum that is usually bright metallic blue, though individuals may occasionally exhibit bluish-green, golden-green, bronze-green, or coppery coloration. In contrast, *C. orientalis* displays a dull dark golden-green dorsal surface, with elytral

punctures that are large, bluish-black, and encircled by a purple aureole. The pronotal disc is densely and finely punctate, with lateral furrows deep and narrow in the basal third but replaced by broad shallow impressions in the anterior two-thirds. The species *C. vernalis* and *C. orientalis*, both belonging to the subgenus *Ovasoma*, are distinguished from each other by these morphological characters (Bieńkowski, 2019). *Chrysolina gypsophilae*, belonging to the subgenus *Stichoptera,* elytra black with reddish-yellow lateral margins and epipleura, irregularly punctate, bearing a rufous lateral stripe across 2–3 external intervals with denticulate upper edge; pronotal lateral impression basally deep and wrinkled, lateral callus convex; dorsum bluish-black, hind wings normally developed; length 8.2–11.3 mm. The common ancestry of Ovosoma and Stichoptera, as demonstrated by Garin et al. (1999) and Jurado-Rivera & Petitpierre (2015), was confirmed in this study. Besides, *Chrysolina gypsophilae* is distinguished from *C. herbacea*, *C. coerulans* and *C. didymata* by its black elytra with reddish-yellow lateral margins and epipleura, irregularly punctate, whereas in the latter species the elytra are concolorous and entirely metallic green, blue, or black (Bieńkowski, 2019; Kasap, 1988). Moreover, *C. herbacea* and *C. coerulans*, both belonging to the subgenus *Synerga*, can be distinguished by the structure of the prosternal process and body coloration. In *C. herbacea*, the prosternal process bears a shallow, large, and rugose depression, and the body is metallic light green. In contrast, *C. coerulans* exhibits a deeper and smooth depression on the prosternal process, with a metallic green body and elytra marked by sutural and sublateral bands showing bluish reflections. Finally, *C. didymata*, belonging to the subgenus *Hypericia*, shares the common character of concolorous elytra, entirely metallic green, blue, or black, with *C. coerulans* and *C. herbacea*, indicating a close branching relationship. It is distinguished from these species by the presence of sublateral incisions on the pronotum behind the middle, elytral punctures arranged in five paired rows, and a metallic blue body with elytra finely punctured, the punctures distinctly aligned in rows and the interstices very finely punctate (Kasap, 1988; Warchalowski, 2010a, b; Bieńkowski, 2019). In light of these identification keys (Kasap, 1988; Warchalowski, 2010a, b; Bieńkowski, 2019), the topology recovered in the gene trees is well supported. The species are consistently grouped at the subgenus level, and the clusters observed in the trees are corroborated by the shared morphological characters. Among the samples, only “CC3didymata,” identified morphologically as *C. didymata*, showed a separate branching pattern from the clustered *C. didymata* specimens (Figure 2). The comparative photographs in Figure 2 demonstrate that no distinct morphological differences can be observed between the “CC3didymata” specimen and other *C. didymata* specimens. This finding indicates that the “CC3didymata” specimen exhibits a level of similarity that cannot be distinguished from intraspecific morphological variation. Therefore, it is considered that the specimen in question may represent a ‘cryptic species’. Furthermore, the specimen “CE4herbacea” clustered with *C. coerulans*, a species belonging to the same subgenus, indicating a close phylogenetic relationship (Figure 2).

The GMYC analyses conducted with different markers and threshold models revealed considerable variation in the inferred species boundaries. Analyses of the *COI* gene and *ITS2* region separately produced clustering patterns largely consistent with the six morphologically defined species (Supplementary Figure 6,8) though both markers yielded a higher number of entities under the multiple-threshold model (Supplementary Figure 7,9). In particular, *ITS2* under the multiple-threshold approach identified up to 13 entities (Supplementary Figure 9). This outcome may be explained, as noted by Fujisawa & Barraclough (2013), by the tendency of the multiple-threshold approach to overestimate the number of delimited species. By contrast, concatenated analyses of *COI* and *ITS2* reduced the number of clusters to three or four (Supplementary Figure 10, 11). This finding may be explained by limited sample representation, hindering the full reflection of genetic diversity and, consequently, leading to narrower delineation of species boundaries. Therefore, the results should be interpreted within the scope of the available sampling, and future studies incorporating broader datasets will contribute to a more reliable resolution of species boundaries. These findings highlight the sensitivity of GMYC results to both marker choice and threshold model, underscoring the importance of integrating morphological evidence with multiple independent molecular datasets to achieve robust species delimitation.

The primary aim of this study was to determine whether the *ITS2* marker exhibits a level of effectiveness comparable to that of the *COI* marker in molecular identification and species delimitation. When viewed in light of all findings, the rDNA *ITS2* region shows comparable utility to the mtDNA *COI* marker in resolving genetic diversity among insect taxa, underscoring its potential as a complementary or alternative marker in taxonomic studies. The generalizability of these findings is limited to the *Chrysolina* species analyzed within the scope of the current study. Future research aims to expand taxonomic sampling and incorporate additional molecular markers to enhance phylogenetic resolution.

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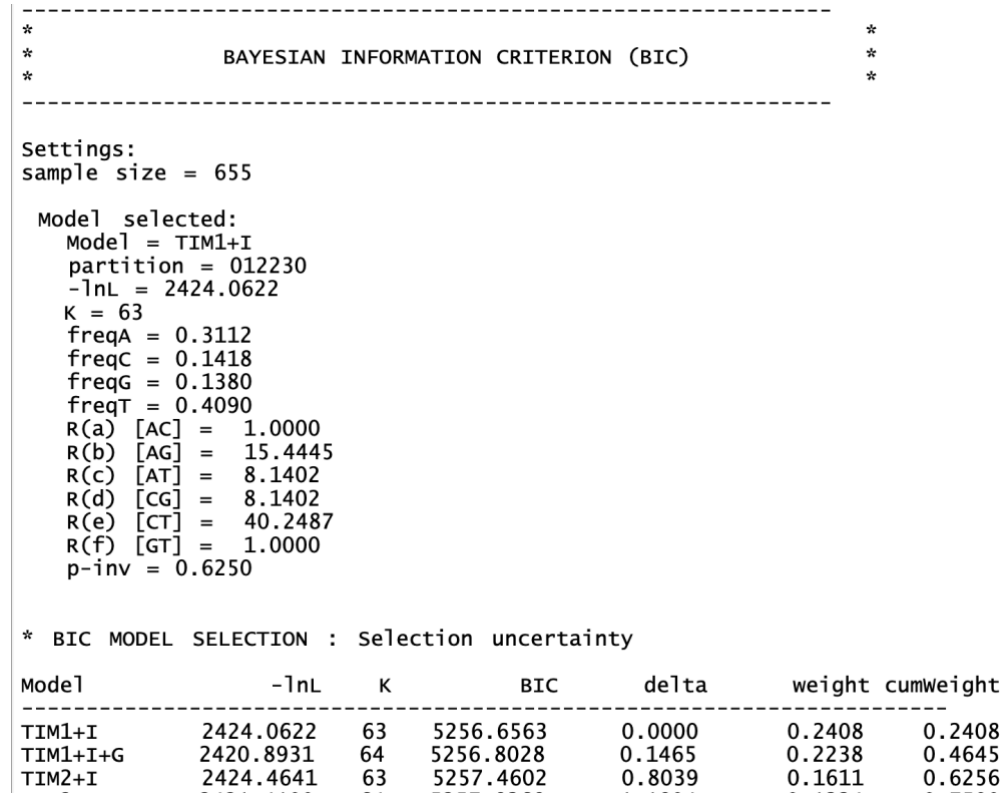
Supplementary Data

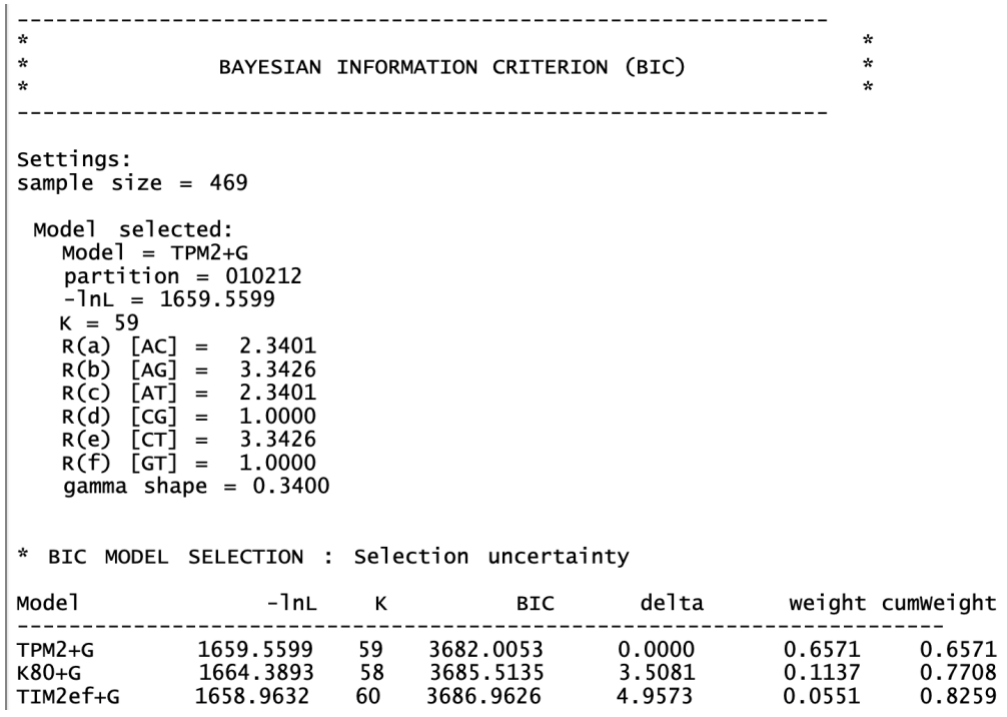
DnaSP v6.12.03	Parameter	*COI*	*ITS2*
Haplotypes	Number of haplotypes	H: 17	H: 18
	Haplotypes	H1:CB1coerulans H2:CB5coerulans H3:CB4coerulans H4:CE4herbacea H5:CB7coerulans H6:CB2coerulans H7:CB9,CC4,CC5,CB10 didymata H8:CC6didymata H9:CB11didymata H10:CB12didymata H11:CC3didymata H12:CA3, CA6 orientalis H13:CA8,CA9,CA7,CA5,CA4,CA2 orientalis H14:CD7vernalis H15:CD9vernalis H16:CE2gypsophilae H17:GH8, GH9, GH10, GH11 fornicata	H1:CA2,CA3,CA6 orientalis H2:CA4,CA5,CA7,CA8 orientalis H3:CA9orientalis H4:CE2gypsophilae H5:CD7vernalis H6:CD9vernalis H7:CB1coerulans H8:CB2coerulans H9:CB5coerulans H10:CB7coerulans H11:CB4coerulans H12:CE4herbacea H13:CB9,CC4 didymata H14:CC6,CB10 didymata H15:CC5didymata H16:CB11,CB12 didymata H17:CC3didymata H18:GH8,GH9,GH10,GH11 fornicata
	Haplotype diversity	Hd: 0,9310	Hd: 0,9557
Conserved Regions	Conserved Region	2	6
	Net number of analyzed sites	L: 655	L: 430
	Number of variable/polymorphic sites	S: 204	S: 146
	Sequence conservation	C: 0,689	C: 0,660
DNA Polymorphism	Number of polymorphic (segregating) sites	S: 204	S: 86
	Total number of mutations	Eta: 251	Eta: 105
	Haplotype (gene) diversity	Hd: 0,931	Hd: 0,936
	Variance of Haplotype diversity	0,00086	0,00045
	Standard Deviation of Haplotype diversity	0,029	0,021
	Nucleotide diversity	Pi: 0,11849	Pi: 0,09558
	Theta (per site) from Eta	0,09758	0,0791
	Theta (per site) from S	Theta-W: 0,07931	Theta-W: 0,06479
	Variance of theta (no recombination)	0,0006224	0,0004409
	Standard deviation of theta (no recombination)	0,02495	0,021
	Variance of theta (free recombination)	0,0000308	0,0000488
	Standard deviation of theta (free recombination)	0,00555	0,00699
	Theta (per site) from Pi	0,14072	0,10954
	Theta (per site) from S	0,09705	0,07616
	Theta (per site) from Eta	0,1097	0,08689
	Average number of nucleotide differences	k: 77,608	k: 32,305
	Stochastic variance of k (no recombination)	Vst(k): 1101,243	Vst(k): 195,895
	Sampling variance of k (no recombination)	Vs(k): 82,633	Vs(k): 14,676
	Total variance of k (no recombination)	V(k): 1183,877	V(k): 210,571
	Stochastic variance of k (free recombination)	Vst(k): 25,869	Vst(k): 10,768
	Sampling variance of k (free recombination)	Vs(k): 1,848	Vs(k): 0,769
	Total variance of k (free recombination)	V(k): 27,717	V(k): 11,538
	Theta (per sequence) from S	Theta-W: 51,946	Theta-W: 21,899
	Variance of theta (no recombination)	267,016	50,375
	Variance of theta (free recombination)	13,227	5,576
	Sites with alignment gaps or missing data	0	131
	Invariable (monomorphic) sites	451	252
	Variable (polymorphic) sites	204	86
	Total number of mutations	251	105
	Protein Coding Region assignation	Yes	No
InDels Polymorphism	Number of sites with missing data	0	72
	Total number of InDel sites	0	59
	Number of InDel Haplotypes	0	6
	InDel Haplotype Diversity	0	0,8
	InDel Diversity, k(i):	0	5,31
	InDel Diversity per site, Pi(i):	0	0,01439
	Tajima's D:	0	1,03755
	Statistical significance:	0	P > 0.10
Syn-NonSyn. Substitutions	Genetic Code	mtDNA Drosophila	Standart
	Number of codons analyzed	218 (654 sites)	No coding region
	Number of protein coding regions (exons)	1	
	Number of noncoding regions	0	
	Protein coding region, from site	1 to 655	
	Number of Synonymous sites	152,22	
	Number of NonSynonymous sites	501,78	

Supplementary Figure 1. *COI* and *ITS2* markers comparison in DnaSP.



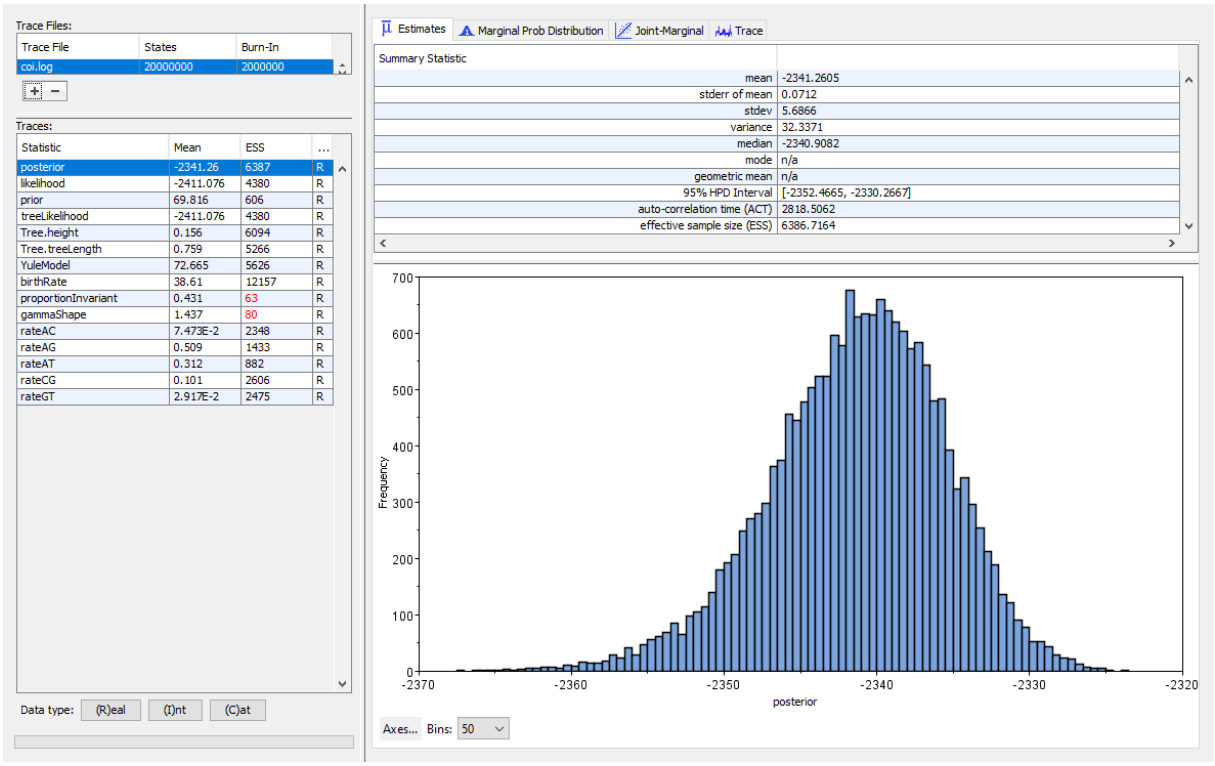
```
-------------------------------------------------------------
*                                                           *
*               BAYESIAN INFORMATION CRITERION (BIC)        *
*                                                           *
-------------------------------------------------------------

Settings:
sample size = 655

 Model selected:
   Model = TIM1+I
   partition = 012230
   -lnL = 2424.0622
   K = 63
   freqA = 0.3112
   freqC = 0.1418
   freqG = 0.1380
   freqT = 0.4090
   R(a) [AC] =  1.0000
   R(b) [AG] =  15.4445
   R(c) [AT] =  8.1402
   R(d) [CG] =  8.1402
   R(e) [CT] =  40.2487
   R(f) [GT] =  1.0000
   p-inv = 0.6250


* BIC MODEL SELECTION : Selection uncertainty

Model              -lnL     K          BIC       delta       weight cumWeight
-------------------------------------------------------------------------
TIM1+I         2424.0622    63    5256.6563      0.0000       0.2408     0.2408
TIM1+I+G       2420.8931    64    5256.8028      0.1465       0.2238     0.4645
TIM2+I         2424.4641    63    5257.4602      0.8039       0.1611     0.6256
```

Supplementary Figure 2. The result of JModelTest 0.1.1 for the mtDNA *COI* gene region based on BIC parameters.

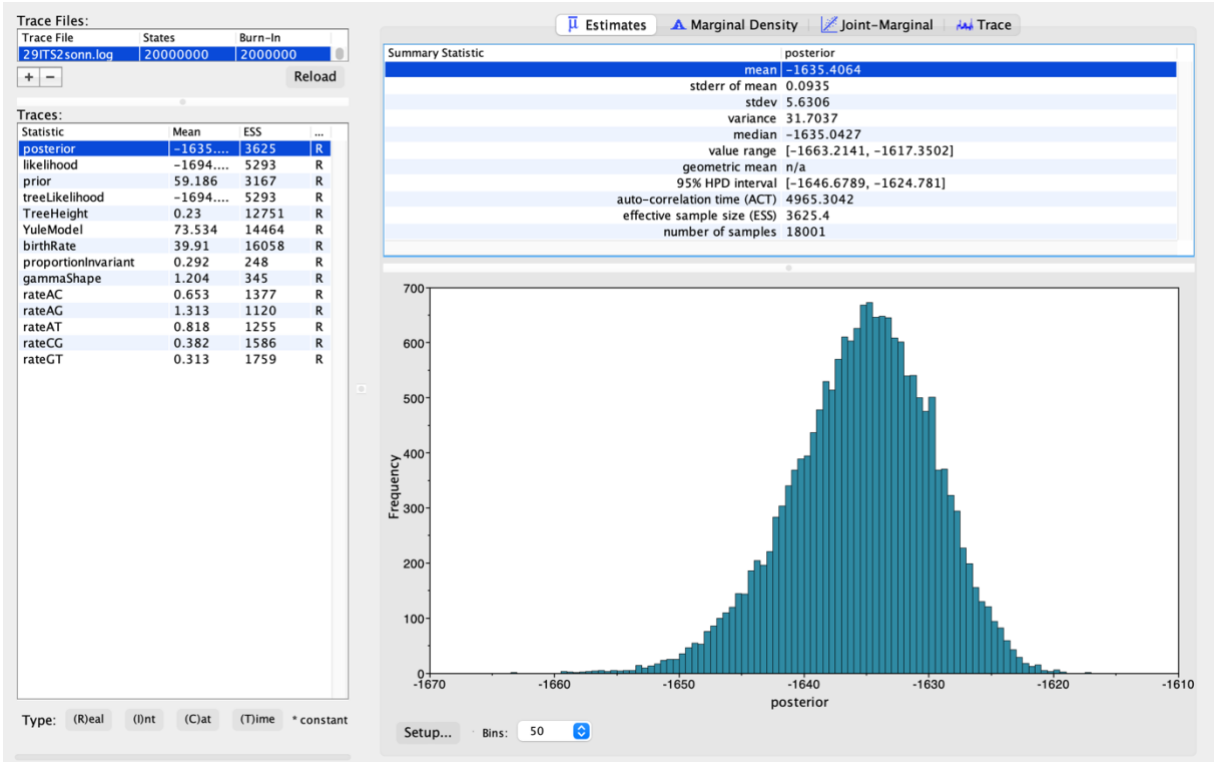


```
-------------------------------------------------------------
*                                                           *
*               BAYESIAN INFORMATION CRITERION (BIC)        *
*                                                           *
-------------------------------------------------------------

Settings:
sample size = 469

 Model selected:
   Model = TPM2+G
   partition = 010212
   -lnL = 1659.5599
   K = 59
   R(a) [AC] =  2.3401
   R(b) [AG] =  3.3426
   R(c) [AT] =  2.3401
   R(d) [CG] =  1.0000
   R(e) [CT] =  3.3426
   R(f) [GT] =  1.0000
   gamma shape = 0.3400


* BIC MODEL SELECTION : Selection uncertainty

Model              -lnL     K          BIC       delta       weight cumWeight
-------------------------------------------------------------------------
TPM2+G         1659.5599    59    3682.0053      0.0000       0.6571     0.6571
K80+G          1664.3893    58    3685.5135      3.5081       0.1137     0.7708
TIM2ef+G       1658.9632    60    3686.9626      4.9573       0.0551     0.8259
```

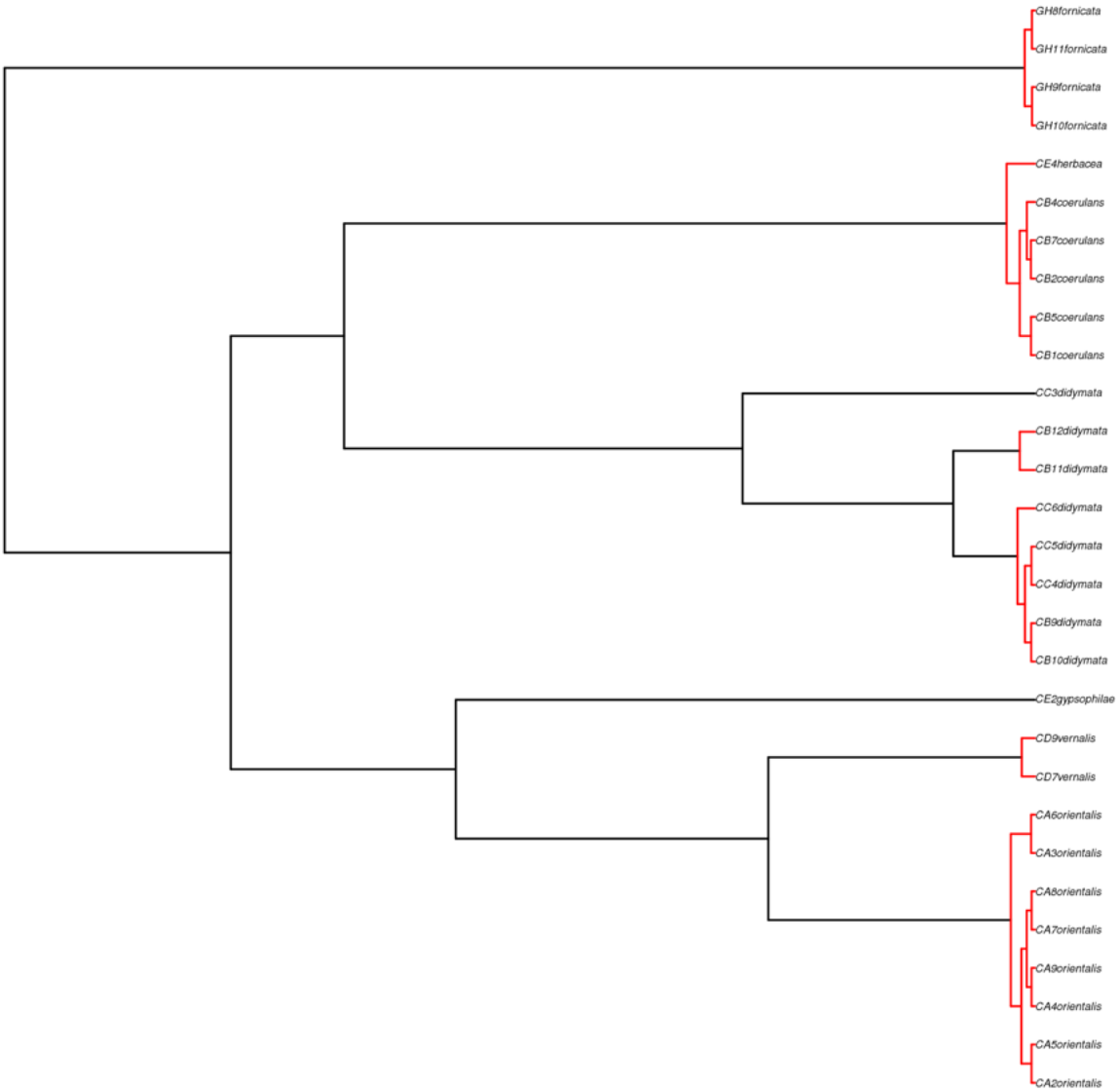
Supplementary Figure 3. The result of JModelTest 0.1.1 for the rDNA *ITS2* region based on BIC parameters.



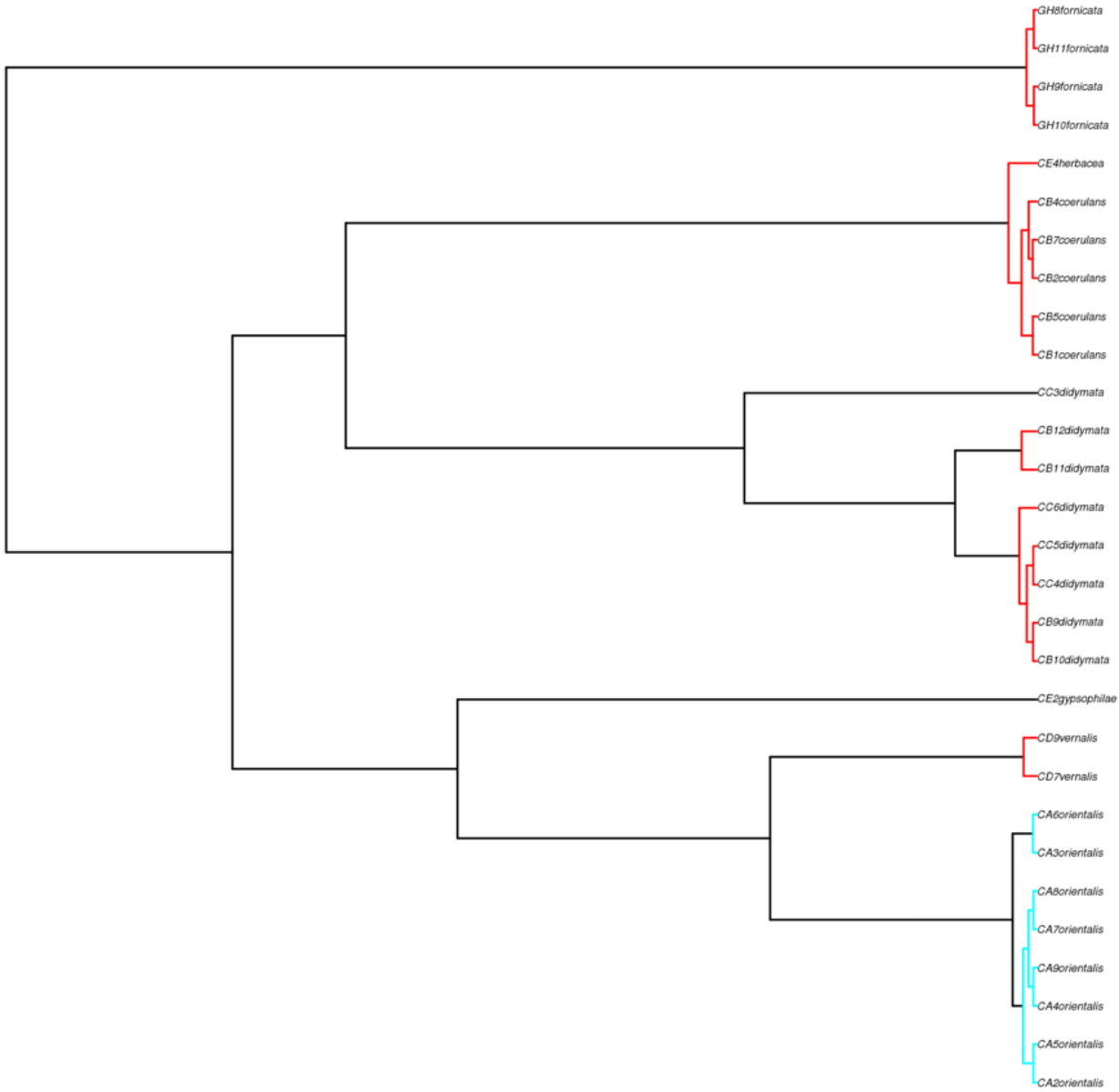
Supplementary Figure 4. Tracer's accuracy results for the mtDNA *COI* gene region.



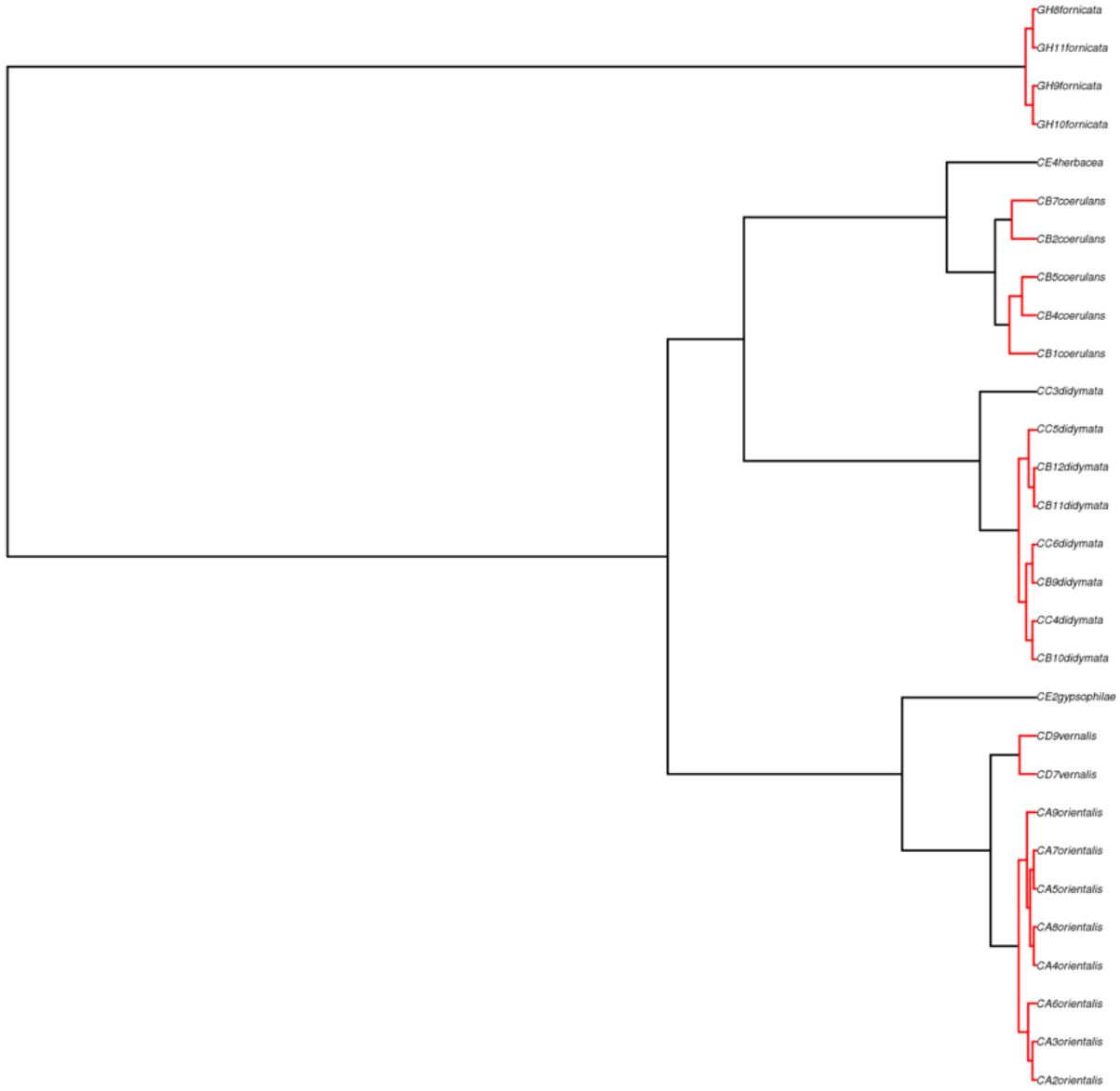
Supplementary Figure 5. Tracer's accuracy results for the rDNA *ITS2* region.



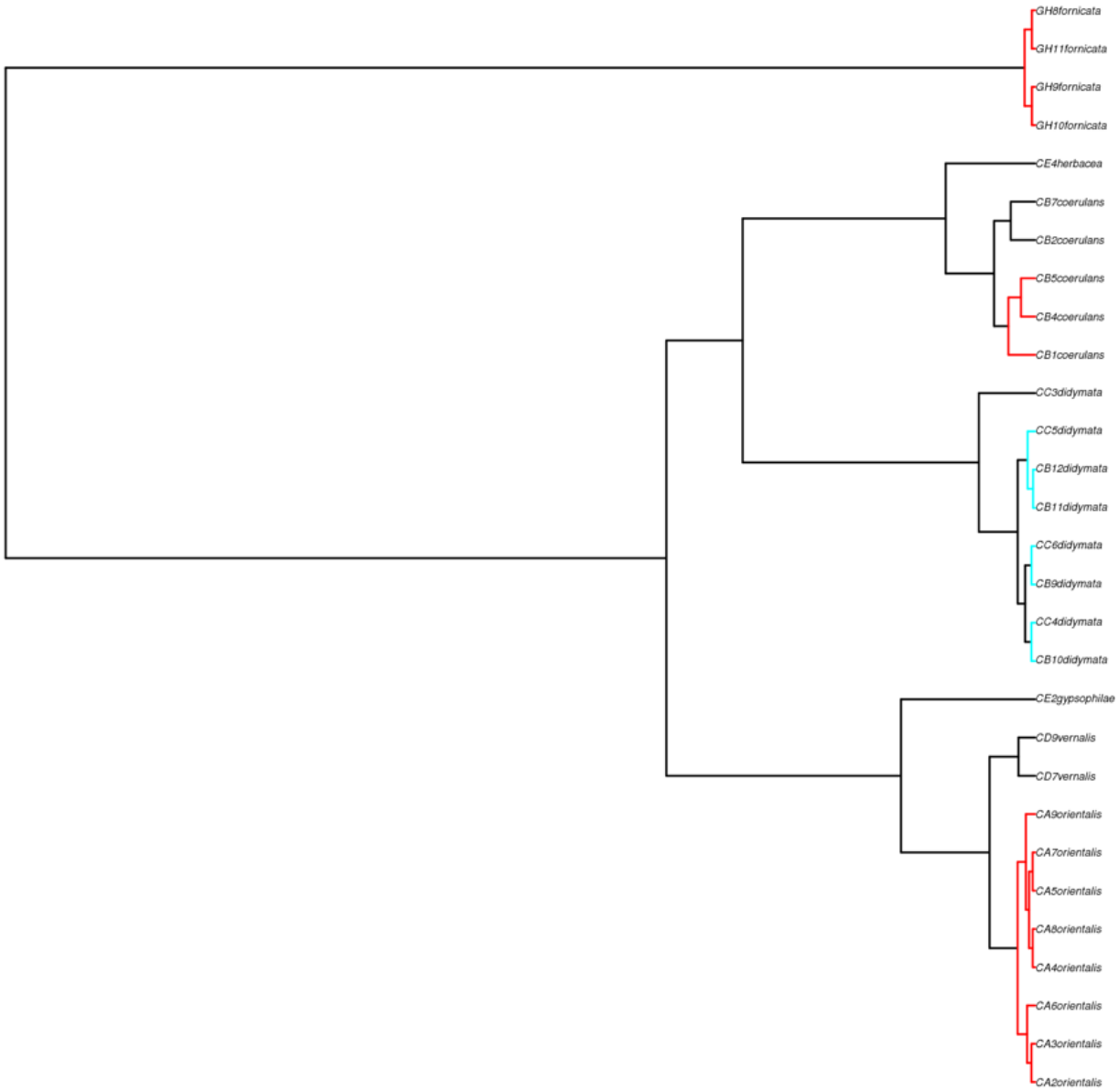
Supplementary Figure 6. mtDNA *COI* gene suggests 6 clusters – 8 entities using the single threshold method.



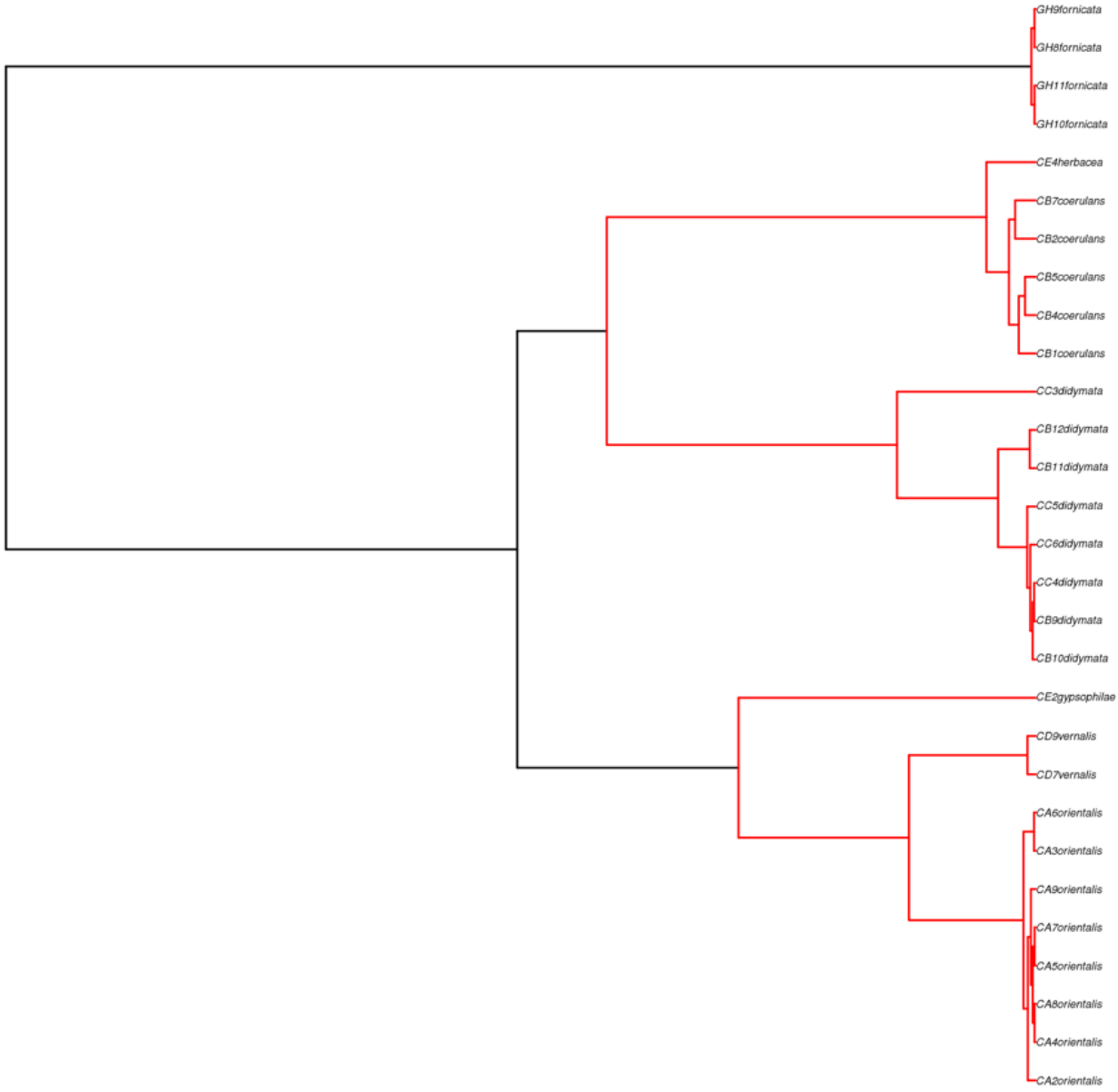
Supplementary Figure 7. mtDNA *COI* gene suggests 7 clusters – 9 entities using the multiple threshold method.



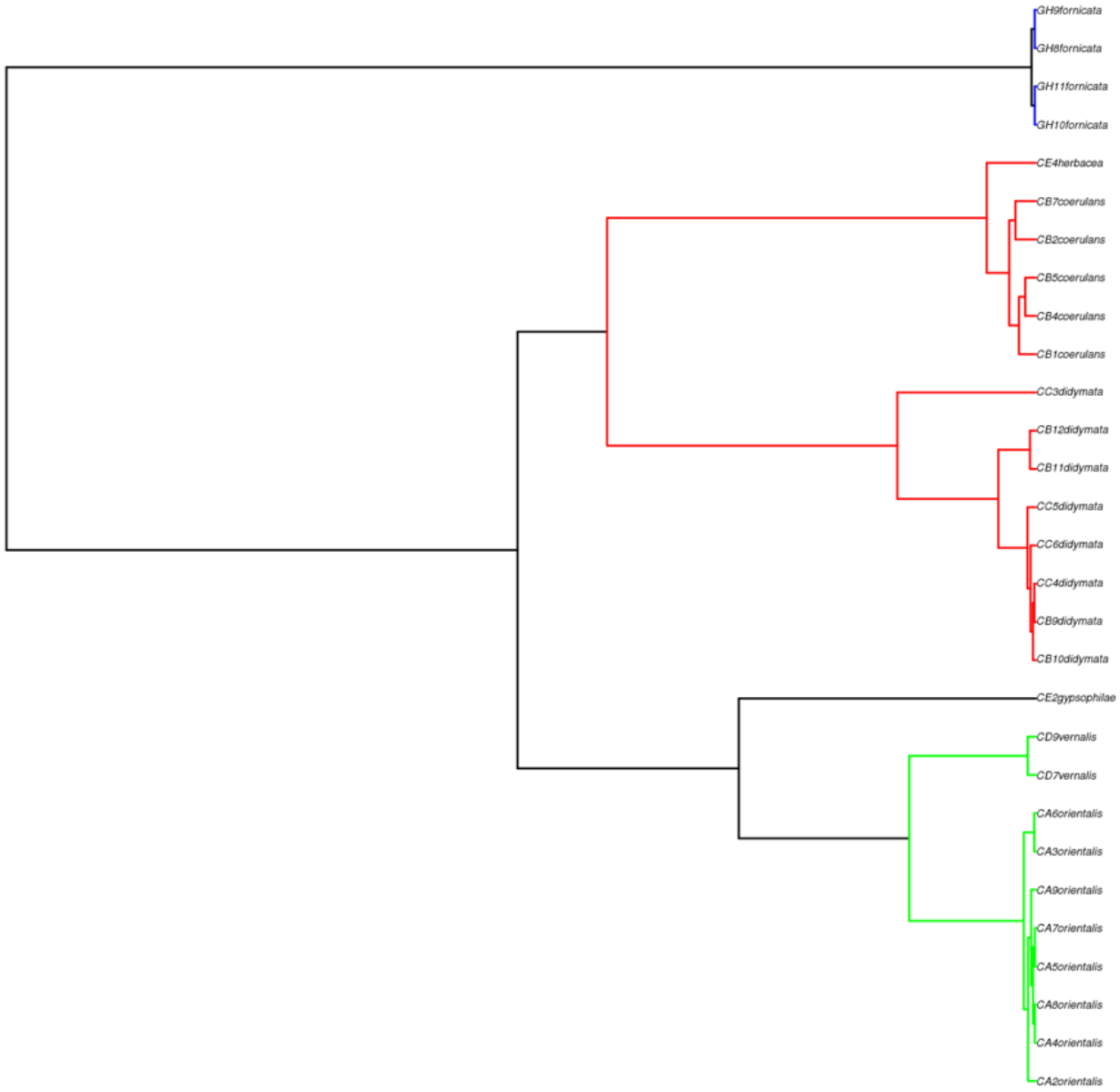
Supplementary Figure 8. rDNA *ITS2* region suggests 6 clusters – 9 entities using the single threshold method.



Supplementary Figure 9. rDNA *ITS2* region suggests 6 clusters – 13 entities multiple threshold method.



Supplementary Figure 10. Concatenated sequence data (*COI* and *ITS2*) suggests 3 clusters – 3 entities using the single threshold method.



Supplementary Figure 11. Concatenated sequence data (*COI* and *ITS2*) suggests 4 clusters – 5 entities using the multiple threshold method.

Supplementary Figure 12. The specimen “CA5orientalis” as a representative of *Chrysolina orientalis*.

Supplementary Figure 13. The specimen “CD7vernalis” as a representative of *Chrysolina vernalis*.

Supplementary Figure 14. The specimen “CE2gypsophilae” as a representative of *Chrysolina gypsophilae*.

Supplementary Figure 15. The specimen “CE4herbacea” as a representative of *Chrysolina herbacea*.

Supplementary Figure 16. The specimen “CB11didymata” as a representative of *Chrysolina didymata*.

Supplementary Figure 17. The specimen “CC3didymata” as a representative of *Chrysolina didymata*.

Supplementary Figure 18. The specimen “CB5coerulans” as a representative of *Chrysolina coerulans*.