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Species delimitation and phylogenetic relationships among some morpho-species belonging to *Thymelicus* Hübner, 1819 (Lepidoptera: Hesperiidae) from eastern Anatolia of Türkiye

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Abstract

Taxonomic studies combining morphological and molecular characters have become increasingly important in recent years for estimating species diversity and evolutionary relationships. The presented study includes the result of the morphological and molecular analyses of *Thymelicus* Hübner, 1819 (Lepidoptera: Hesperiidae) specimens collected from Bitlis, Hakkari, Malatya, Tunceli, and Van provinces and preserved in the CESA collection between 2015 and 2022. Based on morphological examinations, the appearance and male/female genitalia of *Thymelicus* butterflies were determined and four morphological species were recorded: *Thymelicus sylvestris* (Poda, 1761), *T. lineola* (Ochsenheimer, 1808), *T. hyrax* (Lederer, 1861) and *T. novus* (Reverdin, 1916). Mitochondrial cytochrome oxidase-I gene (*COXI*) sequences, used as a universal barcode, were obtained for the first time from Eastern Anatolian populations, and comprehensive evolutionary algorithms were used to determine the species diversity of the genus and understand their phylogenetic relatedness to congeners. The results showed that the taxa *T. sylvestris*, *T. lineola*, *T. hyrax*, and *T. novus* are separate species, but some *T. acteon* populations are closer to *T. christi* populations than to their morphological counterparts, and similarly, some *T. lineola* populations are closer to *T. novus* populations. This situation suggests that the elongated branch lines of the *T. acteon* and *T. lineola* main clades, due to the high genetic diversity of populations clustered in subclades, may indicate putative species.

Keywords: COI, Hesperiidae, Phylogeny, Species delimitation

1. Introduction

Thymelicus (Hübner, 1819) is a genus of the skippers' butterfly family, Hesperiidae (Lepidoptera), with bright orange-brown wings, distributed throughout the Palearctic region in a variety of habitats, including abundantly flowering forests, tall grassy thickets, and even along highways and railways. Currently, the genus is the sole member of the tribe Thymelini, but this information is currently incomplete because many hopping butterflies have not yet been assigned to tribes (Warren et al., 2009). The genus comprises 13 recognized species, whose external morphology and genitalia are well-studied and whose diagnostic features are well-studied. These include *Thymelicus sylvestris (Poda, 1761), T. acteon (Rottemburg, 1775), T. lineola (Ochsenheimer, 1808), T. hyrax (Lederer, 1861), T. sylvaticus (Bremer, 1861), T. hamza (Oberthür, 1876), T. leonina (Butler, 1878), T. christi (Rebel, 1894), T. alaica (Filipjev, 1931), T. flavus (Müller, 1776.), T. nervulata (Mabille, 1876), T. novus (Reverdin, 1916)* and *T. stigma (Staudinger, 1886)*. Although members of the genus are restricted to the Palearctic Region, *T. lineola* skippers were accidentally introduced to North America via London, Ontario, in 1910 and are now widespread in Eastern Canada and the northeastern United States, with recent occurrences in Western Canada (Jong, 1984; Lohse et al., 2024). Additionally, *Thymelicus lineola* has been reported as a pest of hay and pasture grasses in Southern Ontario (Pengelly, 1961). Gene flow between the populations that constitute a species is the most important factor maintaining species integrity. Partial genetic isolation or lack of gene flow among populations sharing a common ancestor among species sharing a common area triggers sympatric differentiation. Therefore, the intermittent distribution of species within a given geographic area allows for the discovery of new species-level lineages thanks to rich geographic sampling (Joshi et al., 2024). Recently, comparative molecular sequencing combined with morphological characterization has become a new standard in taxonomy for species identification and delimitation. Sequences of the mitochondrial cytochrome c oxidase subunit I (*COXI*) gene are widely used in taxonomic and phylogenetic analyses of butterflies. Due

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to its higher resolution than other descriptive genes, this gene is used as a "species barcode" to delimit taxa at and below the species level of many insect lineages (Dinca et al., 2011; Haussman et al., 2011; Huemer et al., 2014; Dinca et al., 2015; Mutanen et al., 2016; Huemer et al., 2017). While the differences among currently known *Thymelicus* species appear sufficient for species separation, a detailed phylogenetic study is lacking. Therefore, the genetic diversity, speciation and relatedness of *Thymelicus* species are not yet fully understood (Lohse et al., 2024). The current numbers of hesperiid species identified in Türkiye have increased from 40 to 43 (Hesselbarth et al., 1995; Koçak & Kemal, 2006; Baytaş, 2007). Among the hesperiid genera, members of *Carcharodus*, *Muschampia*, *Pyrgus*, and *Thymelicus* represent the richest species in Türkiye (Baytaş, 2007). The *Thymelicus* species recorded in Türkiye and the subject of this study are *T. lineola*, *T. hyrax*, *T. acteon*, and *T. sylvestris* (Shapiro, 1971; Van Der Poorten, 1990, Gençer et al., 2008). However, there are currently no studies comparing the four morphospecies with their populations from different geographical locations or with congeners, testing species boundaries, or demonstrating phylogenetic relationships.

In the presented study, detailed morphological data were obtained from populations of four *Thymelicus* species from eastern Anatolia of Türkiye. To investigate *COXI* sequence variation among morphospecies, each barcode obtained in the study was compared with all other taxa recorded in the genus based on phylogenetic analysis of gene sequences and discussed in detail.

2. Materials and methods

The materials of this study, which are some *Thymelicus* butterflies, were selected from the CESA (Centre for Entomological Studies Ankara) collection and used for morphological diagnoses and molecular analyses in Table 1. Firstly, the external morphology, and the genital organs for species identification were examined and photographed in the samples from which DNA barcodes would be extracted.

Table 1. Collection information for *Thymelicus* specimens obtained from Eastern Anatolia

no	Species/Populations	K2P-Genetic Distances	CESA ID*
1	*Thymelicus sylvestris* (Poda, 1761)	Bitlis, Mutki, Aydemir 1760m (13F73), 24 vii 2017, M.Kemal ve A.Koçak leg.1♂; Hakkari, Taşbaşı Tüneli K., 960m (30Ad), 9 vi 2022, M.Kemal ve H.Uçak leg. 2♂; Van, Bahçesaray, Paşaköy 1600m (65Ai), 12 vi 2016, M.Kemal ve A.Koçak leg. 2♂ (GP3396, GP3400); Başkale, Ziyanis 2500m (65Bo), 09 vii 2015, 1♂ (GP3393); Çatak, Saklıvadi, 2030m (65Df), 21 vi 2017, 1♂ (GP3399); Edremit Elmalık, 1750m (65Le), 01 vii 2017, M. Kemal, H.Uçak ve A.Koçak leg. 1♀ (GP3388).	Lep-Thym01Cesa
2	*T.lineola* (Ochsenheimer, 1808)	Bitlis Adilcevaz Harmantepe K., 2290m (13Af), 04 vii 2015, M.Kemal ve A.Koçak leg. 1♂ (GP3392); Hakkari Çukurca KD., 1580m. Dez Vadisi 1600m (30Bc5), 17 vi 2018, M.Kemal ve A.Koçak leg. 2♂; Malatya Akçadağ Gürkaynak 1540m, 08 vi 2013, M.Kemal ve A.Koçak leg. 1♂ (GP3398); Tunceli, Ovacık, Yaziören 1450m (62Eö), 04 vi 2021, 1♂; Ovacık, Gözeler (Yılanlıköy) 1410m (62Ey1), 03 vi 2022, 2♂; Van, Başkale, Ziyanis 2400m (65B), 07 vii 2015, 1♀ (GP3394); Gevaş, Elmalık 1750m (65Le), 04 vi 2017, 01 vii 2017, 2♂ (GP3391, GP3390); Gevaş, Artos Dağı, 2300m (65Fv), 24 vii 2020, M.Kemal, H.Uçak ve A.Koçak leg. 1♂ (GP3389).	Lep-Thym02Cesa
3	*T.hyrax* (Lederer, 1861)	Van, Gürpınar, Başet Dağı 2660m (65Gü), 07 vii 2019, M.Kemal ve A.Koçak leg. 1♀ (GP3395)	Lep-Thym03Cesa
4	*T.novus* (Reverdin, 1916)	Hakkari, Yükseova, Dağlıca, Bozkaya, 1310m, 11 vi 2021, 1♀1♂ (GP3402, GP3401); Şemdinli, Karaağaç (Öveç) 1400m, 1507m (30Cb), 11 vi 2021, 2♂; Yüksekova, Doğanlı, 1000m (30Af), 09 vi 2022, M.Kemal ve H.Uçak leg. 1♂	Lep-Thym04Cesa

Note: * – http://www.cesa-tr.org/

2.1. DNA extraction, PCR amplification, sequencing, and barcoding

For barcoding of populations of four species in the Eastern Anatolia Region, DNA representing the entire genome was isolated from the leg femur of dried individuals using a special tissue DNA isolation kit (REDExtract-N-Amp™ Tissue PCRKit/XNAT-100RXN).

The widely used LepF1: 5′-ATTCAACCAATCATAAAGATATTGG-3′ and LepR1: 5′-TAAACTTCTGGATGTCCAAAAAATCA-3′ primer sets were used to amplify the mitochondrially encoded *COXI* gene sequence from Lepidoptera members by Polymerase chain reaction (PCR) (Hajibabaei et al., 2006). The cycling conditions for the amplification reactions are as follows : 94 °C for 2 min at initial denaturation, followed by 5 cycles of 40 s denaturation at 95°C, 40 s annealing at 45°C, 1 min extension at 72°C, and additional 36 cycles of 40 s denaturation at 94°C, 40 s annealing at 51°C, 1 min extension at 72°C, with a final extension of 72 °C for 10 min. Purification and bidirectional sequencing of amplified barcode sequences were performed by Macrogen Company (Macrogen, Amsterdam, Netherlands). The quality of nucleotide reads and the sequence length of each barcode were checked with chromatograms using Codon Code Aligner program v.8.0.2.

2.2. Species delimitation analysis

The comparison of existing barcodes downloaded from online portals of *Thymelicus* species with barcodes of the populations from Türkiye presented in the study was aligned in MEGA 6.0 software using *Clustal*W pairwise comparison method (Chenna et al., 2003). In the same computer program, *Kimura 2-parameter distance* model was used to determine the genetic diversity among *COXI* barcodes of *Thymelicus* species and populations (Kimura, 1980). Additionally, barcodes from eleven *Thymelicus* morpho-species were used to determine maximum intraspecific divergence, speciation rates or species boundaries using the Kimura (K80) distance measure by the ABGD calculation method in abgdph 0.1 software. The smallest intraspecific distance threshold was set at 0.001 and the largest genetic distance at 0.05 for 10 steps. To identify genetic breaks between samples, the species separation line was initiated at 1.5 levels to account for the possibility of hybridization tendencies in different species. To determine the final number of species, the graph and table of the column with P=0.01 to P=0.02 values from the initial partition result were selected.

2.3. Phylogenetic analysis

Phylogenetic relationships of butterflies were calculated using two evolutionary algorithms: Maximum Likelihood (ML) and Bayesian inference (BI). *Ochlodes sylvanus* was selected as the outgroup taxon. Maximum Likelihood analysis (ML) was performed in CIPRES Science Gateway XSEDE v.8.2.4 using 1000 bootstrap iterations of the RAxML Blackbox calculation. (Miller et al., 2010). Markov Chain Monte Carlo (MCMC) simulations were run for 3,000,000 generations, with two sets of four chains sampling at a frequency of 100 generations in MrBayes 3.2. software (Ronquist &Huelsenbeck, 2003). The first 7,500 unstable trees were burned, and the posterior probability was calculated for all remaining trees by checking the majority rule consensus.

3. Results

Species identified by morphological characteristics were barcoded, and both species boundaries were tested and their status was determined through molecular taxonomic analysis. In the presented study, the external morphology and genital characteristics of 27 individuals, 23 females and 4 males, were examined, and four species were identified according to their morphological taxonomy: *Thymelicus sylvestris* (Poda, 1761), *T. lineolas* (Ochsenheimer, 1808), *T. hyrax* (Lederer, 1861), and *T. novus* (Reverdin, 1916) in Figures 1-2. Looking at the external morphology of *T. sylvestris* in Figure 1, the wingspan is 25-30 mm, and the upper part of the wings and body, as well as the tips of the antennae, are dark orange. A silvery white color is noticeable on the underside of this species. The external morphology of this butterfly was determined to be quite similar to that of *Thymelicus lineola*. It was determined that the underside of the antenna tip of *T. sylvestris* is yellow to orange, while that of *T. lineola* is black. Furthermore, differences were observed between these two species in the upper wings, especially in the lower regions, where black areas were present. Again, a characteristic black stripe formed by scent scales on the wings of male *T. sylvestris* individuals was identified as a distinguishing feature. Looking at the external morphology of the *Thymelicus hyrax* species, differences in

wing morphology are particularly noticeable. The upper part of the wing is yellowish-brown, the front part of the wing is brighter orange, it has a longer forewing compared to *T. sylvestris*, and the lines around the wing form a much thinner black line. Members of the *T. novus* species, on the other hand, were found to have a distance of 23-24 mm between the forewings, yellow wings, whitish structures resembling tassels on the edges of the wings, and black wing veins in Figure 1.

Figure 1. Morphology of some *Thymelicus* species whose DNA has been barcoded, male individuals (1-9) and female individuals (10-13): 1-2. *T. sylvestris* (GP3393, GP3399), 3-7. *T. lineola* (GP3389, GP3390, GP3398, GP3392, GP3391), 8- *T. sylvestris* (GP3400), 9- *T. novus* (GP3401). 10-*T. sylvestris* (GP3388), 11-*T. lineola* (GP3394), 12-*T. novus* (GP3402), 13-*T. hyrax* (GP3395).

The genital organs were found to be attached to the tenth or most distal part of the abdomen. Identification of *Thymelicus* species was made by identifying two species (*Thymelicus sylvestris* and *T. lineolus*) in males with a wide variety of complex spines, hairs, scales, and tufts, and four species (*T. sylvestris*, *T. lineolus*, *T. novus*, *T. hyrax*) in females with different types of pincers and varying modifications of duct bursae in Figures 2.

Species identified by morphological characteristics were barcoded, and both species boundaries were tested and their status was determined through molecular taxonomic analysis. Species boundaries and speciation ranges for thirteen taxa belonging to the genus *Thymelicus* were tested through comparative analyses using barcode sequences. For these analyses, only *T. lineola* populations were included for intraspecific comparison with the current study population due to the availability of previous data from Türkiye. According to the K2P comparative genetic distance analysis of eleven *Thymelicus* species, the genetic distances between the species were determined to be in the range of 0.02-0.06. The genetic distance between the eastern Anatolian population of *T. sylvestris* and China population of *T. sylvaticus* (HSPED353_11) was 0.02. The distances between the eastern Anatolian population presented in this study and the other Türkiye populations of *T. lineola* from the provinces of Kars (BPAL3220_16) and Artvin (LOWAB286_09) recorded in Boldsystems were measured at 0.01. Similarly, the genetic distance between the *T. lineola* Artvin population and the *T. novus* Hakkari population was 0.02 in Table 2.

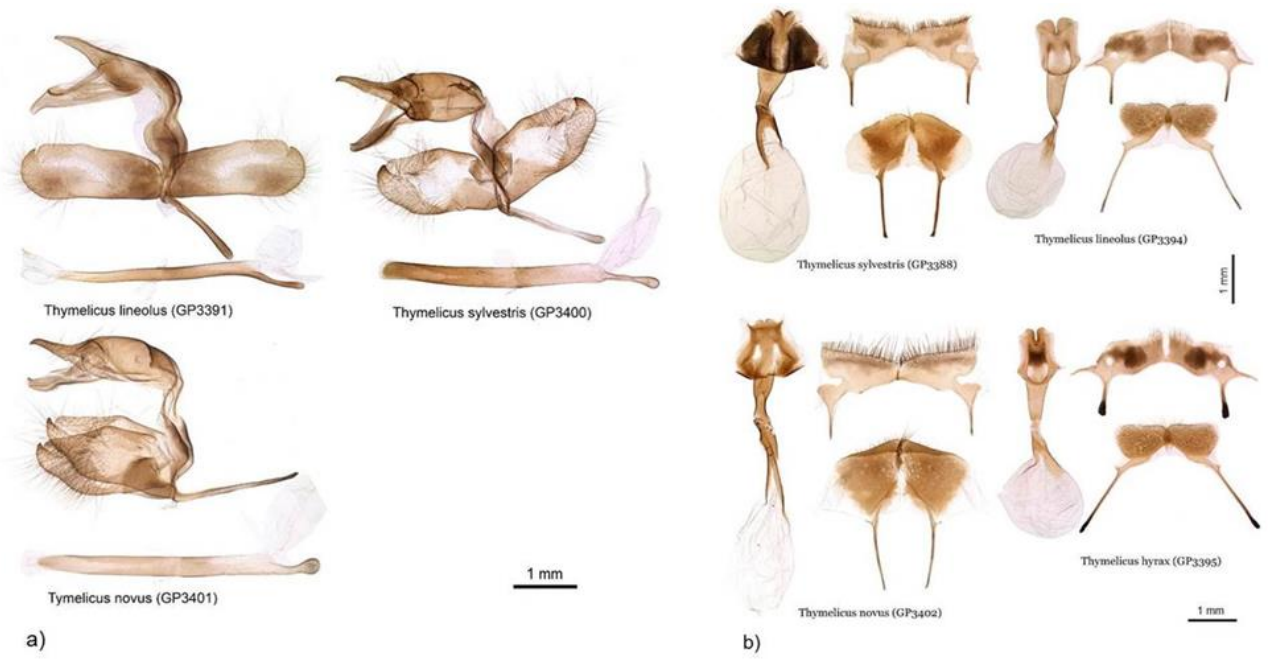

Figure 2. Genital morphology of DNA barcoded some *Thymelicus* species, male (a) and female (b) individuals.

Table 2. Genetic distance results among *Thymelicus* species according to the *Kimura*-2 parameter

Code/Species/Populations	**K2P-Genetic Distances**											
*T. sylvestris*_East_Anatolia Pop.												
HSPED353_11_*T. sylvaticus*	0.02											
GMCHL029_14_*T. leonina*	0.03	0.02										
BPALB219_17_*T. alaicus*	0.03	0.04	0.05									
BPALB278_17_*T. stigma*	0.03	0.04	0.04	0.03								
*T. novus*_ East_Anatolia_Pop.	0.04	0.04	0.05	0.05	0.05							
IRANB198_08_*T. acteon*	0.04	0.05	0.06	0.05	0.05	0.05						
BDE776_21_*T. christi*	0.04	0.04	0.05	0.05	0.05	0.05	0.03					
WMB1340_13_*T. hamza*	0.04	0.05	0.05	0.06	0.05	0.06	0.05	0.04				
*T. hyrax*_East_Anatolia_Pop.	0.05	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.06			
*T. lineola*_East_Anatolia_Pop.	0.05	0.05	0.06	0.06	0.06	0.03	0.05	0.05	0.05	0.04		
BPAL3220_16_*T. lineola*	0.05	0.05	0.05	0.06	0.06	0.03	0.05	0.04	0.05	0.04	0.01	
LOWAB286_09_*T. lineola*	0.05	0.05	0.05	0.06	0.06	**0.02**	0.05	0.04	0.05	0.04	0.01	0.00

In the schematic representations of the Automatic Barcode Divergence (ABGD) generated using for eleven valid species and some populations of the genus shows that divergence less than 0.02 represents the assumed intraspecific divergence, while higher divergence represents the interspecific divergence in Figure 3. For the *Thymelicus COXI* sequences in our dataset, the most stable species separation line was calculated as 0.02. Therefore, according to the initial partition results corresponding to this threshold value, the samples in the dataset were divided into eleven molecular operational taxonomic (MOTUs) units. These obtained molecular species boundaries largely agree with our morphological identification results.

The phylogenetic relationships of *Thymelicus* species were examined using BI/ML analyses two different cladistic algorithms. The tree topologies of both algorithms were very similar, indicating consistent results. Therefore, the bootstrap values and Bayesian posterior probability values are shown together in the maximum-likelihood tree in Figure 4. In the phylogenetic tree, *Thymelicus* taxa were divided into two main clades. The first main clade contains the clade consisting of *T. hamza* populations, with the branching shown in light green. The other main clade is divided into three subclades, two resolved and one polyphyletic. The first resolved subclade is the blue branched clade, which clusters the *T. sylvestris* populations. Within this subclade, European populations are clustered into monophyletic groups. The Greek, Israeli, Jordanian, and Armenian populations, including the East Anatolian *T. sylvestris* population presented in this study, are polyphyletic.

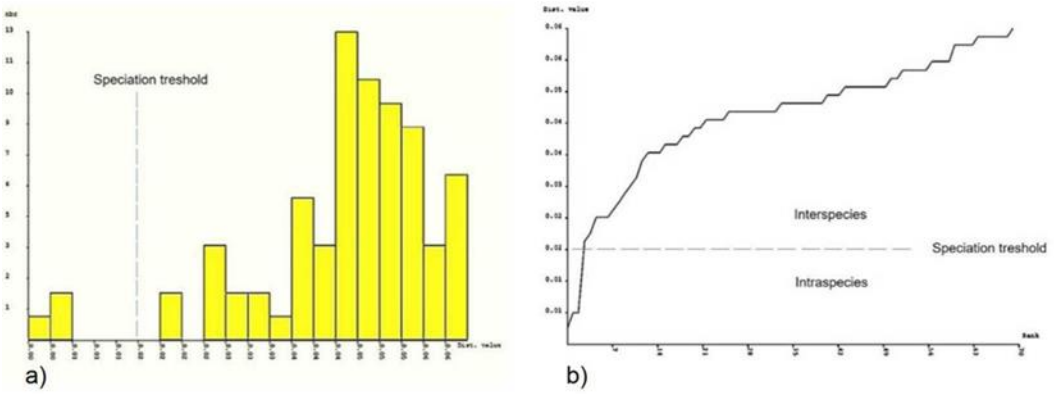

Figure 3. Two schematic representations of the Automatic Barcode Divergence belong to *Thymelicus* spp. a) Histogram of distances for the hypothetical distribution of intraspecific/interspecific divergence. b) Representation of the same data as ordinal values.

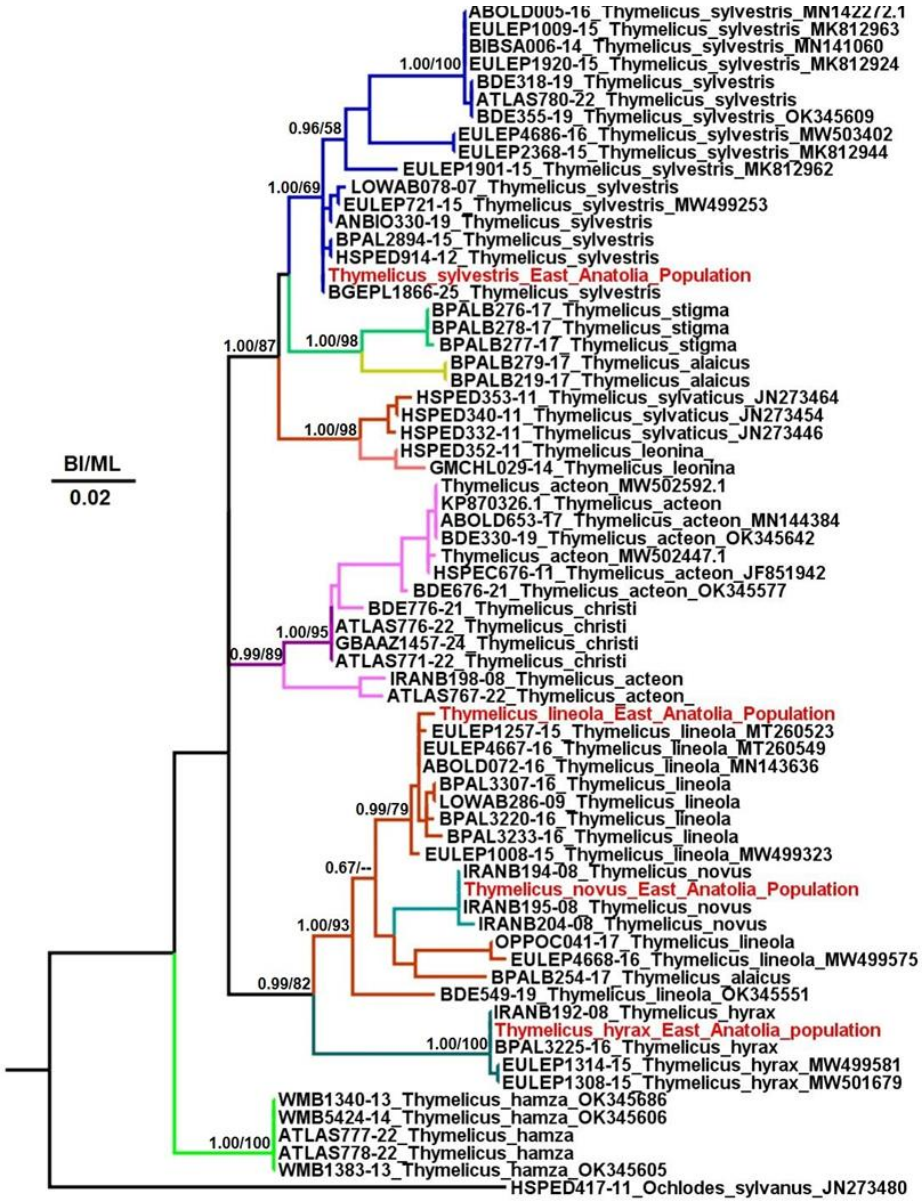

Figure 4. The phylogenetic tree based on COI barcodes of *Thymelicus* species/populations constructed with BI and ML algorithms. The Boldsystems numbers are on the left of the *Thymelicus* specimens, and the NCBI acceptance numbers, if any, are on the right. Numbers at the nodes indicate the posterior probability values for BI and bootstrap values for ML. Two dashes for ML value indicates a value less than 50%. The scale bar represents 2 substitutions per 100 nucleotide positions.

The dark green branched *T. stigma* populations, which are sister to the blue clade, are placed alongside the closely related yellow branched *T. alaicus* populations. These subclades, divided into two sister branches, blue and green, are connected to the red branched monophyletic clade, consisting of *T. sylvestris* and *T. alaicus* populations. Within the second resolved subclade, *T. lineola* populations formed three paraphyletic dark red branched clades. The first consisted of the Eastern Anatolian population, closely related to the Greek, Bulgarian, and Austrian populations, and sister to other Turkish populations (Artvin and Kars provinces), as well as the Azerbaijani population.

The second consisted of the cyan-branched *T. novus* clade, sister to the Canadian and French *T. lineola* populations, and the closely related *T. alaicus* population in Tajikistan. The Spanish population of *T. lineola* was basally connected to all of these. The dark red-branched *T. hyrax* populations were sister to the forest-branched clade. The unresolved lineage is shown in the purple and lilac branched clade. This clade consists of the paraphyletic *T. acteon* and *T. christi* populations. The first group consists of the acteon+christi clade, and the second group consists solely of the *T. acteon* Iranian and Cypriot populations.

4. Discussion and conclusions

The morphology of *Thymelicus* species is well studied (Shapiro, 1971; Prins et al., 1992). Therefore, the presented morphology and genitalia are consistent with previous studies. Based on molecular taxonomic analyses conducted by including all taxa in the genus and genetic distances and species boundaries determined by species barcodes of four morphologically identified *Thymelicus* species from eastern Anatolian populations, each population was clustered within its own species clade. Therefore, morphological diagnoses and molecular taxonomic findings are consistent with each other. Phylogenetic relationships of *Thymelicus* species have been partially reported for five species (*T. christi*, *T. acteon*, *T. hyrax*, *T. sylvestris*, and *T. lineola*, only from European populations) (Mutanen et al., 2016). Comparison of barcodes with other species of the genus and species delimitation analyses have not been performed until this study. Here, we report the barcodes and species delimitation results of four *Thymelicus* species from Turkish populations (previously present northeastern populations of *T. lineola*). Genetic distance analyses and phylogenetic relationships among eleven species of the genus based on *COXI* barcode sets are presented for the first time in this study. The mitochondrial COXI gene is a universal standard barcode used in species identification for butterflies and moths due to its high copy number, lack of an intronic region, and clarity in tracing a maternally inherited lineage. Many studies using this gene region have determined that intraspecific variation is low and interspecific variation is high. These results enabled the species boundaries of this barcode to be clearly delineated (Hajibabaei et al., 2006; Hausmann et al., 2011; Huemer et al., 2014). According to the ABGD method and the *Kimura*-2 parameter model, the genetic distances among all species range from 0.02 to 0.06 in Figure 3 and Table 2. These references indicate that the species are clearly genetically distinct. Results, not previously presented in studies and for the first time in this study, based on the *Kimura*-2 parameter model, highlighted in red in the genetic distance table, show that *T. alaicus*, *T. stigma*, and *T. novus* species are genetically distinct from other species within the range of 0.02 to 0.05. The same table also shows a genetic distance between the *T. novus* eastern Anatolian population and the *T. lineola* Artvin province population of 0.02 (bold red). The results of the analyses based on the ABGD approach and the Kimura-2 parameter were found to be highly consistent with the phylogenetic tree results.

In the consensus tree constructed with Bayesian inference and Maximum Likelihood algorithms, 11 species (except for 2) were observed to form single clades with strong support values. Within the polytomic branched acteon+christi clade, shown in purple, the first acteon group is closely related to the *T. christi* clade, while the second acteon group consists of the *T. acteon* populations from Cyprus and Iran, and sister position with acteon+christi clade. Another paraphyletic taxon was observed in the lineola+novus clade. The dark red branched *T. lineola* populations were divided into three groups. The first group clustered with the European populations of eastern Anatolia and the previously existing populations from Artvin and Kars provinces presented in this study. The second group comprised the *T. lineola* populations from Canada and France, and the *T. alaicus* (BPALB254-17) population, which are sister to them. The third group was the *T. lineola* population from Spain, which is basally connected to them. We believe that the *T. alaicus* taxon placed within this lineola+novus clade was misdiagnosed because all the samples whose barcodes were used in the tree were from Tajikistan populations. The distribution of *T. lineola* in this region is reported, and the other *T. stigma* and *T. alaicus* are barcodes of specimens recorded from this region. *T. acteon* and *T. lineola* are not monophyletic, while the others clustered in discrete clades. We observe that when data from different

geographical regions are collected for the species, and considering the overlapping habitat, the phylogeny signals increase the likelihood of paraphyletic taxa. Therefore, interspecific gene transfer via hybridization is quite common among Lepidoptera between species that share a common ancestor or have recently diverged from the ancestral lineage (having recently completed speciation) (Zakharov et al., 2009; Dasmahapatra et al., 2012; Hinojosa et al., 2022). In particular, maternally driven mitochondrial gene traits should be considered as a possible cause of mtDNA paraphyly due to introgression. Almost 16% of the recorded European butterfly species can hybridize with a closely related species in their natural habitat. It has been determined that 8% of these matings result in the production of surviving hybrid offspring (Descimon & Mallet, 2009). Similarly, DNA barcodes (mitochondrial *COXI* gene) of 217 species of Swiss moths and forest moths (Rhopalocera and Zygaenidae) were obtained for species identification. It was reported that approximately 90% of the genetic differentiation analysis results of the DNA-barcoded Swiss species were consistent with morphology. The remaining 10% were emphasized as non-monophyletic due to the possibility of introgression or incomplete lineage divergence, as they were closely related taxa and may even present examples of potential hidden diversity. Among these species, *Melitaea athalia* and *M. nevadensis* were recorded to have come into contact in a narrow region, and some hybridization occurred in this region. Furthermore, it has been suggested that *T. sylvestris* and *T. lineola*, despite their morphological similarity, may harbor two or more cryptic species due to their potentially high genetic diversity within themselves (Litman et al., 2018). In the presented phylogenetic tree, consistent with the results of the referenced study, the *T. sylvestris* clade (blue clade) is divided into significant subclades based on branch lengths. More detailed morphological, ecological, and molecular analyses of the populations within these subclades should be considered to explore the possibility of cryptic species. Similarly, *T. lineola* (red clade) populations, exhibiting high genetic diversity and identical morphology, are clustered into three distinct lineages. The genetic differentiation within them is clearly visible. Considering three distinct putative species within the lineola clade in this way could be a key to resolving the paraphilia problem with *T. novus* and *T. alaicus*.

In conclusion, the barcoding of four *Thymelicus* species from Eastern Anatolia was performed for the first time, and species boundaries with other species of the genus were determined, supported by morphological data. Furthermore, phylogenetic relationships of eleven species were calculated using two evolutionary algorithms and identified at the species level. However, the fact that the database only contains barcode information for European species/populations of this genus, with no data from the eastern Palearctic and North Africa, presents the biggest challenges for comparative analyses. This study marks the first time that *Thymelicus* species of the Eastern Anatolian region have been barcoded and species boundaries tested using modern taxonomic research. Therefore, we believe that sufficient sampling of the distribution areas of the species in different geographical locations will reveal intertaxon boundaries and uncover high genetic diversity that has not yet been recognized, providing more robust evidence in the future for solving problems such as interspecies hybridization, incomplete lineages, or unknown parologies.

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Author contribution

Conceptualization, S.K.; Methodology, S.K. and M.K.K.; Validation, S.K. and M.K.K.; Investigation, S.K.; Resources, S.K.; Data Curation, S.K.; Writing—Original Draft, S.K.; Writing—Review & Editing, S.K.; Supervision, S.K.

Declaration of ethical code

The authors of this article declare that the materials and methods used in this study do not require ethics committee approval and/or legal-special permission.

Conflicts of interest

The authors declare that there is no conflict of interest.

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