Contents lists available at Dergipark
Journal of Scientific Reports-A

journal homepage: *https://dergipark.org.tr/tr/pub/jsr-a*

E-ISSN: 2687-6167 ***Number 56, March 2024***

RESEARCH ARTICLE

Receive Date: 24.10.2023 *Accepted Date: 10.01.2024*

Determination of the species boundaries of genus *Dolerus* (Hymenoptera: Tenthredinidae) using the *COI* gene

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Abstract

New generation molecular approaches and methods are being developed to identify species and determine species boundaries. There are many different approaches of species delimitation used to assess the species richness of poorly studied and highly diverse invertebrate taxa. The basis of these approach is DNA barcoding studies. DNA barcoding has been used as a powerful tool for species identification and delimitation. Although DNA barcoding studies have been carried out on the family Tenthredinidae, there are no studies on species delimitation. Herein, we compare species delimitation analyzes belong to *Dolerus* genus based on *cytochrome c oxidase I* (*COI*) region. In this context, it was used five species delimitation approaches (ABGD, ASAP, DNA Taxon, PTP and GMYC). Thirty-six morphotypes were used in the study. These morphotypes separated into six species (*Dolerus triplicatus*, *Dolerus germanicus*, *Dolerus puncticollis*, *Dolerus nigratus*, *Dolerus* sp1 and *Dolerus* sp2) in ABGD, ASAP and DNA Taxon approaches. Two additional species were introduced because of the tree-based PTP and GMYC approaches. These species were named as *Dolerus* sp3 and *Dolerus* sp4 which were separated from *Dolerus puncticollis* clade and *Dolerus nigratus* clade, respectively. These analyzes were supported by the phylogenetic tree and CBC entities that constitute the ITS2 data.

Keywords: COI; Dolerus; Hymenoptera; Species Delimitation; Tenthredinidae.

1. Introduction

Hymenoptera, one of the 'big four' megadiverse insect orders, has more than 153,000 described and one million estimated species [1, 2, 3]. Along with species richness, the lifestyles of Hymenoptera are extremely diverse, ranging from feeding on or in plants to a wide variety of parasitic and predatory species [4, 5]. Symphyta (Gerstacker, 1867), commonly known as sawflies [6], is a small suborder of Hymenoptera represented with 4,396 species. [7]. Tenthredinidae is the largest of the nine families of Symphyta suborder and includes 415 genera comprising 5721 species [7]. *Dolerus* (Panzer, 1801), is a genus belonging to Tenthredinidae, has 259 species distributed in the Palearctic and Nearctic regions [7, 8]. Adults and larvae of *Dolerus* are found in different habitats: open vernal pools like sedges (Cyperaceae), horsetails (Equiseataceae), open, wet, grass communities (Poaceae)

and rushes (Juncaceae) [9].

The morphological identification problems and inadequate taxonomic studies of sawflies lead to difficulties in identification of these taxa. Although, there are many studies on the order Hymenoptera involving both DNA barcoding and species delimitation approaches [10-17], the number of studies on phylogenetic relationships of Symphyta is still limited [1]. Both conventional taxonomy and molecular marker investigations have been conducted on the Tenthredinidae [18-25], however none of them have employed species delimitation techniques.

The finding of unique morphological differences in identification keys was the foundation of traditional taxonomy, which is still in widespread use today. However, modern approaches are being developed every day to identify species and determine species boundaries [26]. Integrative taxonomy, which includes DNA data and morphology-dependent analyses, is now utilized for efficient taxonomic identification [27, 28]. DNA barcoding [29, 30] refers to the utilization of the *cytochrome c oxidase I* (*COI*) region, located on mitochondrial DNA (mtDNA), to efficiently and precisely identify species of taxa that are challenging to discern based on their morphology. These studies mostly use mitochondrial gene (*COI*) or nuclear region (ITS2) which known as molecular markers [31]. For insects, an approximately 650 bp fragment of the *COI* is used as the standard "barcode region" [32, 33]. The relatively high mutation rate of mitochondrial genes compared to nuclear genes allows us to reveal phylogenetic relationships and incompatibilities such as geographic variation [34, 35]. The *COI* gene has an important role in revealing the taxonomy and evolutionary relationships in the DNA barcoding studies, due to its comprising both highly conserved and variable regions [36]. Because of all these advantages, the *COI* gene is preferred in barcoding studies by many researchers. The *COI* gene has been used for species delimitation approaches also in many Hymenoptera families, including diverse groups such as Braconidae [37, 38], Formicidae [39], Gasteruptiidae [40, 41], Eurytomidae [42], Vespidae [43], Ichneumonidae [44]. The barcoding and species delimitation studies can also show unsolved diversity [45], reveal lineages or point out new species [46].

Contemporary molecular-based species delimitation analyses consist of procedures for classifying individuals as either members of an existing species or as new species [47]. These analyzes are now widely used in a variety of taxa to support traditional taxonomy [48, 49]. A single locus is considered ideal in these analyses, while multiple loci may sometimes be preferred. Single-locus species delimitation methods are still widely applied in both DNA barcoding and species delimitation studies involving organisms like bacteria, fungi, vertebrates, and invertebrates [50, 51]. Species delimitation approaches can also use processed data such as distance or phylogenetic trees. The aim of the using different data is to verify consistency of results [52-54].

It is important to use different genes or additional data such as morphology in integrative taxonomic analyzes to delimit the species more accurately [27, 28]. Over the last 20 years, ITS2 region together with *COI*, has been the most popular marker for phylogeny and species identification from different perspectives [55, 56]. However, high variation of ITS2 prevent its safe use in species delimitation and it has been understood that the sequences of ITS2, are not informative enough for species-level comparisons for some insect genera [57]. Therefore, revealing the species diversity needs further use of DNA barcoding and species delimitation approaches with different gene or regions [6].

Many of the species groups in the genus of *Dolerus* have not been yet resolved taxonomically [58]. Therefore, we preferred species delimitation approaches used in taxonomic and molecular studies here. The aims of the present study: a) to compare distance- and tree-based species delimitation approaches on *Dolerus* genus, b) to compare the results of the species delimitation analyzes with those of our previous ITS2-based study [25]. For this purpose, we utilized the *COI* phylogenetic tree, genetic distances, and comparison of various species delimitation approaches. At the same time, our study represents the first evaluation of comparing species delimitation approaches based on partial *COI* data of the genus *Dolerus* using molecular data.

2. Materials and Methods

2.1. Molecular analysis

DNA extracts of 36 morphospecies from the genus *Dolerus* identified in our previous study, obtained by using

Salting out protocol [59], were preserved at -20ºC in Entomological Collection of Cumhuriyet University, Sivas. These samples were used for the amplification of the *COI* region by using primer pairs s1859 (5'-GGA ACI GGA TGA ACW GTT TAY CCI CC -3') and a2590 (5'-GCT CCT ATT GAT ARW ACA TAR TGR AAA TG-3') [60]. PCR reactions and cycling conditions were taken from Gülmez et. al. 2022 [25] except for the annealing stage which is conducted at 46ºC for 30 s. The obtained PCR products were visualized by electrophoresing on the 1% agarose gel. PCR products were then sequenced using Sanger technology (Macrogen Ltd., Seoul, Korea) in both directions.

2.2. Phylogenetics analysis

The raw sequences of 36 samples from the genus *Dolerus* were generated for this study and each sequence with the forward and reverse direction were assembled, edited, and manually checked by eye using Geneious R9 [61]. Each partial *COI* sequence was checked whether belonging to the genus *Dolerus* using "blastn" algorithm [62]. The sequences were deposited to GenBank under the accession numbers OR721886- OR721921. Alignments of partial *COI* sequences of the 36 samples of *Dolerus* were performed using the MAFFT algorithm [63]. Pairwise genetic distances of the partial *COI* dataset were determined using Kimura-2 (K2P) [64] and uncorrected distance (p-distance) parameters in MEGA11 [65]. These distance data were exported as a MEGA file to be used in Automatic Barcode Gap Discovery (ABGD) analysis, one of the species delimitation tests [36]. The best-fit model of nucleotide substitution was determined using jModelTest 2.1.7 [66] and fasta file were created using only 1st and 2nd codon positions by MEGA11 due to the substitution saturation in 3rd codon positions [65]. The dataset was used both for the construction of Maximum Likelihood (ML) tree using Randomized Axelerated Maximum Likelihood-High Performance Computing (RAxML-HPC) v.8 with 1000 bootstrap replications in CIPRES portal [67] and construction of Neighbor-Joining (NJ) tree with 1000 bootstrap replications in MEGA11. ML and NJ tree files in newick format were visualized using FigureTree (v 1.4.4) [68].

2.3. Species delimitation analyzes

Five different approaches were preferred for species delimitation analyzes: The General Mixed Yule Coalescent (GMYC) model [69] with a single threshold, (ABGD) [36], the Poisson Tree Processes (PTP) (https://species.h-its.org/) [70], Assemble Species by Automatic Partitioning (ASAP) (https://bioinfo.mnhn.fr/abi/public/asap/) [71] and TaxonDNA [72]. However, it was performed two different analyzes using p-distance and K2P distance parameters in ABGD approach. So, this study was planned a total of six analyzes based on five different approaches. For ABGD analysis, which is a distance-based method, the model setting was set as follows: TS/TV (ratio of translation to translation) is 0.967, variability (P) is between 0.001 (P-min) and 0.132 (P-max), K2P and P distance, minimum gap width (×) of 0.1-1.5. To apply the GMYC delimitation method, an ultrameric tree was constructed with "force.ultrametric" command and was checked using "is.ultrametric" command in R [73]. The obtained ultrameric tree for GMYC was used with single threshold method using the "gmyc" function under the "SPLITS" package (R Development Core Team, www.R-project.org). For PTP, the RAxML tree was employed as input file and analyzed via the PTP web server (https://species.h-its.org) with all parameters given by default, except for the number of generations, which was set to 100,000 generations. The most proper group was found by objective clustering with p-distance thresholds at 1–6% using TaxonDNA 1.8. Best Close Match (BCM) test in TaxonDNA/Species Identifier 1.8 was used to select the best threshold value and to evaluate the potential of the *COI* dataset for species identification. ASAP approach [71] is distance-based method like ABGD, and this analysis has performed in web interface. In this method, p distance parameter was preferred simple distance (p- distances).

3. Results and Discussion

Six analyses with five different methodologies (tree-based and distance- based) were conducted in this study. The compared methods used in this study all rely on a single locus for identifying species boundaries. Information

of thirty-six *Dolerus* samples identified according to these analyzes is given in Table 1. These species are *Dolerus triplicatus* (Klug, 1818), *Dolerus germanicus* (Fabricus, 1775), *Dolerus puncticollis* Thomson, 1871, *Dolerus nigratus* (Müller, 1776), *Dolerus* sp1, *Dolerus* sp2, *Dolerus* sp3 and *Dolerus* sp4. They were determined that the putative *Dolerus* sp3 and *Dolerus* sp4 species were separated from *D. puncticollis* and *D. nigratus* species, respectively.

Table 1. İnformation of *Dolerus* samples.

Specimens	Localities of specimens	Identification according to ITS2 (Gülmez et al, 2022)	ABGD-p, ABGD-K2P, ASAP, DNA Taxon	PTP, GMYC
spcmn1	Erzurum-Tortum	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn2	Erzurum-Tortum	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn3	Erzurum-Tortum	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn4	Erzincan-Refahiye	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn5	Erzincan-Refahiye	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn6	Erzincan-Refahiye	*D. triplicatus*	*D. triplicatus*	*D. triplicatus*
spcmn7	Kütahya-Altıntaş	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn8	Kütahya-Altıntaş	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn9	Uşak-Banaz	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn10	Ankara-Bala	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn11	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn12	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn13	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn14	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn15	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn16	Erzincan-Refahiye	*D. germanicus*	*D. germanicus*	*D. germanicus*
spcmn17	Erzurum-Tortum	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn18	Erzurum-Tortum	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn19	Nevşehir-Ürgüp	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn20	Nevşehir-Ürgüp	*D. puncticollis*	*D. puncticollis*	*Dolerus* sp3*
spcmn21	Nevşehir-Ürgüp	*D. puncticollis*	*D. puncticollis*	*Dolerus* sp3*
spcmn22	Ankara-Beyşehir	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn23	Sivas-Gürün	*Dolerus* sp1	*Dolerus* sp1	*Dolerus* sp1
spcmn24	Ankara-Beyşehir	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn25	Niğde-Çamardı	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn26	Niğde-Çamardı	*D. puncticollis*	*D. puncticollis*	*D. puncticollis*
spcmn27	Kastamonu-Tosya	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn28	Kastamonu-Tosya	*D. nigratus*	*D. nigratus*	*Dolerus* sp4*
spcmn29	Kastamonu-Tosya	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn30	Erzincan-Refahiye	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn31	Erzurum-Oltu	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn32	Erzincan-Refahiye	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn33	Erzurum-Oltu	*D. nigratus*	*D. nigratus*	*D. nigratus*
spcmn34	Kütahya-Altıntaş	*Dolerus* sp2	*Dolerus* sp2	*Dolerus* sp2
spcmn35	Kütahya-Altıntaş	*Dolerus* sp2	*Dolerus* sp2	*Dolerus* sp2

spcmn36	Kütahya-Altıntaş	*Dolerus* sp2	*Dolerus* sp2	*Dolerus* sp2

*: As a result of the species delimitation analysis, it was determined that the putative species.

The percentage of the average nucleotide composition of *COI* sequences of each species is given in Table 2. Ratio of nucleotide compositions of the *COI* sequences of each species are variable. AT contents of the examined sequences ranged between 71.80% (*Dolerus* sp1) and 73.10% (*D. nigratus*) (Table 2). The average AT content of the *COI* region mentioned in the study of Hebert (2003) [74], which is considered as the DNA barcode region and used in the analyzes, showed an AT content, like other Hymenoptera members that have been reported [75-80]. Moreover, additional proof that the sequences are *COI* comes from the fact that the "Blastn algorithm" [62] produced Per-Identities scores for the genus *Dolerus* ranging from 93 to 98%.

Table 2. Average nucleotide content of COI gene belongs to each species.

Specimens	Species Name	T%	C%	A%	G%	AT%
spcmn1-6	*D. triplicatus*	39.15	14.02	33.23	13.58	72.38
spcmn7-16	*D. germanicus*	38.85	14.55	32.96	13.65	71.81
spcmn17,18,19,22,24,25,26	*D. puncticollis*	38.64	13.93	33.64	13.79	72.29
spcmn20,21	*Dolerus* sp3	38.2	13.95	33.9	14	72.10
spcmn23	*Dolerus* sp1	38.6	14.9	33.2	13.3	71.80
spcmn27,29,30,31,32,33	*D. nigratus*	39.10	13.67	34.00	13.25	73.10
spcmn28	*Dolerus* sp4	38.9	13.7	33.9	13.4	72.80
spcmn34-36	*Dolerus* sp2	39.73	13.40	32.97	13.87	72.70

A=Adenine T=Thymine, C=Cytosine, G=Guanine, AT= Adenine – Thymine content

As a result of genetic distance, the interspecies distance in eight species was designated as a maximum of 9.7% (*D. nigratus-Dolerus* sp1 vs *D. germanicus*) and a minimum of 1.6% (*D. puncticollis* vs *Dolerus* sp1) (Table 3). In the intra-species genetic distance results, *D. puncticollis* samples have the maximum distance (0.872%) (Table 4). Since *Dolerus* sp1 and *Dolerus* sp4 are represented by one sample each, their interspecies genetic distances could not be calculated. According to Hebert et al (2004) [81], a 10-fold difference between mean intraspecific and interspecific differences is specified as the standard *COI* threshold for identifying animal species. This Figure. is over the designated threshold value, as evidenced by the fact that it was 13 times in the study (the difference between the average interspecies (4%) and intraspecific divergence (0.30%)). Comparison of average intraspecific and interspecific genetic distances is widely used in species delimitation, as well as in barcoding studies. Maximum distances between *Dolerus* species reflect the pattern seen in species delimitation analyses, where well-supported clusters (clades) consist of more than one species.

Table 3. Interspecific genetic distance.

No	Species	Genetic Distance							
		1	2	3	4	5	6	7	8
1	*D. triplicatus*								
2	*D. germanicus*	7.5%							
3	*D. puncticollis*	6.8%	7.8%						
4	*Dolerus* sp1	6.9%	7.6%	1.6%					
5	*Dolerus* sp3	6.4%	8.9%	3.5%	4%				
6	*Dolerus* sp4	6.6%	8%	4.5%	4.4%	4.9%			
7	*D. nigratus*	7.7%	9.7%	7.1%	6.9%	6.5%	6.6%		

8	*Dolerus* sp2	8.3%	9.7%	6.4%	6.6%	6%	4.3%	2.9%

Table 4. Intraspecific genetic distance of *Dolerus* species.

Species	d	SE
D. triplicatus	0	0
D. germanicus	0.2%	0.122%
D. puncticollis	0.9%	0.251%
Dolerus sp3	0.3%	0.232%
Dolerus sp1*	n/c	n/c
Dolerus sp4*	n/c	n/c
D. nigratus	0.06%	0.055%
Dolerus sp2	0.04%	0.228%

* *D.* sp1 and *D.* sp4 are represented by one sample each.

To compare the species delimitation analyses of the Dolerus genus, a total of six analyses based on five different approaches were conducted. In addition, we employed comparison analyzes to utilize the ITS2 results (phylogenetic tree and CBCs) from our previous research [25]. Comparison analyses summarizing the results of the six different species delimitation analyses and the results of Gülmez et al. (2022) [25] (ITS2) are shown on a RaxML tree (Fig. 2). These analyses led to the identification of eight groups from tree-based analyses (PTP and GMYC) and six groups from distance-based ones (ABGD-p, ABGD-K2P, ASAP, and DNA Taxon) (Fig.s 2). The reason for the variability in the number of species is the use of approaches with different algorithms. The recursive partitioning of data using ABGD and ASAP techniques, which are computationally and time-efficient, involves comparing sequence differences to identify a "barcode gap" that may indicate the boundaries of different species [40]. Tree-based methods identify species boundaries by calculating branch variation using a phylogenetic tree.

Two different inputs, P-dist and K2P distance, were used in four distance-based analyzes. In the consequence of ABGD-P-dist analysis and ASAP analysis, it was observed that there were respectively 0.036% and 0.045% barcode gaps between the maximum intraspecific distance and minimum interspecific distance values in the COI data set of *Dolerus* species (Fig. 1). Despite using the same distance data, the barcode gaps were different. However, both analyses grouped the same number of species. Similarly, DNA-Taxon analysis which a species delimitation tool that clusters using intraspecific genetic distances [72], also found that same number groups as other distance-based analyses. The six groups identified by ASAP, ABGD-p, ABGD-K2P, and Taxon DNA analyses yielded identical species groups to those reported in our earlier study [25]. Moreover, for detailed comparison of intraspecific relationships, a distance-based NJ tree was also examined. The NJ tree exhibited the same topology with RAxML.

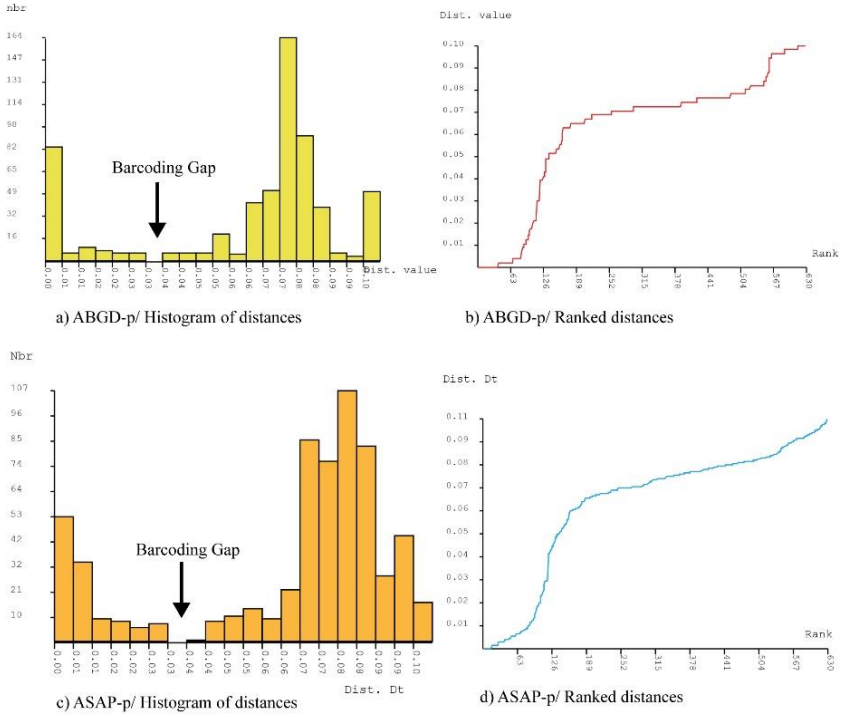

Fig. 1. a) ABGD-p/ Histogram of distances. b) ABGD-p/ Ranked distances. c) ASAP/ Histogram of distances. d) ASAP/ Ranked distances

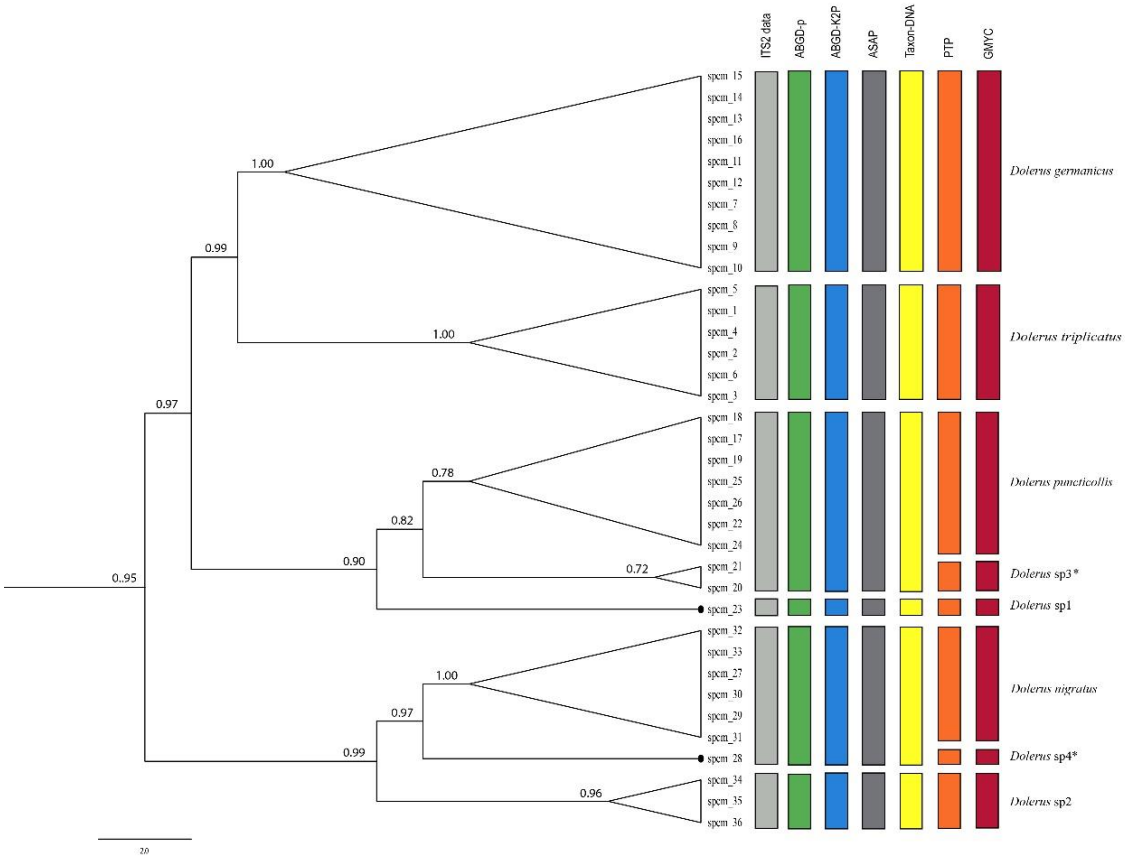

Fig. 2. Comparative all species delimitation analyzes on consensus tree of *COI* region conducted by RaxML.

Samples placed between spcm1 and spcm6 are grouped together in both distance and tree-based analyses. Since these samples represent the group defined as *D. triplicatus* according to ITS2 results (Grey color in Fig. 2), they gave similar results in both studies. Similarly, in all analyses, samples between spcm7 and spcm16 were assigned to a single group and were compatible with the *D. germanicus* species represented in ITS2 results (Grey color in Fig. 2). The spcm23 sample, which was named *Dolerus* sp1 in the previous study (Fig. 2), was in a different group in all species delimitation analyses. Its appearance in the different group supported the previous study. Consistent with the prior study's designation of these specimens as *Dolerus* sp2, all species delimitation analyses included spcm34, spcm35 and spcm36 in same group (Fig. 2). These results support comparison of all species delimitation analyzes and the ITS2 results (Grey color in Fig. 2). Distance-based analyzes (ABGD, ASAP and Taxon DNA) have given spcm20 and spcm21 with *D. puncticollis* in the same group. The distance-based analyzes of COI data and the tree topology of ITS2 results support each other. However, GMYC and PTP analyses grouped these two samples separately from the *D. puncticollis* group. As seen in Fig. 2, spcm20 and spcm21 samples were separated from the *D. puncticollis* species group and formed a different clad (*Dolerus* sp3). When the results of the previous study are examined, it is seen that spcm20 and spcm21 samples are separated from other samples by two CBCs. The existence of these CBCs are supported by this study [25]. For this study, it is thought that the ITS2 phylogeny and CBC presence together with species delimitation analyzes will provide more informative species-level identifications. However, since both ITS2 results and the groups given by tree-based approaches do not support

each other, it was named as the putative *Dolerus* sp3.

The spcm28 sample was found in the same group with *D. nigratus* in distance-based approaches, which shows its similarity with the previous study. Tree-based analyses, however, revealed that this species belonged to a distinct single group. Although, there is no CBC presence between them, the spcm28 sample showed separate branching from the *D. nigratus* clade in the ML tree. The ML tree and species delimitation analyses supported each other, and therefore it was named as the putative species *Dolerus* sp4. GMYC approach is a coalescent-based phylogenetic method that sets thresholds between coalescent and species-level processes to species boundaries. PTP approach models speciation events by the number of substitutions in each branch, which equates to a higher expected number of substitutions between species than within species. In this context, the tree based GMYC model is an analytical approach that an ultrameric phylogenetic tree as the most likely point of transition from merging to speciation branching models [69, 80]. These models continue to be used successfully in recent times to delimit species in a wide variety of little-known insect taxa. [82-85].

GMYC and PTP analyzes generally produce similar estimates of species boundaries [86-89]. Same species groups were determined from PTP and GMYC analyzes, using the RAxML and ultrameric tree as input. Branching points or nodes in a tree are considered to indicate speciation. In monophyletic trees, each node represents the last common ancestor of two lineages that diverged from that node [85]. Therefore, the fact that each of the two main clades containing *D. puncticollis* and *D. nigratus* species have three nodes, as well as the presences of CBCs shown on the tree topology in the previous study, supported the existence of putative species groups emerged in these analyses (Fig. 2). GMYC and PTP also offer some distinct advantages over the other four types of delimitation analysis. The main benefit of these approaches is that they are far less reliant on the threshold value and integrate evolutionary theory [28]. The CBCs identified in the previous investigation support the suggested species boundaries in this analysis. Although analysis of single-locus mtDNA data and decisions based on small sample sizes pose interpretation risks, processing the data with species delimitation analyses can provide accurate estimates of the number of species [90].

4. Conclusion

The species delimitation methods correctly group known species into clusters in most cases. The grouping of the ASAP analysis [71], which is based on the best scoring algorithm, is supported by other distance-based analyzes. In addition, PTP and GMYC analyzes are internally consistent. The main reason for this difference is the use of the ultrameric tree in the PTP and GMYC analyzes. On this tree, rates of branching events are estimated to reveal patterns of speciation (interspecific relation) and coalescence (intraspecific relation) [69, 71]. Therefore, tree-based analyzes take longer to complete than distance-based analyzes in terms of time. Although this is stated to be a disadvantage by some researchers, these analyzes among the most popular approaches to provide reliable results.

There was no consensus on the number of common species in both distance and tree-based analyzes. However, the reliability of tree-based analyzes interpreted using additional data such as ITS2 and CBC, is one step forward. Puillandre et al., (2020) [71], reported that the performance of ABGD was similar to that of ASAP, and although PTP did not perform very well, GMYC performed very well as long as the number of species was not too high. Since GMYC and PTP analyzes are based on evolutionary relationships, we named the groups separated from *D. puncticollis* and *D. nigratus* as *Dolerus* sp3 and *Dolerus* sp4, respectively. As this is the first study with this taxon group, testing species delimitation analyzes will serve as a resource for future studies for this important family.

Acknowledgments

This study was financially supported by the TÜBITAK (The Scientific and Technological Research Council of Turkey) via a research project with grant number 113Z753. The members of Cumhuriyet University Evolutionary Bioinformatics Research Group (EBRG) are thanked for their contributions in bioinformatic work. Mehmet GÜLMEZ who first author is supported by the Council of Higher Education (CoHE) of Turkey with 100/2000 PhD Scholarship

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