PERIODICUM BIOLOGORUM
VOL. 124, No 3–4, 107–114, 2022
DOI: 10.18054/pb.v124i3-4.24754

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original Research Article

Status of the BOLD reference library of DNA barcodes of caddisflies (Insecta: Trichoptera) from the Western Balkans

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Keywords: *COI* gene; BOLD; BAGS; diversity assessment; biomonitoring; aquatic insects

Abstract

Background and purpose: *Available data in research literature suggest that the Western Balkan countries hold a rich diversity of caddisflies. Assessment and biomonitoring of such rich diversity could be facilitated through DNA-based high-throughput approaches like DNA metabarcoding that depend on the availability of comprehensive reference libraries.*

Materials and methods: *We assessed the status of the* COI *barcode sequence data for a total of 112 caddisflies species in the investigated region by determining the gaps in representative sequences in the Barcode of Life Data System (BOLD) and examining the accuracy of available records using the Barcode, Audit and Grade System (BAGS).*

Results: *Results revealed a considerable underrepresentation of surveyed geographic region in BOLD records for the target insect group. Moreover, the large majority of the species records were rated "discordant" (72.80% grade E), and only 15.20% were classified as "consolidated concordance or basal concordance" (3.20% grade A and 12.00% B). Approximately 3.20% of the records pertaining to species occurring in multiple BINs (Barcode Index Number) and 8.80% were poorly represented (i.e., less than three specimens, grade D). A fraction of the species graded discordant were deemed concordant after detailed inspection of individual data, decreasing by 14.07%.*

Conclusions: *The assessment of the current state of BOLD entries indicated that DNA barcoding is still not widely applied in Albania, Bosnia and Herzegovina, Montenegro, North Macedonia, Serbia, and Slovenia, emphasizing that Croatia has the most barcoded caddisflies species. The finding that available BOLD Trichopteran records for investigated countries were mainly graded as "discordant" indicates the need for better quality control of reference libraries.*

Received December 23, 2022
Revised February 20, 2023
Accepted March 1, 2023

INTRODUCTION

Countries of the Dinaric Balkan region have high biodiversity, especially regarding freshwater fauna *(1,2,3,4)*. Many cryptic and endemic species have been reported in this region *(2,5,6,7)*.

Balkans is recognized as one of the biodiversity hotspots of Europe and endemism globally *(3)*. Due to the process of speciation and physical isolation, unique fauna can be found in its mountain streams, karst sources, and canyons *(8)*. A diverse hydro network has conditioned the

fauna of the Western Balkans to be especially rich in freshwater invertebrates. However, the research intensity has not been equal across all these organism groups. The most investigated group during the late 1900s was Trichoptera (caddisflies) *(9,10,11,12,13,14)*. Entomologists are still reporting new records on this order in the Western Balkan countries. New data on larvae morphology, ecology, and distribution patterns have been found for various species *(15,16,17,18,19,20,21,22,23,24)*. Additionally, new species are still being found *(25,26)* and described *(2,5)*, as well as new subspecies *(27)*.

Caddisflies are greatly important because of their utilization in freshwater biomonitoring. Prommi and Thani *(28)* showed that the number of caddisflies species correlates with the physicochemical parameters of the aquatic ecosystem. Uncovering caddisflies' biodiversity is an important prerequisite for their application in freshwater assessment and biomonitoring. In addition to the standard morphological approach, a trend of using DNA barcoding *(29)* as a method in aquatic biomonitoring has been increasingly growing since the late 2000s *(30)*.

DNA barcoding *(29)* became one of the widely used tools in studying species biodiversity in the last decade, aiding in solving taxonomic problems and providing a new perspective to biodiversity research *(31)*. Different databases were developed to support DNA barcode data generation, archiving, and application. The leading and most comprehensive database is the Barcode of Life Data System (BOLD), developed by the Centre for Biodiversity Genomics in Canada, containing four main modules: Data portal, Educational portal, Registry of Barcode Index Numbers (BINs), and Data collection and analysis workbench *(32)*. Similar to National Center for Biotechnology Information (NCBI) GenBank *(33)*, authors can directly submit data to the BOLD database. While this approach allows for uninterrupted growth of the database content, it also allows for errors or discordances due to insufficient supervision or quality control of the submitted reference dataset. Since the process largely relies on the expertise of the submitter, errors can occur at multiple points along the barcoding pipeline *(34)*. Using such corrupted records as references in DNA barcode-based studies (e.g., environmental DNA (eDNA) or metabarcoding) may lead to recurring misidentifications *(35,36,37,38)* and invalid conclusions. Although metadata standards have been recommended *(39)*, no comprehensive automated system for auditing and annotating the taxonomic consistency of the deposited DNA barcode records has been implemented. To help end-users of reference libraries to assess the taxonomic reliability of the barcode entries, Fontes et al. *(40)* developed the "Barcode, Audit and Grade System" (BAGS). It is an R-based application for automated auditing and annotation of DNA barcode reference libraries based on a previously proposed ranking system *(41,42)*. It allows researchers to sort out and annotate species confidently identified with current data from ambiguous or inaccurate records that need revision, or to flag cases of suspected hidden diversity *(40)*.

Therefore, we aimed to assess the representation of caddisflies' DNA barcodes from the Western Balkan countries in the BOLD database, assess their quality using BAGS, and inspect the potential discordances in grades assigned by BAGS.

MATERIALS AND METHODS

We mined all BOLD entries (BOLDSystems accessed on the October 28, 2022, at 16:00) for caddisfly species collected in Bosnia and Herzegovina and six other regional countries, including Croatia, Serbia, Montenegro, North Macedonia, Albania (Western Balkan countries) and Slovenia (regional, but Central European country). We used BOLD's Workbench for detailed sequence analysis that included building Neighbor-Joining (NJ) Taxon ID Tree for every country (Supplementary 2–8).

In BAGS, sequence entries from BOLD can be checked by applying a list of species provided by users or using large taxon-specific datasets composed of all available cytochrome oxidase subunit I (*COI*) barcode sequences in BOLD. With this, BAGS facilitates the audit and maintenance of reference libraries with barcodes, improving their quality.

Table 1. *Criteria for A to E grade assignment implemented in BAGS (40).*

Grade	Designation	Criteria
A	Consolidated concordance	The morphospecies is assigned a unique BIN, which is also assigned uniquely to that species, plus the species has more than 10 specimens present in the library
B	Basal concordance	The morphospecies is assigned a unique BIN, which is also assigned uniquely to that species, plus the species has 10 or less specimens present in the reference library
C	Multiple BINs	The morphospecies is assigned to more than one BIN, but each of those BINs is assigned exclusively to that species
D	Insufficient data	Species is not assigned discordantly, but it has less than 3 specimens available in the reference library
E	Discordant species assignment	Species assigned to a BIN that is assigned to more than one species.

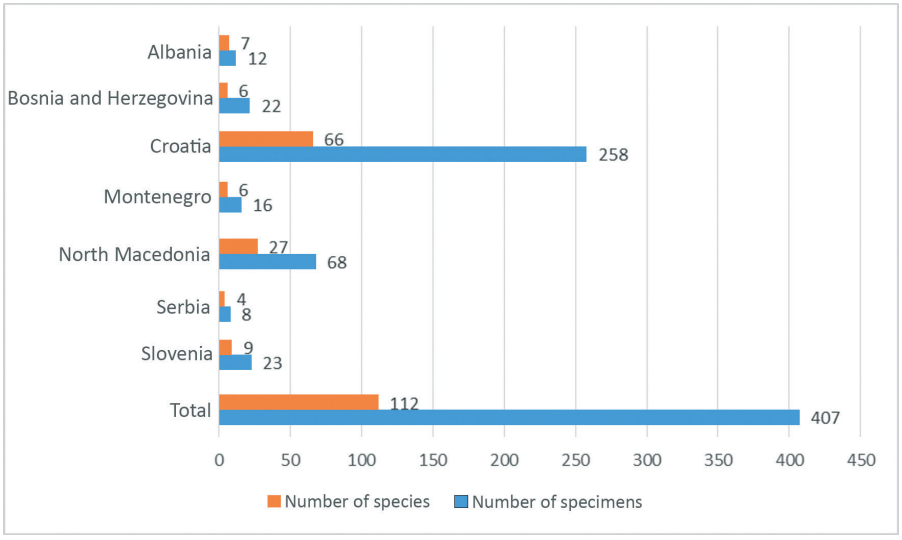

Figure 1. *Number of DNA barcoded species and specimens of caddisflies collected in the Western Balkans, and their distribution per country where specimens were collected.*

We tested retrieved BOLD's entries in BAGS by providing lists of 112 species characteristic for surveyed countries in the *.txt* format as an input file. Results were saved as a *.tsv* library checklist file. Furthermore, we filtered that file by location to avoid data for species found in countries that are not the focus of this study.

The BAGS annotation system assigns a grade (from A to E; Table 1) to each species entry based on the amount of sequence data for each species and the congruence between morphospecies and operational taxonomic units (OTUs) consisting of BINs *(43)*.

To fully assess the assigned grades, it was necessary to carefully observe the NJ Taxon ID tree for every country to find a potential mismatch between species and assigned BIN (BIN oversplitting or overlumping) and the possible existence of synonymous species names. A detailed review of the trees and data obtained from BAGS may indicate discordances in the tree and any grades incorrectly assigned. Also, there could be grades that, albeit considered correctly assigned from the viewpoint of BAGS workflow, in reality, may constitute analysis artifacts (e.g., pseudo-discordances in grade E, BIN oversplitting, or overlumping).

RESULTS AND DISCUSSION

Public data showed 64,781 barcode entries for Trichoptera in the Barcode of Life Data Systems submitted from 113 countries. Of these records, 53,572 have species names and represent 5,229 species. Library checklist retrieved from BAGS showed a total of 407 specimens of caddisflies for 112 species in the analyzed region (Figure 1). Moreover, it showed minimal activity in DNA barcoding of caddisflies in Bosnia and Herzegovina and nearby countries (1-2% of described caddisflies species were barcoded in Serbia, B&H, Albania, Slovenia, Montenegro, and 9% in North Macedonia), except the Croatia where 22% of the described species were barcoded (Figure 1). This Croatian scientific contribution to uncovering caddisflies' biodiversity should encourage other Western Balkan countries to intensify DNA barcoding efforts to build a complete picture of trichopteran biodiversity. The number of DNA barcoded species per country follows the trend of specimen number per country where they were collected (Figure 1). The low proportion of sequenced species indicates that DNA barcoding is still not widely applied in Albania, Bosnia and Herzegovina, Montenegro, North Macedonia, Serbia, and Slovenia.

Grades assigned to species in each of the analyzed countries are displayed in Figure 2. The most frequent grade was consistently grade E (72.8%). Generally, grades A and C made only 3.2%, while grades B and D made 12% and 8.8%, respectively, of all analyzed species. The grade of each species individually is shown in Supplementary 1.

The detailed inspection of grouping sequences in the NJ trees (Supplementary 2–8) and data obtained from BAGS, including grades, their descriptions, and BINs, did not confirm true discordances in E-graded species from Bosnia and Herzegovina (Supplementary 2), Montenegro (Supplementary 4), and Serbia (Supplementary 6). Fontes et al. *(40)* noted that often species graded discordant emerge as concordant after detailed individual inspection of the data due to artifacts such as synonyms and misspelling or obvious misidentifications, contamination, or mislabeling of the records. Similar findings have been reported by Leite et al. *(44)* and Radulovici et al. *(34)* through the analyses of discordant records in a large num-

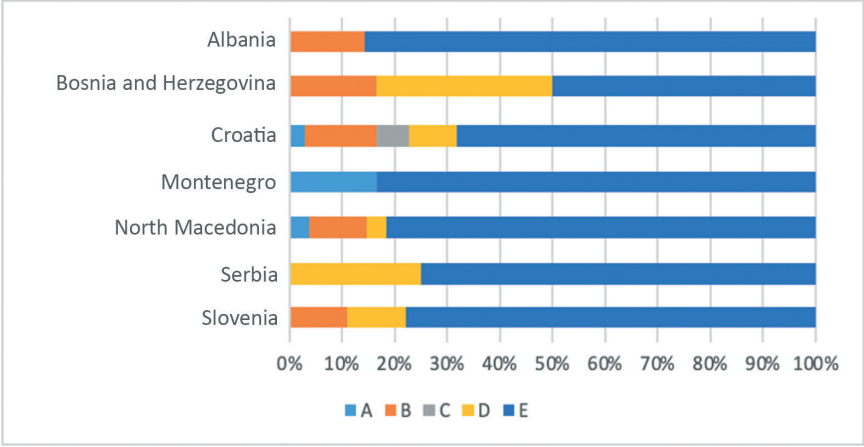

Figure 2. *Attributed BAGS' grades to DNA barcode data of caddisflies species of Western Balkans*

Table 2. *Species/barcode discrepancies in the four countries they were found in.*

Country	Species	Discrepancies
Albania	*Drusus arbanios*	Share the same BIN
	Drusus dacothracus	
	Drusus illyricus	
	Drusus pelasgus	
	Drusus juliae	Share the same BIN
	Drusus kerek	
Croatia	*Chaetopteryx rugulosa*	Assigned to two different BINs
	Micropterna sequax	Assigned to two different BINs
	Sericostoma flavicorne	Assigned to two different BINs
North Macedonia	*Drusus cf. plicatus*	Share the same BIN
	Drusus discophorus	
	Hydropsyche incognita	Share the same BIN
	Hydropsyche instabilis	
	Philopotamus montanus	Assigned to two different BINs
	Potamophylax latipennis	Assigned to two different BINs
Slovenia	*Chaetopteryx clara*	Share the same BIN
	Chaetopteryx goricensis	

ber of marine invertebrate species. We noticed some discordance for the other four analyzed countries (Table 2).

For Slovenia (Supplementary 7), disagreements encompass species *Chaetopteryx clara* and *C. goricensis* (non-synonymous names) sharing the same BIN, which could be a reason for grade E. Both *C. clara* and *C. goricensis* belong to *C. rugulosa* species group *(45,46,47)*. Oláh et al. *(46)* described a new species subgroup called *C. irenae* that includes both of these species based on their morphological characteristics. In addition, Malicky *(47)* reported evidence of hybridization among species of the genus *Chaetopteryx*, including *C. goricensis* and *C. clara*. In general, caution should be applied when analyzing DNA barcode data from *C. rugulosa* and *C. villosa* group, which consists of *C. villosa*, *C. gessneri*, *C. fusca*, *C. sahlbergi*, C. *atlantica*, *C. bosniaca*, *C. vulture*, and *C. trinacriae (48)*, and conclusions must be heavily supported with morphological analysis. For North Macedonia (Supplementary 5), we noticed that species *Hydropsyche incognita* and *H. instabilis* share the same BIN, which possibly caused the grade E. *H. incognita* was probably assigned to different BINs because of potential morphological misidentification. Both specimens of *H. incognita* under BOLD:AAB1966 clustered with *H. instabilis*, while *H. incognita* under BOLD:AAD1254 are clustered separately. A similar situation was noticed with species *Drusus cf. plicatus, D. discophorus and D. pelasgus*. On the contrary, *Philopotamus montanus* was assigned to different BINs, as well as *Potamophylax latipennis*, and both were graded with E. From a Croatian tree (Supplementary 3), we noticed that *C. rugulosa rugulosa* and *C. rugulosa mecsekensis* were assigned to different BINs and clustered separately. Still, BAGS recognized them as a single species as they indeed are and graded them with C. Moreover, the subspecies *C. rugulosa rugulosa* has different BINs, as well as *Micropterna sequax* and *Sericostoma flavicorne*. The reason could be geographically structured high intraspecific variability or the presence of potentially new taxa within *M. sequax*, as has been published in Kučinić et al. *(49)*. In the NJ tree for the Albania (Supplementary 8), *Drusus juliae* and *D. kerek* have the same BIN. The same BIN was also assigned to species *D. dacothracus*, *D. illyricus*, *D. pelasgus*, *D. arbanios*. In none of the above examples are synonymous names involved. After inspection of individual data, a fraction of the species graded discordant were

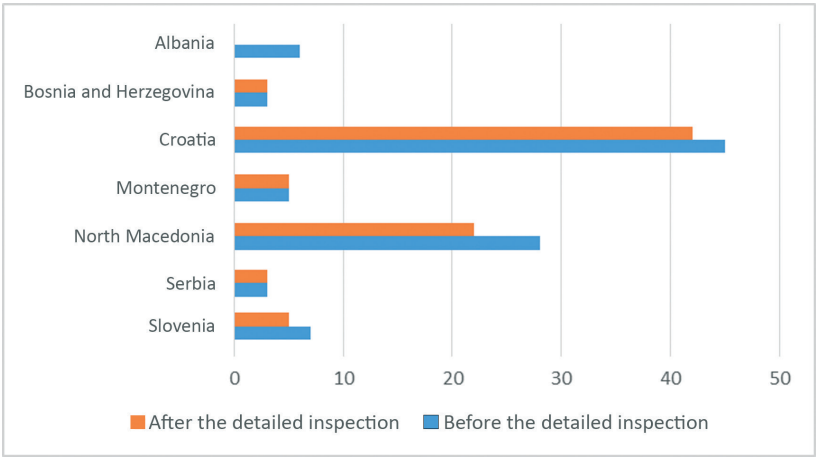

Figure 3. *Decrease of the discordant species after detailed inspection according to countries.*

deemed concordant, decreasing by 14.07%, the final percentage of "discordant" species (Figure 3).

The discrepancies (Table 2) highlighted above may result from misidentification. To create reliable records in the BOLD database and ultimately gain a comprehensive and applicable database for countries of the Western Balkans, it is necessary to cooperate with narrowly specialized taxonomists. Sequential data should be accompanied by morphological, distributional, and ecological species data. The integrative taxonomic approach should be chosen since misidentification may result from identification based only on genetic sequence or morphology. This is especially a problem in groups with different life stages, such as Trichoptera. The stages of larva and pupa remain morphologically undescribed for many species. Therefore, assigning the correct species name to the sequential data gained solely from such specimens is impossible. This is one of the main problems regarding the DNA barcoding research of caddisflies in the Western Balkans. There are potential misidentified samples in the BOLD database. Kučinić et al. *(49)* pointed out the example of *Glossosoma discophorum* and *Glossosoma neretvae* as the potential consequences of misidentification in the BOLD database.

Observations of BOLD's and BAGS' results led us to the cognition that Croatia, compared to other regional countries, is the most active in DNA barcoding of caddisflies, and it has continuity in this research field. Other Western Balkan countries are at the beginning point of establishing DNA barcoding. The reasons for insufficient activity in DNA barcoding could vary and occur at different points of the DNA barcoding workflow. The major obstacle is still the correct morphological identification of the samples, which is a critical step in creating an applicable reference library of DNA barcodes. The reason is that most of the caddisflies specimens collected during field studies are in the larval stage, and many have not yet been described *(50)*. The larvae of some species are phenotypically very similar in this developmental stage when observed with the naked eye, which further complicates correct identification. This is especially a problem for early career researchers, so inclusive collaboration of established morphologists and early career researchers should be the priority and would aid in solving this obstacle. Another major caveat to large-scale barcoding campaigns in the investigated countries regards the infrastructural demands. Some countries lack adequately equipped laboratories and trained staff to perform DNA barcoding, and lack of funding is the most common hindrance. Wherefore many regional research groups still see morphology as the more realistic identification option for individual specimens *(51)*.

More entries in the BOLD will increase the credibility of sequences and correlated grades. BAGS will still attribute a grade E to morphospecies that displays even a single record (e.g., a single misidentification) in discordant BIN, so the morphospecies-centered approach is more advantageous. However, it is advantageous to apply BAGS to sort out taxonomic incongruences, point out possible cases of human error during the generation of the barcodes, and uncover potential cases of hidden diversity among species *(41)*. Joint studies of caddisflies in surveyed countries would facilitate retrieval of relevant BOLD data for this group, thus enabling a complete insight into their diversity in the Western Balkans. It is a prerequisite for applying novel methods in biomonitoring and bioassessment, such as DNA metabarcoding or environmental DNA. This study's findings reinforce that networking among countries is essential for bioassessment since countries with budget limitations would benefit from being part of large research groups and networks to overcome insufficient national funding, limitations to access international funding, and the most up-to-date biotechnological infrastructures and training programs *(37)*.

Supplementary material is available on-line at https://hrcak.srce.hr/pb

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