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Original article (Orijinal araştırma)

Characterization and taxonomic utility of ITS2 in *Dolerus* Panzer, 1801 (Hymenoptera: Tenthredinidae)[1]

Dolerus Panzer, 1801 (Hymenoptera: Tenthredinidae) cinsinde ITS2'nin karakterizasyonu ve taksonomik kullanımı

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Abstract

The widespread use of ITS2 as a potential marker in insects has accelerated species-level phylogenetic studies. Reliable and quality data can be obtained thanks to the features such as rapid evolution and secondary structure of this marker. This paper presents the phylogenetic relationship among 36 individuals of *Dolerus* Panzer, 1801 (Hymenoptera: Tenthredinidae) collected between 2002 and 2018 to obtain the first data on ITS2 secondary structure. Aligned of ITS2 data were analyzed by application of maximum likelihood method to reveal phylogenetic relationship among the specimens. Also, the structural properties, length variation and presence of compensatory base changes make the ITS2 useful marker in determination of species boundaries of closely related species. Four species (*Dolerus triplicatus* (Klug, 1818)*, Dolerus germanicus* (Fabricius, 1775)*, Dolerus puncticollis* Thomson, 1871 and *Dolerus nigratus* (Müller, 1776) and two putative species (*Dolerus* spp. 1 and 2) were determined from 36 individuals belonging to *Dolerus*.

Keywords: Compensatory base changes, *Dolerus*, Hymenoptera, ITS2, molecular marker

Öz

Böceklerde ITS2'nin moleküler belirteç olarak kullanılmasının yaygınlaşması, tür düzeyindeki filogeni çalışmalarına hız kazandırmıştır. Bu belirtecin hem hızlı evrime sahip olması hem de ikincil yapı oluşturması gibi özellikler sayesinde güvenilir ve kaliteli veriler elde edilebilir. Bu makalede, *Dolerus* Panzer, 1801 cinsine ait 2002-2018 yılları arasında toplanmış olan 36 bireyin (Hymenoptera: Tenthredinidae) aralarındaki filogenetik ilişkiyi ve ITS2 ikincil yapı özelliklerine ilişkin ilk verileri sunuyoruz. ITS2'nin hizalanmış verileri, örnekler arasındaki ilişkiyi görmek için maksimum likelihood yöntemi uygulanarak analiz edildi. Aynı zamanda ITS2'nin yapısal özellikleri, uzunluk polimorfizmi ve CBC'lerin olması, yakından ilişkili türlerin tür sınırlarının belirlenmesinde faydalıdır, *Dolerus* cinsine ait 36 bireyden, 4 tür (*Dolerus triplicatus* (Klug, 1818)*, Dolerus germanicus* (Fabricus, 1775)*, Dolerus puncticollis* Thomson, 1871 *ve Dolerus nigratus* (Müller, 1776) ve 2 olası tür (*Dolerus* spp. 1 ve 2) belirlenmiştir.

Anahtar kelimeler: CBC, *Dolerus*, Hymenoptera, ITS2, moleküler belirteç

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Introduction

The order Hymenoptera is one of the large insect orders comprising 153 thousand species described with very diverse life-histories (Niu et al., 2019; Çalmaşur, 2020; Taeger et al., 2021). Symphyta (Gerstacker, 1867) (sawflies) which is economically important insect linage that include major forest and horticultural pests (Heidemaa, 2004; Niu et al., 2021), is a small suborder of Hymenoptera largely distributed in the Palearctic region with 4,396 species (Taeger et al., 2021). Most of these species are members of Tenthredinoidea (Latreille, 1803), the most diverse lineage of non-apocritan Hymenoptera (Çalmaşur & Özbek, 2004a; Katılmış & Kıyak, 2015; Vilhelmsen, 2015). The Tenthredinoidea, known as typical sawflies, is the most well-known superfamily of Symphyta (Taeger et al., 2021). Tenthredinidae, the largest family of Symphyta, is one of seven families in Tenthredinoidea (Çalmaşur & Özbek, 2004b). This family comprises more than half of symphytan species mostly distributed in the Palearctic (Taeger et al., 2021). *Dolerus* Panzer, 1801 (Hymenoptera: Symphyta: Tenthredinidae) with 259 species, is a widespread genus in the Palearctic and Nearctic regions (Barker, 1998; Taeger et al., 2021).

Hymenoptera have diverse life-histories that range from feeding on or inside plants to highly variable forms of parasitism, social life and predation (Malm & Nyman, 2015; Niu et al., 2021). Adult sawflies feed on sap, such as maples, apples, pears, and nectar, such as willows, cherries and plums (Çalmaşur & Özbek, 2004b). Also, some larvae feed on plant in different ways such as holes or notches in the leaves, tunneling through the plant stem and making galls on the foliage (Goulet, 1986; Çalmaşur & Özbek, 2004b).

The two most common species of sawflies in rural habitats are graminivore *Dolerus* and *Pachynematus* (Barker et al., 1999). In recent studies, it has been reported that the genus *Dolerus*, known as wheat-sawflies, is not only found in wheat, but also in seed-grass plants and has become a more important pest, especially *Dolerus nigratus* (Müller, 1776) and *Dolerus puncticollis* Thomson, 1871. The larvae of *Dolerus* generally feed on plants in the Cyperaceae, Equisetaceae, Juncaceae, Poaceae (Haris, 1995). There are numerous reports of *Dolerus* larvae feeding on grain or grass crops highlighting their economic importance as pests (Haris, 1995). In addition, *Dolerus* larvae are known as an important food source for juvenile birds in rural habitats (Barker et al., 1999).

There are only a few phylogenetic studies of *Dolerus*. Vilhelmsen (2015), based on a morphological study on phylogeny of the Tenthredinidae, suggested that *Dolerus* is not monophyletic (Vilhelmsen, 2015). Also, Malm & Nyman (2015) using DNA data concluded that Selandriinae and Doleriini were clearly separate groups. However, more studies are required to determine the phylogeny of this group.

There are numerous phylogeny studies using both morphological characters and molecular markers on the family of Tenthredinidae. Cytochrome oxidase subunit I (COI) and internal transcribed spacers 2 (ITS2) genes have been preferred as molecular markers in many studies. Also, ITS2 has conserved primer sequences across many different taxa (Schulmeister, 2003; Prous et al., 2011; Kearse et al., 2012; Leppänen et al., 2012; Budak et al., 2016).

Analyzing DNA (or RNA) sequences is an important method for phylogenetic and taxonomic studies of protistan, plant and animal species (Young & Coleman, 2004; Salvi & Mariottini, 2012; Hong et al., 2019). Given its rapid evolution, ITS2 region is used to phylogenetic analyses of closely related species (Zhao et al., 2018; Verma & Mishra, 2020). Also, it can be used as a molecular marker for species-level phylogeny and molecular clock (Uluar & Çıplak, 2020) like COI (Wagener et al., 2006; Schwarzfeld & Sperling, 2015). ITS2 is located between 5.8S and 28S ribosomal genes and has a function in the regulation and maturation of rRNA genes (Caisová et al., 2013; Jørgensen et al., 2013; Poczai et al., 2015; Fagan-Jeffries et al., 2019). ITS2 commonly has a conserved secondary structure with four helices around a loop. This structure has regions both conserved (Helix II and III) and quite variable (Helix I and Helix IV) (Coleman, 2009; Salvi & Mariottini, 2012; Poczai, et al., 2015; Fagan-Jeffries et al., 2019).

Given secondary structure of ITS2, compensatory base changes (CBCs) are formed on the helices (Torres-Suárez, 2014). CBCs are defined as mutations in both nucleotides of a paired position in a double-stranded structure of the transcribed RNA (Ponce-Gordo et al., 2011; Salvi & Mariottini, 2012), for example, when G-C mutates to A-U (Gutell et al., 1994; Coleman, 2003; Schill et al., 2010). Hemi-CBCs consist of a change of one of the nucleotides in the pair. If a CBC occurs between the two organisms, there is a 93% probability that these organisms are different species (Müller et al., 2007; Ruhl et al., 2010; Pawłowska et al., 2013; Torres-Suárez, 2014). Thus, the use of the secondary structure of ITS2 is an important method in phylogeny and species delimitation (Vandivier et al., 2016; Verma & Mishra, 2020). Helix I is variable in sequence unlike Helix II which has more conserved structure and almost represents at least one pyrimidine-pyrimidine mismatch (UxU, UxC and CxC). Helix III is usually much longer and branched with conserved region in its apical region and includes a conserved four nucleotide motif (YGGY). Helix IV is a short structure that highly variable and may not be present in all species (Caisová et al., 2011; Coleman, 2015).

In this study, the secondary structure of ITS2 was investigated in *Dolerus* for the first time. This region was sequenced and then characterized the predicted secondary structure of ITS2 for 36 specimens of *Dolerus* representing four morphotypes identified using existing morphological keys. Then, phylogenetic analyses were applied based on the structural alignment and CBCs were defined between all samples.

Materials and Methods

Sample preparation

The specimens, collected with sweeping netting between 2002 and 2018 years from different locations in Turkey (Table 1), were provided from the Entomological Collection of Cumhuriyet University, Sivas which was protected in 99% ethanol at -20ºC. A total of 36 *Dolerus* specimens were identified to species using identification keys of Haris, (2000).

Molecular analysis

Molecular analyses of all specimens were performed during 2019. Whole genomic DNA was extracted from the hind legs of the specimens by the salting-out method of Aljanabi & Martinez (1997). The primers used for amplification of ITS2 region were CAS5p8sFc (5'-ATG AAC ATC GAC ATT TCG AAC GCA CAT-3') and CAS28sB1d (5'-TTC TTT TCC TCC GCT TAG TAA TAT GCT TAA-3') (Ji et al., 2003). Amplifications were performed in 50-ml volumes containing 0.5 U of Taq polymerase, 5 ml of 10x reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P-40), 10 pmol of each of the primers, 0.2 mM of each of the four dNTPs, 1.5 mM $MgCl_2$ and 1 ml of DNA template (50-100 ng). PCR cycle conditions were: 94ºC for 5 min; 35 cycles of 94ºC for 30 s, 46ºC for 30 s and 72ºC for 60 s., and finally 72ºC for 5 min. The resulting PCR products were electrophoresed on 1% agarose gel and viewed. The purification and sequencing of amplification products were performed using a commercial sequencing service (Macrogen Ltd., Seoul, Korea.). Sequencing was performed in both directions using the same primers as in PCR reactions. The forward and reverse nucleotide sequences were assembled, edited and aligned by eye using Genious R9 (Kearse et al., 2012) and Mega7 (Kumar et al., 2016). The sequences were deposited to GenBank under the accession numbers OK642104-OK642139.

Inference of secondary structure

ITS2 sequences were identified using the ITS2 database V (Ankenbrand et al., 2015). E-value of <0.01 and metazoan HMMs with ITS2 minimum size of >150 nt were used to describe the borders of ITS2. Then defined sequences were progressed in the RNA Mfold Server folding program (Zuker, 2003). As parameters, linear sequence, RNA version 2.3 energy rules, 25ºC were applied. The structures and sequences were synchronously aligned by 4SALE (Seibel et al., 2006; Wolf et al., 2014) in locally implemented Clustal W (Larkin et al., 2007). The secondary structures were redrawn and annotated to improve visualization using VARNA 3.9 (Darty et al., 2009).

Phylogenetic analyses and mapping of synapomorphic compensatory base changes

ITS2 aligned sequences were used to show phylogenetic relationship of 36 *Dolerus* specimens. *Tenthredopsis tessellata* (Klug, 1817), is most closely related to *Dolerus* in database, was used as an outgroup because ITS2 data of Selandriinae was not available in the database. As a model, p-distance parameter was used to determine intra- and interspecific distance in Mega7 (Kumar et al., 2016). Phylogenetic tree was built by application of maximum likelihood (ML) using phangorn (Schliep, 2011) as performed in the statistical framework R (R core team, 2014). The R script used in the construction of ML structure tree was reached from the 4SALE website (4sale.bioapps.biozentrum.uni-wuerzburg.de) and for further details see Wolf et al. (2014). The robustness of the ML trees was tested by 1,000 bootstrap replicates.

Results and Discussion

The thirty-six specimens identified according to morphological characters are given in Table 1. The identified species were *Dolerus triplicatus* (Klug, 1818) (spcmn1-6), *Dolerus germanicus* (Fabricus, 1775) (spcmn7-16), *D. puncticollis* (spcmn17-26) and *D. nigratus* (spcmn27-36).

Table 1. Locality information and ITS2 secondary structure characteristic features of the specimens

Specimens	*Dolerus* morphospecies	Collection localities	Length (nt)	% GC content	ΔG value kcal/mol	Length (nt)			
						Helix I	Helix II	Helix III	Helix IV
spcmn1-3	*Dolerus triplicatus*	Erzurum-Tortum	647	57,0	-318,96	10	108	498	-
spcmn4-6	*Dolerus triplicatus*	Erzincan-Refahiye	647	57,0	-318,96	10	108	498	-
spcmn7	*Dolerus germanicus*	Kütahya-Altıntaş	640	55,9	-316.14	84	60	481	-
spcmn8	*Dolerus germanicus*	Kütahya-Altıntaş	640	55,6	-314.31	84	60	481	-
spcmn9	*Dolerus germanicus*	Uşak-Banaz	640	55,9	-313.77	84	60	481	-
spcmn10	*Dolerus germanicus*	Ankara-Bala	640	55,8	-317.95	84	60	481	-
spcmn 11-12-14-15-16	Dolerus *germanicus*	Erzincan-Refahiye	640	55,6	-313.15	84	60	481	-
spcmn13	*Dolerus* germanicus	Erzincan-Refahiye	640	55,8	-313.20	84	60	481	-
spcmn17	*Dolerus puncticollis*	Erzurum-Tortum	622	55,5	-321.39	48	63	475	15
spcmn18	*Dolerus puncticollis*	Erzurum-Tortum	619	55,6	-321.33	47	63	472	9
spcmn19	*Dolerus puncticollis*	Nevşehir-Ürgüp	619	55,6	-322.51	47	63	472	9
spcmn20	*Dolerus puncticollis*	Nevşehir-Ürgüp	621	54,6	-293.92	46	68	473	14
spcmn21	*Dolerus puncticollis*	Nevşehir-Ürgüp	621	55,4	-307.90	47	68	474	9
spcmn22	*Dolerus puncticollis*	Ankara-Beyşehir	620	55,5	-319.87	47	63	473	9
spcmn23	*Dolerus puncticollis**	Sivas-Gürün	559	55,5	-282.82	47	70	380	18
spcmn24	*Dolerus puncticollis*	Ankara-Beyşehir	622	55,5	-320.21	47	63	475	13
spcmn25	*Dolerus puncticollis*	Niğde-Çamardı	619	55,6	-322.51	47	63	472	9
spcmn26	*Dolerus puncticollis*	Niğde-Çamardı	624	57,2	-308,47	47	68	476	15
spcmn27	*Dolerus nigratus*	Kastamonu-Tosya	605	56,7	-280.35	90	80	394	27
spcmn28	*Dolerus nigratus*	Kastamonu-Tosya	605	56,9	-279.62	91	80	394	27
spcmn29	*Dolerus nigratus*	Kastamonu-Tosya	605	56,7	-282.61	88	74	394	27
spcmn30	*Dolerus nigratus*	Erzincan-Refahiye	605	56,5	-282.53	88	74	395	27
spcmn31	*Dolerus nigratus*	Erzurum-Oltu	607	55,7	-275.23	94	80	404	27
spcmn32	*Dolerus nigratus*	Erzincan-Refahiye	605	56,9	-279.62	91	80	395	27
spcmn33	*Dolerus nigratus*	Erzurum-Oltu	605	56,7	-282.61	88	74	395	27
spcmn34	*Dolerus nigratus***	Kütahya-Altıntaş	621	55,7	-321.15	9	152	432	9
spcmn35	*Dolerus nigratus***	Kütahya-Altıntaş	621	55,6	-321.06	9	149	432	9
spcmn36	*Dolerus nigratus***	Kütahya-Altıntaş	624	55,1	-316.27	9	152	432	9

*,** From the molecular analysis, these species was defined as *Dolerus* sp. 1 and 2, respectively.

The average GC content of the ITS2 region of 36 samples in the study was 56.0%. The GC contents of the examined sequences were ranged between 54.6% (spcmn20) and 57.2%. (spcmn26). These values were consistent with previous studies on eukaryotes (Mullineux & Hausner, 2009). The ITS2 sequence lengths were ranged between 559 and 647 bp (Table 1). Length polymorphism was not observed in *D. triplicatus* (647 bp) and *D. germanicus* (640 bp). The intra-specific length variation of ITS2 was found mostly in *D. puncticollis* (619-624 bp). The length variation of ITS2 in all studied animals was ranged between 100 and 2052 bp (Budak et al., 2016).

Inter-specific genetic distance was determined as a maximum of 28.8% (*D. nigratus* vs *D. triplicatus)*, and a minimum of 14.8% (*D. germanicus* vs *D. triplicatus*). Also, interspecific standard error was at most maximum 2% (*D. triplicatus* vs *D. nigratus)* and down to 1.4% (*D. triplicatus* vs *D. germanicus*) (Table 2). Intraspecific genetic distance was determined as a maximum of 3% (*D. puncticollis*) and a minimum of 0% (*D. triplicatus*) (Table 3). The results show that despite the high genetic distances between species, the absence of nucleotide variation within the species such as *D. triplicatus* strongly suggests that ITS2 evolved through concerted evolution (Dover, 1982). Concerted evolution is defined as a process in which related genes within a species experience genetic exchange, leading to their nucleotide evolution to be concerted over some period of time (Liao, 1999).

Table 2. Interspecific genetic distance

	Standard error			
Genetic	*D. triplicatus*	*D. germanicus*	*D. puncticollis*	*D. nigratus*
Dolerus triplicatus	-	0.0147	0.0173	0.0202
Dolerus germanicus	0.1480	-	0.0164	0.0187
Dolerus puncticollis	0.2352	0.2148	-	0.0177
Dolerus nigratus	0.2883	0.2629	0.2793	-

Table 3. Intraspecific genetic distance

Species	n	d	SE
Dolerus triplicatus	*6*	0.00000	0.00000
Dolerus germanicus	*10*	0.00531	0.00170
Dolerus puncticollis	*10*	0.03012	0.00397
Dolerus nigratus	*10*	0.00500	0.00157

The ITS2 secondary structures were predicted by the approach of energy minimization (Mathews et al., 1999) for all sequences. Predicted thermodynamic energy values of the putative secondary structures varied from -275.23 to -322.51 kcal/mol (spcmn31 vs spcmn19 and -25). The ITS2 structures in the database for eukaryotes display a common core structure (Ankenbrand et al., 2015). However, in the previous study of the same family (Budak et al., 2016), the ITS2 structures showed numerous branching. Despite diverged nucleotide sequences of ITS2 for studied taxon, secondary structures displayed a similar pattern. *Dolerus triplicatus* and *D. germanicus* have three helices (Helix I, II and III) and, *D. puncticollis* and *D. nigratus* have four helices (Helix I, II, III and IV) in their ITS2 secondary structures. Similar secondary structures were found in *Tenthredopsis* by Budak et al. (2016).

The length of Helix I ranged from 9 to 94 bp (*D. nigratus*). Helix I forms a non-dichotomous structure as seen in eukaryotes (Caisová & Melkonian, 2014). The length of Helix II changed between 60 bp (*D. germanicus*) and 152 bp (*D. nigratus*). Although these helix lengths show similarities with the study by

Budak et al., (2016), they are longer than helix length of many eukaryotes (Coleman, 2007). Helix III was the longest, as in most eukaryotes. The length of Helix III varied between specimens (380 bp in *D. puncticollis* and 498 in *D. triplicatus*). Despite the variation in length between species, Helix III had similar branching and folding structures. Also, Helix III had the conserved motifs, the sequences of these motifs were 5 'AUCGUCCGCGG (11 bp) and GUCGUUCCGUGAAU (15 bp). These conserved sequences have been suggested both as a protein binding site and a cleavage site because of their locations, lengths as well as conserved nucleotides (Coleman, 2007). The previously reported YGGY motif as a likely division site on the 5' side and near the apex (Coleman, 2007; Caisová et al., 2013) was also found in *Dolerus* spp. Helix IV structure of *D. nigratus* and *D. puncticollis* was similar to the genus *Tenthredopsis* (Tenthredinidae) (Budak et al., 2016). However, this helix was not found in *D. triplicatus* and *D. germanicus*. The length of Helix IV ranged from 9 to 27 bp and had no branching in its secondary structure (Figure 1).

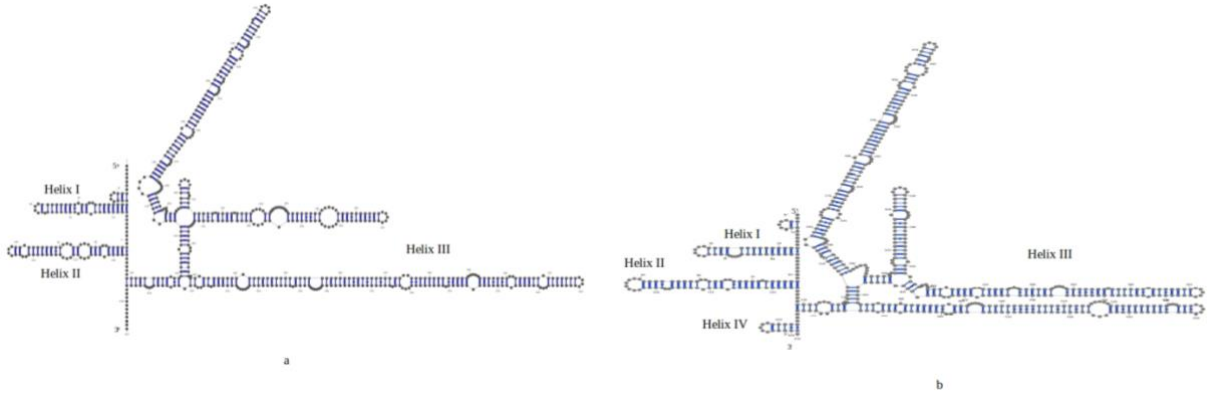

Figure 1. Secondary structures of ITS2 in *Dolerus:* a) the three-helix structure, and b) the four-helix structure.

The presence of a CBC between two specimens indicates that they belonging to different species with a probability of 93% (Coleman, 2007). The CBC formation was observed on Helix II and III of ITS2, but for the exceptional cases, the CBCs were equally distributed over all four helices (Müller et al., 2007). Also, the length differences of ITS2 are important in distinguishing species (Tang et al., 1996; Kitthawee, 2003; Nelson et al., 2008). Morphology, CBCs and length polymorphism data were used to define species boundaries.

Prior to phylogenetic analyses, the samples were designated numerically with the prefix *spcmn* (Table 1). The ML tree was built using the 36 ITS2 secondary structures (Figure 2). Only apomorphic CBCs were shown on the ITS2 tree (Figure 2). In total, 24 CBCs were identified among the six morphologically distinct taxa belonging to four identified species: three in Helix I, six in Helix II and fifteen in Helix III as given Table 4. In the ML tree, 36 *Dolerus* samples were divided into two main clades. The split of these two clades was supported with five CBCs (H1CBC3, H3CBC12, H3CBC13, H3CBC14 and H3CBC15) (Figure 2). The basal covered the specimen of *D. nigratus* without any CBCs. The second clade contained other taxa. This clade was divided into two subclades and each subclade was supported by the intra and interspecific CBCs (H2CBC4, H2CBC5, H2CBC6, H2CBC7, H3CBC6, H3CBC7, H3CBC8, H3CBC9, H3CBC10 and H3CBC11). Although the first subclade contained *D. germanicus* and *D. triplicatus*, *D. puncticollis* and two morphotypes, *Dolerus* sp. 1 and 2, were in the second subclade (Figure 2).

Table 4. Position of compensatory base changes in ITS2 secondary structure

CBC Name	Base Change	Position
H1CBC1	G:C-A:U	24-47
H1CBC2	A:U-G:C	26-45
H1CBC3	U:A-G:C	49-58
H2CBC1	A:U-G:C	122-155
H2CBC2	A:U-G:C	36-46
H2CBC3	C:G-G:U	38-43
H2CBC4	G:C-A:U	110-151
H2CBC5	C:G-A:U	125-138
H2CBC6	C:G-U:A	117-146
H3CBC1	G:C-C:G	185-267
H3CBC2	G:C-A:U	463-472
H3CBC3	U:A-G:C	406-444
H3CBC4	U:A-C:G	316-441
H3CBC5	U:A-C:G	315-338
H3CBC6	G:C-C:G	200-316
H3CBC7	G:C-A:U	434-466
H3CBC8	U:A-A:U	298-311
H3CBC9	A:U-G:C	320-406
H3CBC10	U:A-C:G	477-599
H3CBC11	C:G-A:U	335-340
H3CBC12	C:G-G:C	214-301
H3CBC13	U:A-C:G	225-289
H3CBC14	G:C-U:A	529-571
H3CBC15	G:C-A:U	436-464

Dolerus triplicatus and *D. germanicus* had similar ITS2 secondary structures, length variation and nucleotide similarity data, as reflected in the topology of the ML tree (Figure 2). However, the other two species (*D. puncticollis* and *D. nigratus*) were not monophyletic because of length variation and secondary structure differences of ITS2. It is suggested that these morphotypes (as *Dolerus* spp. 1 and 2) which are thought to have morphological similarities, may be distinct species. Only the spcmn31 in *D. nigratus* clade showed length variation. This length difference can be considered as intraspecific variation. spcmn34, spcmn35 and spcmn36 were morphologically identified as *D. nigratus* according to the morphological keys. However, phylogenetic analyses imply that these specimens could not be placed in the *D. nigratus* clade (Figure 2). Length differences of ITS2 gene and the presence of CBCs support this finding. In the light of these results, these specimens were temporarily designated as *Dolerus* sp. 2. with spcmn23 as species 1 being similar to *D. puncticollis* in terms of morphological characters. However, the high length variation (Table 1) and genetic distance of ITS2 (Table 2) separate *Dolerus* sp. 1 from *D. puncticollis*. The presence of CBCs (H1CBC1 and H2CBC3) between these specimens strongly support. *Dolerus* sp. 1 being considered as a distinct species. Although the presence of CBCs (H1CBC2, H3CBC4 and H3CBC5) between *D. puncticollis* samples, they were not considered as distinct species. Both the phylogenetic analyses and morphological similarities supported the monophyly of *D. puncticollis*. As a result, these CBCs can be considered as intraspecific variations. The sequence data of *D. triplicatus* samples were identical for all specimens. This is compatible with concerted evolution thus it can be said that ITS2 undergo genetic exchange between populations of the species (Table 3). All the samples of *D. triplicatus* had monophyletic relationship as expected. The presence of three CBCs (H2CBC2, H2CBC3 and H3CBC1) among the sister clade can be seen as the main feature that distinguishes this species from *D. germanicus*.

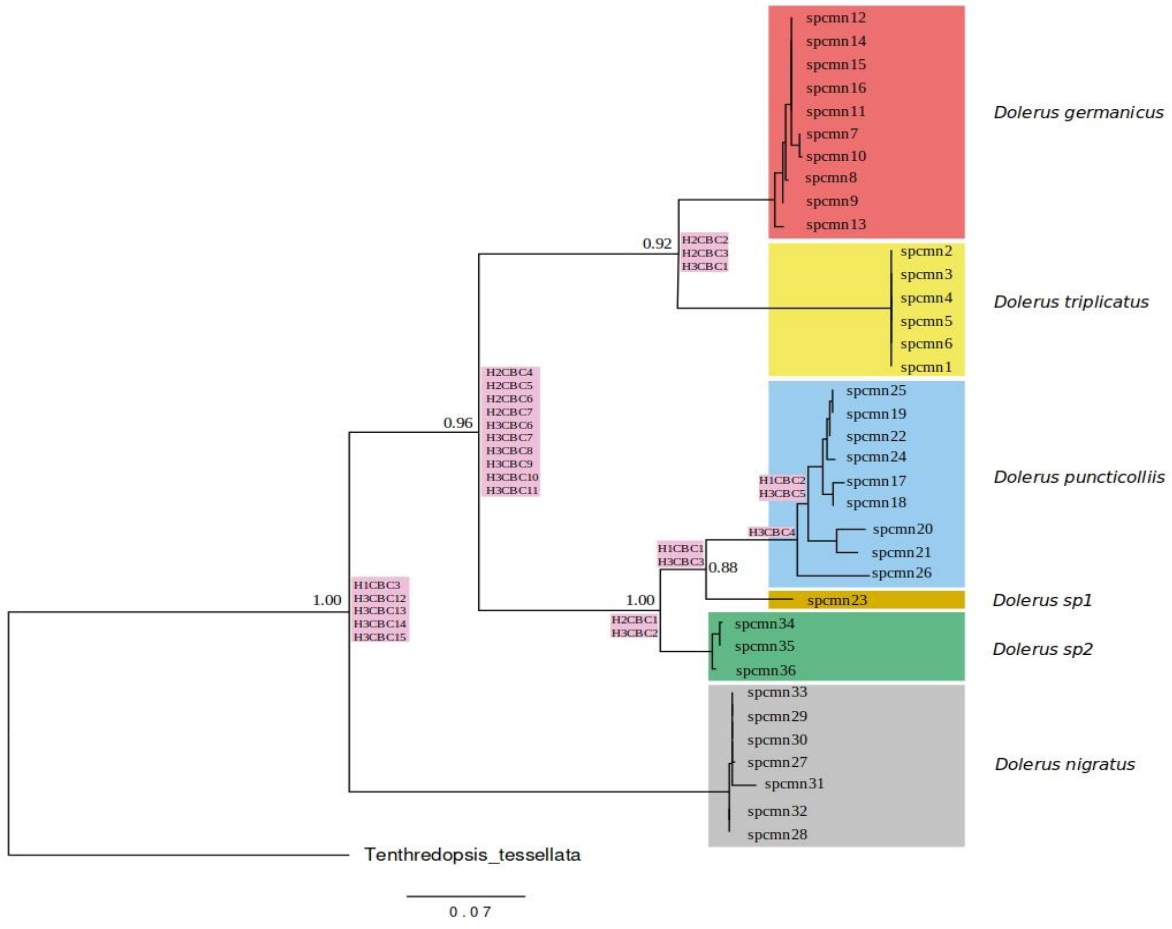

Figure 2. ML tree based on the structural alignment data of ITS2 in *Dolerus*.

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