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Genetic and Morphological Diversity of *Tenthredopsis* (Tenthredinidae: Symphyta: Hymenoptera) Species: A Case Study in Anatolia

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ABSTRACT

The genus Tenthredopsis (Tenthredinidae: Hymenoptera), characterized by a Palearctic distribution, is widely recognized as a taxonomic challenge due to its limited morphological variation. This study aims to evaluate the phylogenetic and biogeographical characteristics of Tenthredopsis species in Anatolia by analyzing their morphological and molecular traits. Initially, morphotypes were defined based on morphological characters, and the taxonomic validity of each morphotype was assessed through phylogenetic analyses using three mitochondrial gene regions. The findings revealed congruence between morphological and molecular data. The study identified the distribution of 27 taxa within Anatolia, comprising 17 confirmed and 10 potential taxa, represented by 250 individuals belonging to the genus Tenthredopsis. The results underscore Anatolia's significance as a hotspot for genetic and morphological diversity within the genus Tenthredopsis and highlight its critical role in the evolutionary adaptations of these species. Additionally, the observed distribution patterns further support the importance of Anatolia as a refugium during the last glacial periods.

Keywords: Tenthredopsis, COI, COII, Cytochrome b, Biogeography.

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Introduction

The understanding and classification of biological diversity are of paramount importance for elucidating the fundamental mechanisms underlying natural systems [1]. The field of systematics provides a framework for comprehending the evolutionary relationships of organisms, thereby offering essential insights into the foundations of biodiversity. Taxonomic studies not only facilitate the identification of existing species but also contribute to understanding their ecological roles and devising effective conservation strategies [2]. Within this context, an in-depth understanding of biodiversity dynamics enables the formulation of more robust policies to address environmental changes.

Anatolia occupies a unique position globally due to its exceptional biodiversity. Its geographic location at the intersection of Asia, Europe, and Africa has resulted in a mosaic of habitats that are both in floristic and faunistic rich. This distinctive geographic structure has accelerated species adaptation processes in the region, leading to a high degree of genetic diversity [3]. The exceptional biodiversity of Anatolia renders it an ideal model for investigating evolutionary processes in species adapted to diverse ecological conditions. This unique status of Anatolia is critical not only for the survival of endemic species but also for their adaptation to environmental stressors. Geographic barriers have shaped species distributions and genetic divergence, thereby enhancing regional biodiversity. For instance, Anatolia's mountainous terrain and varied microclimatic zones are significant factors supporting the genetic variation observed in resident species. This phenomenon offers a pertinent example of adaptive evolution and biogeographical distribution within the genus *Tenthredopsis*.

The *Tenthredopsis* species, which exhibit broad distribution across the Palearctic region, demonstrate considerable variation in response to the unique environmental conditions of Anatolia. During past glacial periods, Anatolia functioned as a refugium for numerous species. Following the retreat of glaciers, these species expanded to different regions, thereby increasing genetic diversity. The geological history of Anatolia offers critical insights into the current genetic structure and distribution of species in the region [1]. Within this framework, *Tenthredopsis* species represent an ideal model group for understanding the biogeographical and evolutionary processes of Anatolia. Their adaptation processes integrate both morphological and genetic characteristics.

Systematic studies of *Tenthredopsis* species necessitate the integrated application of genetic and morphological methods. These species are notable for their high adaptive capacity to environmental factors. Limitations encountered in morphological analyses are effectively addressed through molecular biology techniques, leading to more precise results. For instance, species exhibiting similar morphological traits can be distinctly identified using DNA sequencing methods. In particular, mitochondrial DNA markers play a crucial role in elucidating phylogenetic relationships taxonomic units. Gene regions such as COI, COII, and cytochrome b serve as effective tools for assessing genetic distances among species.

Anatolia is considered a transition zone for both plant and animal species in terms of biogeography. This perspective not only facilitates an understanding of current species distributions but also provides critical insights into their origins and evolutionary trajectories. In this regard, *Tenthredopsis* species serve as pivotal examples for investigating the nexus between biogeography and evolutionary biology. The findings of this study are anticipated to contribute not only to the understanding of *Tenthredopsis* species but also to the development of broader biodiversity strategies for Anatolia.

This study comprehensively examines *Tenthredopsis* species to elucidate the role of Anatolia in fostering biological diversity. The primary objective is to analyze the genetic and morphological characteristics of these species, thereby providing a clearer understanding of their evolutionary processes and biogeographical distributions. Future research should focus on the population dynamics and habitat requirements of these species in greater depth.

Given the ecological significance of Anatolia, developing conservation strategies for these species constitutes a critical step toward ensuring the sustainability of biodiversity at both local and global scales. *Tenthredopsis* species, in this context, emerge as a model group for advancing studies in conservation biology and evolutionary biology.

Materials and Methods

The specimens belonging to the genus Tenthredopsis examined in this study were collected from Anatolia during April - July between 1995 and 2006. DNA isolation was performed on a total of 250 individuals belonging to 27 putative taxa, including one outgroup. COI, COII, and Cyb fragments were amplified using the PCR method. The fragments were sequenced, aligned, and data sets were prepared for analysis. Datasets were analyzed using different contemporary approaches to determine species boundaries.

Preparation of Specimens for Morphological Data

A total of 250 specimens belonging to 27 putative taxa, including one outgroup, were examined in this study. As a result of studies conducted in Anatolia, 18 taxa were recorded by researchers; however, not all of these taxa could be collected during field study. Therefore, some localities of the specimens belong to different countries. For morphological data analysis, one of the 250 selected specimens belonged to the genus *Aglaostigma*, which was selected as the outgroup. Each specimen analyzed in the study was assigned a unique number. Nikon SMZ-645 and Comecta-SQF-E stereomicroscopes were used to describe the characters. Based on the morphological character examination, the specimens were categorized into 27 morphotypes (putative taxa).

Preparation of Specimens for Molecular Data: DNA Isolation, Amplification, and Sequencing

Total genomic DNA was isolated from specimens preserved in 85% ethanol following the DNA isolation protocol of Moritz (1990) [4]. Primer pairs for the gene regions —COI s1859/a2590, C2J3400/C2N3600, and CB1/CB2—were based on studies conducted on the Hymenoptera order [3]. For the PCR reactions, the prepared 50 µl reaction mixture contained 10 µl [100 µM/µl each of dATP, dGTP, dCTP, and dTTP (MBI Fermentas)], 5 µl of 1x Taq DNA polymerase buffer (75 mM Tris-HCl pH 8.8 at 25°C, 20 mM (NH4)2SO4, 0.01% Tween 20), 4 µl (1.5 mM/µl MgCl2, 1 pmol forward primer, and 1 pmol reverse primer), 0.3 U (5 U/µl) Taq DNA Polymerase (MBI Fermentas), and 1 µl (200 ng/µl) template DNA. The final volume was adjusted to 50 µl with distilled water. Sequencing reactions were performed by Macrogen.

Morphological Data Analysis

In the morphological study, characters were coded as “0, 1, 2, 3, 4, ?” depending on their character states. In the coding, “0” indicates the character state observed in the outgroup, while “1, 2, 3, 4” denote states differing from the outgroup. When multiple character states were observed within the same morphotype, the state was coded as polymorphic (e.g., 0 and 1). A total of 28 characters were examined for each morphotype. The morphological data set was analyzed using the PAUP* 4.0b10 software [5]. The data set was analyzed under the Parsimony approach, considering the recommended steps and options [6].

Molecular Data Analysis

The DNA sequence data obtained from the three gene regions consisted of a total of 1278 nucleotides in length. DNA sequence analyses were conducted using the PAUP* 4.0b10 software under Parsimony and Maximum Likelihood approaches.

Findings and Discussion

Morphological and molecular data analyses of Anatolian *Tenthredopsis* species were performed in this study. COI, COII, and cytochrome b gene regions from mitochondrial DNA were used to the molecular analysis of individuals belonging to the genus of *Tenthredopsis*. The generated sequence lengths of the COI, COII and cytochrome b gene regions were 652 bp, 221 bp and 406 bp; respectively.

Morphological Data Analyses

Prior to the analysis, *Aglaostigma* was designated as the outgroup. The "uncertainty" option was selected for multiple character states. The character polarization option was set to "unordered." In the first step of the heuristic method, the "stepwise addition" phase employed the "simple" algorithm. the "branch-swapping" phase and the “Tree Bisection and Reconnection” algorithm was utilized in the second research step. As a result of the analysis, 4485 most parsimonious trees with a length of 108 steps were obtained. The index values for the dataset were calculated as CI: 0.4630, HI: 0.5370, RI: 0.6027, and RC: 0.2790. Subsequently, character weighting was performed 1000 times under the RC and "best-fit" options. The number of trees was fixed at 260. In cases where a large number of alternative parsimonious trees existed, a "Consensus Tree" summarizing the common information of these trees was constructed using the "majority" option. The Maximum Likelihood tree obtained is illustrated in Figure 1a. The majority-rule consensus tree of the 4485 parsimonious trees is shown in Figure 1b.

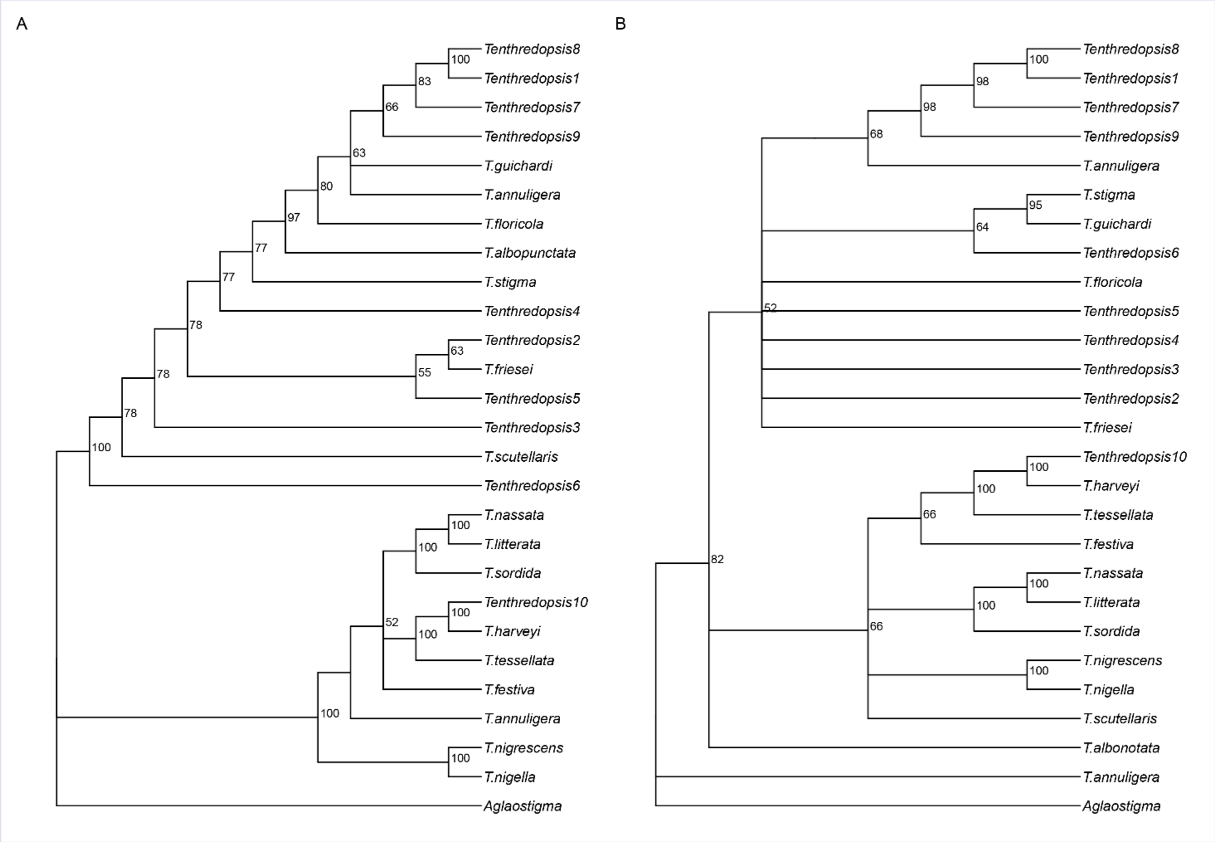

Figure 1. The majority-rule consensus tree obtained from 4485 parsimony trees (A) and the Maximum Likelihood analysis of morphological data based on 260 trees (B).

Molecular Data Analysis

The analysis was conducted on the total molecular data set, which was created by combining data obtained from all three gene regions. **Parsimony Analysis:** For the COII region, the analysis options were applied as in the morphological data analysis. For the COI and Cyb regions, the analysis was performed using the branch-and-bound algorithm. As a result, 40,900 parsimonious trees with a step length of 684 were obtained. Character weighting did not reduce the number of trees. Consensus trees derived from the high number of parsimonious trees were found to be unresolved. Therefore, the analysis was repeated using 1000 parsimonious trees. Post-analysis character weighting did not alter the number of parsimonious trees. To evaluate the reliability of the parsimonious trees obtained from the analysis, the bootstrapping method was applied [6]. The majority-rule consensus trees resulting from the parsimony and Maximum Likelihood approaches are presented in Figure 2.

Lineweaver-Burk plot was drawn according to the data obtained in vitro by using 4 different concentrations of XO enzyme substrate (2, 1, 0.5, 0.3 mM). Vmax and Km values were calculated with the help of Lineweaver-Burk plot (Figures 2) (Table 2).

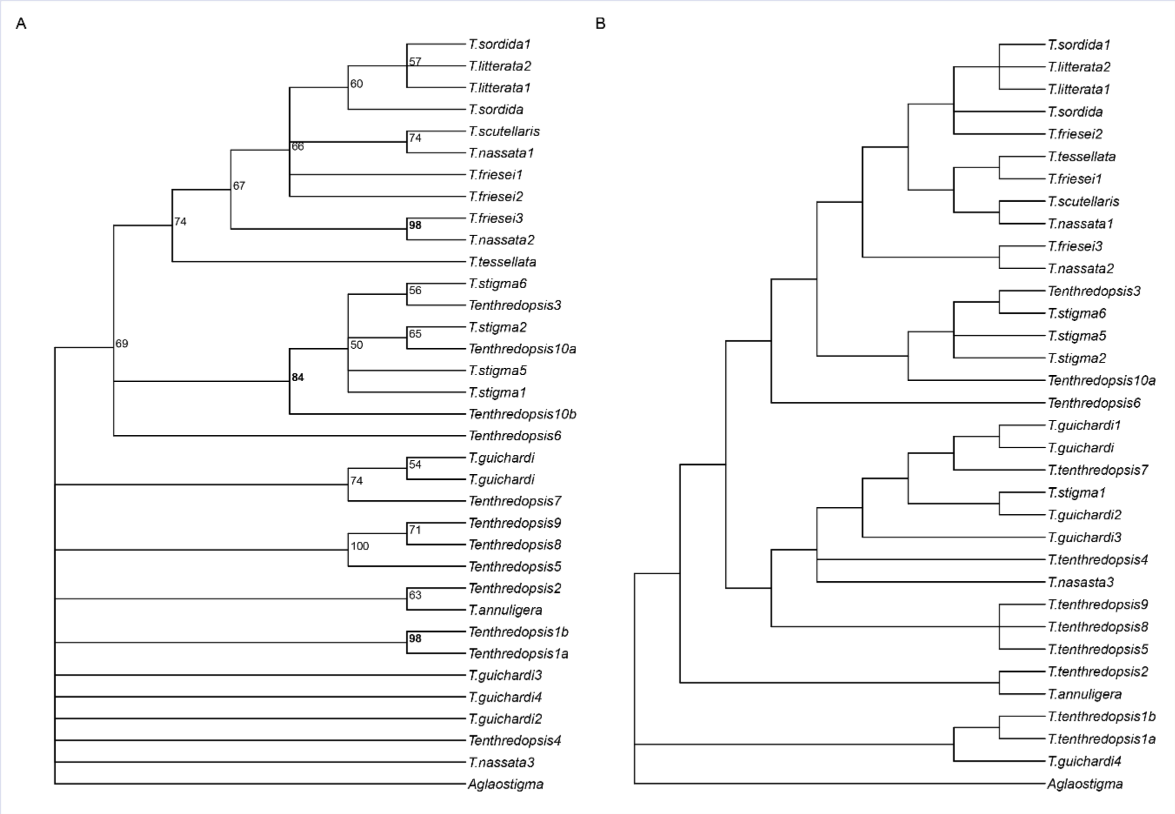

Figure 2. The majority-rule consensus trees resulting from the parsimony (A) and Maximum Likelihood (B) approaches for the total gene sequence.

Morphological Data Assessment

The suborder Symphyta constitutes a model group for systematic and phylogenetic studies. It is of evolutionary interest because it includes primitive groups of the order Hymenoptera with diverse life histories and biology [8-11]. Understanding the evolutionary relationships among the hymenopteran families and the transition from phytophagous to parasitic and predatory feeding modes requires detailed knowledge of this group [12-13-14]. The suborder Symphyta is classified into six superfamilies and 14 families [15]. The family Tenthredinidae, which belongs to the superfamily Tenthredinoidea, exhibits considerable diversity in both habitat and appearance. It contains more species than all other symphytan families combined [15]. This family has seven subfamilies: Selandriinae, Dolerinae, Nematinae, Heterarthrinae, Blennocampinae, Allantinae and Tenthredininae. Adult tenthredinines, members of the largest subfamily Tenthredininae, usually appear in spring or early summer. This subfamily includes ten genera. In the Palearctic region, the genus *Tenthredopsis* A. Costa, 1859, is represented by approximately 40 species [2]. Globally, it includes over 70 species, despite the absence of a recent revision. Five species are recorded in Britain, and 17–20 species are recorded in Russia. No faunistic study on this genus has been conducted in Turkey. However, the examination of materials collected sporadically by researchers has recorded 18 species belonging to this genus in Turkey [2-16-17-18-19-20]. The host plants of this phytophagous genus are members of Apiaceae and *Euphorbia* [21].

The identification of *Tenthredopsis* species presents a significant challenge, primarily due to the genus's limited morphological characters. The utilization of colour characters during identification is a common practice. However, regional variations observed in specimens collected from different geographic regions reduce the utility of these characters, frequently leading to misidentifications. In a study by Benson (1968), the species *Tenthredopsis albonata* (Brulle), recorded from Anatolia, was misidentified due to a typographical error and was treated as a separate species in Turkish records for years. Additionally, Benson synonymized *T. benthini* Rudow, 1871, with *T. albopunctata* (Tischbein, 1852), which was subsequently synonymized with *T. annuligera* (Eversmann, 1847). Consequently, the number of *Tenthredopsis* species recorded from Anatolia was reduced to 14. Of these, *T. guichardi* and *T. harveyi* are endemic to Anatolia. In this study, *T. guichardi* was collected from multiple localities, while *T. harveyi* was not found in any locality. The specimens of *T. harveyi* were therefore requested from the Natural History Museum for descriptions. Similarly, previously recorded but uncollected species such as *T. albonotata*, *T. annuligera*, *T. festiva*, *T. floricola*, *T. litterata*, *T. nigella*, and *T. nigrescens* were examined in the collection of the Deutschland Entomological Institute. However, the species *T. stramineata* Konow, recorded from Anatolia, was not found in any entomological institute contacted. Its descriptions were derived from its original description.

The present study identified ten unnamed taxa. To test whether the morphotypes created during the study represent separate taxa, both morphological and molecular datasets were analyzed using phylogenetic methods. The character states of 28 morphological characters belonging to 27 taxa are presented in the upper part of this section. Majority-rule consensus trees based on the parsimony method of morphological data were evaluated (Figures 1 and 2). Two main clades were observed in the majority-rule consensus tree of 260 parsimonious trees, while four main clades were revealed in the tree derived from 4485 parsimonious trees. However, when *T. albonotata* was included in the first clade and *T. annuligera* in the second clade, three distinct taxon groups were identified in both trees.

In parsimony and maximum likelihood analyses of the COII gene region, three main groups were observed. The only difference between the parsimony tree and the maximum likelihood tree is that *T. friesei1*, *T. nassata2*, and *T. tessellata* are not included in any of the three groups in the parsimony tree. The emergence of *Tenthredopsis2* as a separate lineage in both trees supports the hypothesis that it may represent a new taxon. Similarly, *Tenthredopsis4*, *Tenthredopsis5*, *Tenthredopsis6*, *Tenthredopsis8*, and *Tenthredopsis9* may either represent separate taxa or species groups, or alternatively, they may belong to a single species. *Tenthredopsis10a* and *Tenthredopsis10b* appear to belong within or closely related to *T. stigma*. Similarly, *Tenthredopsis1a*, *Tenthredopsis1b*, and *Tenthredopsis7* are observed within or near *T. guichardi*.

The parsimony and maximum likelihood analyses for the COI gene region resulted in fundamentally similar trees. In the parsimony tree, *T. annuligera* was excluded. Compared to trees from other gene regions, the longer sequence of the COI gene resulted in more resolved trees. Specimens belonging to *T. guichardi* grouped together, as did specimens of *T. stigma*. These findings provide strong support for the distinction of both species as separate taxa. In the maximum likelihood trees, *T. litterata* and *T. friesei* were observed as closely related species. *Tenthredopsis4* and *Tenthredopsis10b*, *Tenthredopsis2* and *Tenthredopsis1b*, and *Tenthredopsis9* and *Tenthredopsis5* were identified as closely related taxa in both trees.

The parsimony and maximum likelihood trees constructed for the cytochrome b gene region were largely consistent with those for the COI and COII regions. While *T. nassata2* and *T. nassata3* were found in different groups, three main groups were observed in the tree. Parsimony-absolute consensus trees, maximum likelihood trees, and bootstrap trees constructed for the combined dataset of COI, COII, and cytochrome b gene regions also revealed three main groups. *T. nassata*, *T. scutellaris*, *T. friesei*, *T. tessellata*, *T. litterata*, and *T. sordida* consistently grouped together in all analyses. Except for *T. guichardi4*, other specimens of *T. guichardi* were also grouped on the same branch.

Among the studied specimens, *T. guichardi* had the longest available sequence. Morphological analysis revealed significant color variations within this species. While *T. guichardi4* emerged as a separate clade in the genetic analyses, the available data are insufficient to confirm its status as a distinct taxon. In the maximum likelihood and bootstrap analyses, *T. stigma*, *Tenthredopsis10a*, *Tenthredopsis10b*, *Tenthredopsis6*, and *Tenthredopsis3* were grouped together. Across all total analyses, *Tenthredopsis5*, *Tenthredopsis8*, and *Tenthredopsis9* consistently grouped together, suggesting the possibility that they represent a single species. Similarly, in all total analyses, *Tenthredopsis7* was placed on the same branch as *T. guichardi*, indicating that they are either two closely related species or a single species.

In Benson's (1968) key for *Tenthredopsis* species, the genus was divided into six species groups based on the shape of the clypeus. The groups observed in our analyses, based on both morphological and molecular data, support Benson’s (1968) species groups. During the identification of specimens in this study, *Tenthredopsis10a* and *Tenthredopsis10b* were observed to be morphologically similar to *T. stigma*. However, due to certain structural differences, they were not classified as the same species. Phylogenetic analyses also placed these taxa in the same groups, suggesting that they may represent either two closely related species or a single species.

T. nassata, *T. scutellaris*, *T. friesei*, *T. tessellata*, *T. litterata*, *T. stigma*, and *T. sordida* are species with a broad Palearctic distribution and are grouped together in phylogenetic analyses based on both morphological and molecular data. The distributions of *T. harveyi* and *T. stramineata*, on the other hand, are restricted to Turkey. *T. guichardi* is the most commonly encountered *Tenthredopsis* species in Anatolia. Similarly, *T. festiva*, *T. nigella*, and *T. nigrescens* are species with a limited distribution across Anatolia and its neighboring countries (Russia and Armenia). When considering regional distribution data, species distributed within the Iran-Turan subregion (*T. nassata*, *T. scutellaris*, *T. friesei*, *T. tessellata*, *T. litterata*, *T. stigma*, *T. sordida*, *T. nigella*, and *T. albonotata*) are found on the same lineage in almost all phylogenetic analyses, except for *T. guichardi*. Similarly, species distributed in the European and Mediterranean subregions, such as *T. annuligera* and *T. floricola*, are grouped together in our morphology-based trees (Figures 1 and 2). Considering the distribution patterns and phylogenetic analyses of these species, as well as evidence from other taxonomic groups with broad Palearctic distributions [22-23-24], it is supported that Anatolia served as a primary refugium during the last glacial period. Additionally, the distribution of certain *Tenthredopsis* species lies along the Balkan-Anatolian corridor. For instance, *T. albonotata* and *T. annuligera* are examples of species distributed across Anatolia, Greece, Macedonia, Croatia, Hungary, Romania, and Ukraine. Three potential routes for their dispersal during interglacial periods are proposed. The first route is the Anatolia–Caucasus corridor, but due to the presence of significant geographical barriers between Anatolia and the Caucasus, this route seems less viable for species with low ecological tolerance and dispersal capability. The second route involves dispersal from eastern Anatolia through Iran, south of the Caspian Sea, and into Russia, eventually reaching Northern Europe. The third potential route suggests dispersal through the straits into the Balkans and subsequently through the Balkans into Europe.

Conclusions

This study comprehensively evaluated both the genetic and morphological characteristics of *Tenthredopsis* species distributed in Anatolia, once again emphasizing the region's importance in terms of the symphytan diversity. The findings of the study demonstrated that species that are difficult to identify morphologically can be classified more precisely using molecular markers, revealing significant genetic diversity among them. The data obtained clearly show that Anatolia has historically served as a biological refuge. During glacial periods, the region functioned as a sanctuary for many species, which also explains its high current biodiversity. Particularly, *Tenthredopsis* species can be considered a model group for understanding Anatolia’s biodiversity strategies due to their ecological adaptations and genetic diversity. Phylogenetic analyses have contributed to understanding the evolutionary relationships among *Tenthredopsis* species and have revealed that some species exhibit genetic variations unique to Anatolia. For instance, the phylogenetic trees constructed from COI and COII gene regions have elucidated the historical distributions and adaptation processes of the species. Additionally, analyses based on the cytochrome b region have provided insights into the geographical distribution patterns and potential evolutionary origins of these species. In conclusion, *Tenthredopsis* species distributed in Anatolia present a unique model for biodiversity conservation and evolutionary biology studies. The region’s biodiversity is of paramount importance, not only for preserving existing species but also for ensuring the sustainability of future ecosystem services. In this context, increasing genetic and ecological research will significantly contribute to biodiversity conservation at both local and global scales.

Conflicts of interest

There are no conflicts of interest in this work.

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