



Contribution to the caddisflies fauna in Bosnia and Herzegovina: The first molecular confirmation and DNA barcode records of two *Synagapetus* species (Trichoptera: Glossosomatidae)

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Abstract

Background and purpose: Three genera and eleven species of Glossosomatidae family have been confirmed in Bosnia and Herzegovina. However, there is limited research on the biodiversity of this family in the region, especially on molecular data. With this paper, we contribute to the knowledge on the Glossosomatidae family with the first molecular records for two species for Bosnia and Herzegovina.

Materials and methods: Larval samples of caddisflies were collected in April 2019 and 2021 at the area of the Natural Monument the Spring of Bosna River followed by morphological identification. Molecular confirmation of species was done using DNA barcoding, targeting the cytochrome c oxidase subunit I (*COI*) region using Folmer's primers and degenerated primers LCO1490-JJ and HCO2198-JJ. All obtained PCR products were bidirectionally sequenced using the same primers as in PCR.

Results: Out of 52 specimens, 18 were morphologically determined to species level. A DNA barcode was successfully generated for 16 specimens. Eight sequences were generated for *Synagapetus krawanyi*, revealing three haplotypes previously absent from the BOLD database. Four sequences were obtained for *S. iridipennis*, with one new haplotype for BOLD database.

Conclusions: We present new data on species distribution and genetic diversity of *S. krawanyi* and *S. iridipennis* from Bosnia and Herzegovina. This study contributes to the knowledge of caddisfly biodiversity and the Global Trichoptera Barcode Initiative by adding new records from two underrepresented species in the BOLD database.

INTRODUCTION

Family Glossosomatidae of the order Trichoptera is known for its specific case morphology in the larval stage. However, their larval forms differ greatly, and hence they were split into separate families (1). In Europe, there are 68 Glossosomatidae species present, specifically, 22 *Agapetus* Curtis, 1834 species, 24 *Synagapetus* McLachlan, 1879 species, two *Catagapetus* McLachlan, 1884 species, and 20 *Glossosoma* Curtis, 1834 species (2). The presence of three genera *Agapetus*, *Synagapetus*, and *Glossosoma* has been confirmed on the territory of Bosnia and Herzegovina (3–5). According to Stanić-Koštroman (6),

eleven species are present in this country: *Glossosoma bifidum* McLachlan, 1879, *G. bunae* Marinkovic-Gospodnetić, 1988, *G. conformis* Neboiss, 1963, *G. discophorum* Klapalek, 1902, *G. neretvae* Marinkovic-Gospodnetić, 1988, *G. boltoni* Curtis, 1834, *Agapetus laniger* Pictet, 1834, *A. ochripes* Curtis, 1834, *Synagapetus iridipennis* McLachlan, 1879, *S. slavorum* Wallengren, 1891, and *S. krawanyi* Ulmer, 1938. In the scientific community, there are opposing opinions on the status of the genera *Agapetus* and *Synagapetus*. Some authors consider *Synagapetus* as a subgenus of *Agapetus* (7), while others regard it to be an independent genus (8). In this paper, they will be treated as separate genera according to Malicky (8). Recent phylogenetic studies of Trichoptera (9) have confirmed that the Spicipalpian families, which include Glossosomatidae, are again found to be paraphyletic, and most closely related to Integripalpia.

Research regarding the biodiversity of the family Glossosomatidae on the territory of Bosnia and Herzegovina is scarce. The most specific paper on species of this family was written in 1988 by Marinković-Gospodnetić (5), in which two microendemic species in Bosnia and Herzegovina were described, *G. bunae* and *G. neretvae*. Although manuscripts contributing to the knowledge of the distribution and diversity of Glossosomatidae in Bosnia and Herzegovina exist, they do not focus specifically on this group of Trichoptera (10, 11). In 2007, Previšić *et al.* (12) found three Glossosomatiade species in Bosnia and Herzegovina, reporting *G. bifidum* in Crna Rijeka, *G. discophorum* and *S. krawanyi* in Bijela Rijeka and Crna Rijeka. In the extensive research of the group of authors (13), species belonging to Glossosomatidae family were

found in Neretva, Radobolja, Lištica and Bunica in Herzegovina and Željeznica, Fojnička Rijeka, Kreševka and Lepenica in Central Bosnia. *G. bifidum* was found in Lištica River, Željeznica, Fojnička Rijeka and Kreševka. *G. bunae* was found in Bunica River. *G. conformis* was found in Željeznica, Kreševka and Lepenica River. *G. discophorum* and *A. ochripes* were found in Neretva, and *G. neretvae* was found in Neretva and Radobolja. In general, the research intensity has not been equal across Trichoptera group, neither across the Western Balkan countries. New species are still being found (14, 13), as well as new subspecies (15).

In this paper, we present findings of two Glossosomatidae species, *S. krawanyi* and *S. iridipennis*, from the Natural Monument the Spring of Bosna River identified through a morphological and genetic approach of species determination.

MATERIAL AND METHODS

Field research

Caddisfly larvae were collected in April 2019 and 2021 at the Natural Monument of the Spring of Bosna River, located southwest of the city of Sarajevo, below the slopes of the mountains Igman and Bjelasnica (Figure 1), with forceps as larvae could be easily spotted on the rocks in the stream. They were stored in 96% ethanol until further analysis. All samples were morphologically identified to the lowest taxonomic category using Olympus SZX10 stereozoom microscope (Olympus Life Science, USA) and the taxonomic key written by Waringer et Graf (16) and then archived in the tissue section of the REBIDA database (17).

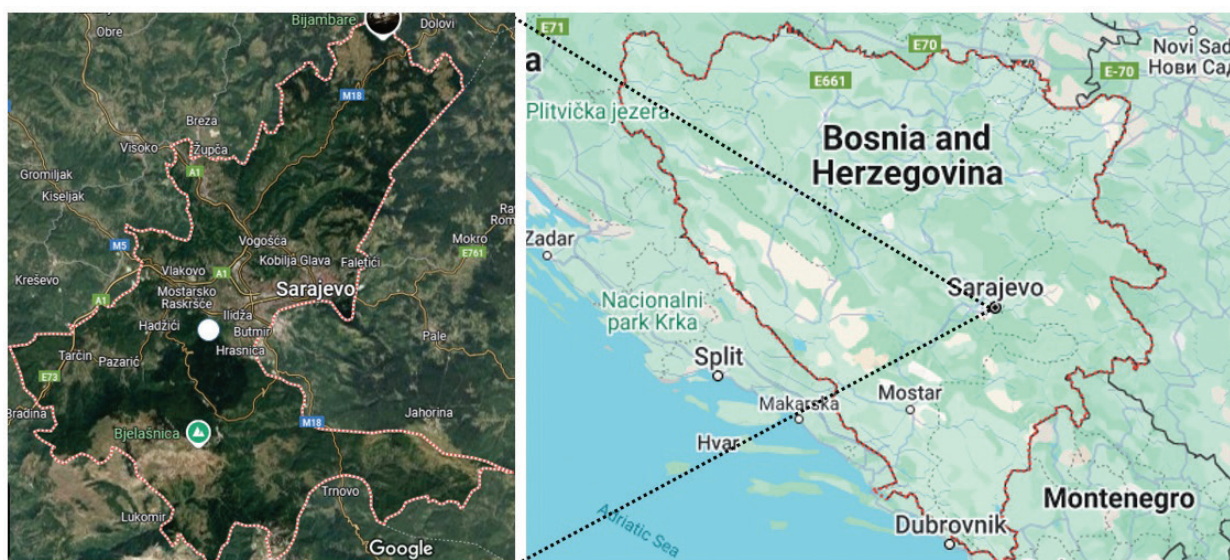


Figure 1. The position of the researched area of the Natural Monument of the Spring of Bosna River, represented by a white circle, in the Sarajevo Canton.

Genetic analysis

Total genomic DNA was extracted according to the modified salting-out protocol (18) using three extremities and thorax tissue. The quality of the extracted DNA was assessed by electrophoresis in 1.5% agarose gel stained with Midori Green (Nippon Genetics, Germany). The cytochrome c oxidase subunit I (*COI*) region was amplified using universal primers LCO1490 and HCO2198 (19). For specimens that could not be amplified with Folmer's primers, we used degenerate primers LCO1490-JJ and HCO2198-JJ (20). The PCR reaction was performed in a total volume of 25 µL, containing 0.5 µM of each primer, 5 µL of Red Load Taq Master (Jena Bioscience GmbH, Germany), and 2 µL of genomic DNA. Thermal cycling conditions comprised the initial denaturation at 95°C for five minutes followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 46°C for 30 seconds, elongation at 72°C for two minutes, and the final elongation at 72°C for five minutes. PCR products were assessed by electrophoresis in 2% agarose gel stained with Midori Green (Nippon Genetics, Germany). Product purification and bidirectional Sanger sequencing were performed in Eurofins Genomics (Ebersberg, Germany) using the same primers as for PCRs.

Bioinformatic analysis

The alignment and editing of bidirectional *COI* sequences were done in the BioEdit software (21). Identification of all sequences was done by the Basic Local Alignment Search Tool (BLAST) (22) on the National Center for Biotechnology Information (NCBI) platform and the *Identification* tool on the BOLD Systems platform (accessed on September 6, 2024). Generated sequences were analyzed further in the DnaSP v6 software and p-distances were calculated for each species (23).

RESULTS AND DISCUSSION

In total, we collected 52 specimens of the subfamily Agapetinae at The Natural Monument of the Spring of Bosna River, including 39 samples from the first and 13 from the second field research. The sample consisted of 50 larvae and two pupae specimens. Eighteen larval specimens were morphologically determined to species level, 12 as *S. krawanyi* and six as *S. iridipennis*. Those two species were found for the first time at The Natural Monument of the Spring of Bosna River, and this finding presents the new distribution data for Bosnia and Herzegovina. Earlier studies confirmed their presence on the other localities in Bosnia and Herzegovina (6, 12). To apply both approaches, morphological and molecular identification, these 18 specimens were selected for further genetic analysis, together with one unidentified pupa and four unidentified Agapetinae larvae. DNA barcodes were successfully generated for 16 specimens. Seven larval and one pupa samples were identified as *S. krawanyi*, four

larval specimens as *S. iridipennis* based on their barcodes that was in accordance with the morphological analysis. Four morphologically unidentified specimens remained unidentified through barcoding. This could be due to the lack of corresponding reference sequences in existing databases. This limitation highlights an ongoing challenge in molecular taxonomy—the incompleteness of databases such as BOLD (24), especially for lesser-known or underrepresented taxa like those in the Agapetinae subfamily. Expanding these databases is essential for more comprehensive species identification, especially in less research-intensive regions like the Balkans, where biodiversity remains underexplored (25). This consistency between genetic and morphological data underscores the reliability of integrating DNA barcoding with morphological approaches, especially in biodiversity studies (26).

In total, eight sequences were generated for species *S. krawanyi* (NCBI accession numbers: MT772019, OK314982-OK314988; BOLD IDs: DTKS003-21-DTKS006-21, DTKS009-21-DTKS012-21). We found three haplotypes, previously absent from the public databases. When sequences were run through the *Identification* tool on the BOLD Systems platform, a >99.67 match with a record of *Agapetus krawanyi* Ulmer, 1938 specimen from Croatia (BOLD ID: CROTR153-19) was retrieved. *Agapetus krawanyi* and *Synagapetus krawanyi* are synonymous species since the status of these genera is not resolved (7, 8). *A. krawanyi* is the taxonomic name used in the BOLD database. Problem of taxonomic ambiguities and usage of taxonomic synonyms in the BOLD database is a wider than this presented here. The application of the “Barcode, Audit and Grade System” has shown that species assessed as discordant are described as concordant after detailed review due to artifacts such as, among others, synonyms or misspelled names (27), that is, also, confirmed on the order Trichoptera (25). There are six public records with sequences for this species. Polymorphism analysis showed that those sequences together with sequences obtained in this study fall into eight distinct haplotypes (overall p-distance is 0.00705, minimum value of p-distance is 0.00, and maximum value of p-distance is 0.01815). A total of 18 polymorphic sites were registered among eight found haplotypes (Haplotype diversity (Hd) is 0.8242).

Four sequences were obtained for *S. iridipennis* (NCBI accession numbers: MT772030, OK314866-OK314868; BOLD IDs: DTKS001-21, DTKS002-21, DTKS007-21, DTKS008-21), with only one haplotype new for the public databases. This haplotype showed the highest similarity to the *S. iridipennis* entry from Austria (>98.68) (BOLD IDs: INTAP191-17). In the BOLD database, there are 12 records of this species but four with barcode sequences. Polymorphism analysis showed that those sequences, together with ones obtained in this study, fall into four distinct haplotypes with nine polymorphic sites (Hd is 0.7143, overall p-distance is 0.00809, minimum

Table 1. Records of *Agapetus*/*Synagapetus* species from Europe in the BOLD database (accessed on September 6, 2024) and records obtained in this study.

C	N	Species (number of records)
NO	201	<i>A. ochripes</i> (200)
DE	46	<i>A. fuscipes</i> (29), <i>A. ochripes</i> (10), <i>A. nimbulus</i> (3), <i>A. laniger</i> (2), <i>S. iridipennis</i> (1), <i>S. dubitans</i> McLachlan, 1879 (1)
AT	46	<i>A. ochripes</i> (30), <i>A. krawanyi</i> (4), <i>A. fuscipes</i> (3), <i>A. nimbulus</i> (3), <i>S. iridipennis</i> (2), <i>S. dubitans</i> (2), <i>A. delicatulus</i> McLachlan, 1884 (1), <i>A. moselyi</i> (1)
GB	73	<i>A. fuscipes</i> (40), <i>A. delicatulus</i> (17), <i>A. ochripes</i> (14), <i>S. dubitans</i> (2)
CH	19	<i>A. fuscipes</i> (4), <i>S. iridipennis</i> (4), <i>A. nimbulus</i> (3), <i>A. laniger</i> (2), <i>A. ochripes</i> (3), <i>S. dubitans</i> (3)
ES	18	<i>A. fuscipes</i> (4), <i>S. lusitanicus</i> Malicky, 1980 (4), <i>A. delicatulus</i> (3), <i>A. segovicus</i> Schmid, 1952 (3), <i>A. incertulus</i> McLachlan, 1884 (1), <i>A. nimbulus</i> (1), <i>A. ochripes</i> (1), <i>A. theischingeri</i> Malicky, 1980 (1)
HR	13	<i>Agapetus</i> sp. (6), <i>A. krawanyi</i> (2), <i>A. ochripes</i> (2), <i>A. laniger</i> (1), <i>A. moselyi</i> Ulmer, 1938 (1), <i>Synagapetus</i> sp. (1)
IT	12	<i>A. padanus</i> Bertuetti, Lodovici & Valle, 2004 (5), <i>A. nimbulus</i> (3), <i>A. cravensis</i> (2), <i>A. delicatulus</i> (1), <i>A. laniger</i> (1)
PT	10	<i>A. fuscipes</i> (2), <i>A. marlierorum</i> Botosaneanu, 1980 (2), <i>S. lusitanicus</i> (2), <i>S. diversus</i> McLachlan, 1884 (2), <i>A. incertulus</i> (1), <i>A. segovicus</i> (1)
FR	10	<i>A. delicatulus</i> (3), <i>A. insons</i> McLachlan, 1879 (2), <i>S. dubitans</i> (2), <i>A. quadratus</i> Mosely, 1930 (1), <i>A. cyrenensis</i> Mosely, 1930 (1), <i>A. placidus</i> Navas, 1918 (1)
BA	12	<i>Synagapetus krawanyi</i> (8), <i>S. iridipennis</i> (4)
GR	7	<i>A. slavorum</i> Botosaneanu, 1960 (5), <i>A. laniger</i> (1), <i>A. iridipennis</i> (1)
BE	3	<i>A. fuscipes</i> (3)
FI	4	<i>A. ochripes</i> (4)
TR	3	<i>A. caucasicus</i> Martynov, 1913 (3)
RS	2	<i>S. iridipennis</i> (2)
MK	1	<i>A. laniger</i> (1)
T	480	

Marks C, N, and T in the table stand for country, number of records, and total number, respectively. In column "C", the two-letter abbreviations of the states are given as follows NO – Norway, DE – Germany, AT – Austria, GB – United Kingdom, CH – Switzerland, ES – Spain, HR – Croatia, IT – Italy, PT – Portugal, FR – France, BA – Bosnia and Herzegovina, GR – Greece, BE – Belgium, FI – Finland, TR – Turkey, RS – Serbia, MK – North Macedonia.

value of p-distance is 0.00 and maximum value of p-distance is 0.01473). Olah (28) suggested the polymorphism could be the very beginning of various speciation processes both in sympatry or allopatry and may represent early stages of speciation combined or not with reproductive isolation.

In the BOLD database, there are 468 records of *Agapetus*/*Synagapetus* specimens identified to species level (accessed on September 6, 2024) from European countries, without records obtained in this study (12 records). These specimens were sampled in 16 countries and included 23 species, listed in Table 1. The most represented *Agapetus* species is *A. ochripes* followed by *A. fuscipes* Curtis, 1834, and *A. nimbulus* McLachlan, 1879. Other species have less than ten records which makes them underrepresented in this database. However, 46 species of *Agapetus*/*Synagapetus* (2) have been described in Europe, which implies that at least 54.35% of species of these genera have yet to be barcoded. It should also be noted that records from the countries in the European biodiversity hotspots have a

low number of records (Portugal, Spain, Italy, Switzerland, Croatia, Greece, Turkey, and North Macedonia).

Based on the results of our analyses and currently available data, DNA barcoding is a valid tool for identifying species within genera *Agapetus* and *Synagapetus*. However, many European species still need to be added to the BOLD database (25). More entries will increase the credibility of sequences. However, the correct morphological identification of the samples is still a very important step in creating an applicable reference library of DNA barcodes (25). Until this research, there were only 13 records in BOLD that represent species-level identified specimens from the Balkans (six from Croatia, seven from Greece, two from Serbia, and one from North Macedonia). Therefore, our results contribute to the data on caddisfly species distribution, genetic variability and species diversity in this region. Due to their high sensitivity to pollution, these two species, *S. krawanyi* and *S. iridipennis*, are valuable indicators of environmental quality, making them significant for biomonitoring or conservation efforts (13,

29). Protecting and maintaining such highly endangered aquatic ecosystems depends heavily on accurately identifying and monitoring the species associated with these ecosystems, and particularly various macroinvertebrate taxa. By adding new records from Bosnia and Herzegovina, an underrepresented country in the BOLD database, this study not only enhances the understanding of caddisfly biodiversity in the region but also supports the broader goals of the Global Trichoptera Barcode Initiative (30).

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