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The first DNA barcode record for *Rhyacophila bosnica* Schmid, 1970 and pairing of adult and larval life stages

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Abstract

Rhyacophila Pictet, 1834 is globally distributed and highly diverse genus of caddisflies (Trichoptera), characterized by numerous regionally endemic species. In the Balkan Peninsula, the highest number of *Rhyacophila* species (23) was recorded for Bosnia and Herzegovina. *Rhyacophila bosnica* Schmid, 1970 is found only in the Balkan Dinaric region, with a *locus typicus* in Vučja Luka, Bosnia and Herzegovina. Like with many species of Trichoptera, the morphology of its larva is still unknown. Therefore, DNA barcoding approach was used to link two developmental stages. In this paper, we report on the first DNA barcode record for this species.

Keywords

Trichoptera, DNA barcode, Rhyacophila bosnica, larva

Introduction

Data regarding the fauna of caddisflies (Trichoptera) in Bosnia and Herzegovina indicate both great diversity and high level of endemism. The first published data were provided by Klapalek (1898, 1900, 1902). Later, other authors (Radovanović, 1935; Botosaneanu, 1960; Marinković-Gospodnetić, 1966, 1973, 1975; Kumanski, 1968, 1971; Obr, 1969; Malicky, 1974) contributed to the knowledge of species diversity and distribution. Recent publications on taxonomy and ecology of Trichoptera corroborated the rich biodiversity of the group in the western Balkan Peninsula (Kučinić & Malicky, 2001; Previšić et al., 2007; Živić et al., 2009; Graf et al., 2008; Kučinić et al., 2008, 2010; Ćuk & Vučković, 2009, 2010; Malicky, 2009; Waringer et al., 2009).

The genus *Rhyacophila* Pictet, 1834 includes more than 700 recognized species (Holzenthal et al., 2007) inhabiting the west and east Palearctic, Nearctic, Oriental and Australasian regions (de Moor &

Ivanov, 2007). When countries of the Balkan Peninsula are considered, the highest number of *Rhyacophila* species (23) was recorded for Bosnia and Herzegovina (Koštroman, 2009) compared to e.g. Slovenia – 19 species (Krušnik & Urbanič, 2002), Serbia – 18 species (Živić et al., 2006), Bulgaria – 18 species (Oláh, 2010) and Kosovo – 15 species (Ibrahimi et al., 2012). However, these species counts constantly grow as new findings of known species are being reported for national checklists (Bilalli et al., 2018; Ibrahimi et al., 2017) or new species described, such as *R. neretva* Oláh, sp. n., found in the Neretva River, upstream of Mostar, Bosnia and Herzegovina (Oláh & Beshkov, 2016).

Trichoptera are important both as a component of national biodiversity as well as indicators of the aquatic ecosystem health. However, many caddisfly species from the Balkan Peninsula and Bosnia and Herzegovina are still under-investigated, with unknown morphology of different developmental stages. One of the species with limited geographic distribution on the Balkan Peninsula and undescribed larva is *Rhyacophila bosnica* Schmid, 1970. *Locus typicus* of this species is Vučja Luka, Bosnia and Herzegovina (Oláh, 2017). The *Rhyacophila* species in Bosnia and Herzegovina dominantly inhabit mountain streams. *R. bosnica* is classified within *Rhyacophila tristis* group; in Bosnia and Herzegovina the group is represented by: *R. tristis* Pictet, 1834, *R. vranitzensis* Marinkovic and Botosaneanu, 1967 and *R. trescavicensis* Botosaneanu, 1960, while species *R. balcanica* Radovanović, 1953 and *R. loxias Schmid, 1970* belong to vulgaris group.

DNA barcoding, a technique that utilizes standardized 658 bp fragment of the cytochrome c oxidase subunit 1 (*COI*) gene proposed by Hebert et al. (2003a), has been growingly used as an auxiliary tool for species identification in cases when morphological approach is insufficiently discriminative. Additionally, the technique has been successfully used to associate different life stages (e.g. adult and larval) in many animals (Ekrem et al., 2006; Webb et al., 2006; Ahrens et al., 2007; Richard et al., 2010; Tang et al., 2010; Ruiter et al., 2013). This study aimed to provide the first DNA barcode sequence for endemic species *Rhyacophila bosnica* and to utilize it to link adult and larval stage, setting up the ground for the morphological description of larvae.

Materials and methods

Adults and larvae of *R. bosnica* were collected from the Rajčevački stream in Vareš municipality, Bosnia and Herzegovina, using an entomological net. The adults were collected in March 2018, while larvae were sampled during October-November 2017 and February 2018 (Figure 1). All samples were stored in 96% ethanol and archived in the tissue database of REBIDA (Kalamujić Stroil et al., 2017). The adults were identified under a stereomicroscope following the identification keys by Malicky (2004).

Figure 1. Collection site at the Rajčevački stream, the fall 2017

Total genomic DNA was extracted from the whole specimens, using ExtractMe DNA Tissue kit (Biolab Innovative Research Technologies), in the sterile environment and following the manufacturer's instructions. The quality of the extracted DNA was assessed by electrophoresis in 1.5% (w/v) agarose gel in 1x SB (sodium borate) buffer, pH8 (Brody & Kern, 2005). Genomic DNA was visualized under UV light after staining with Midori Green (Nippon Genetics Europe).

Initial attempts to amplify 658 bp *COI* barcode region using, first, LCO1490 and HCO2198 primers (Folmer et al., 1994; Hebert et al., 2003a,b) and then primer cocktails CLepFolF and CLepFolR (Hernandez-Triana et al., 2014) failed. Finally, successful amplification was obtained using degenerated primers LCO1490-JJ and HCO2198-JJ (Astrin & Stüben, 2008). Thermal and chemical

parameters of PCR were as reported in Astrin et al. (2016). The product amplification, purification and bidirectional sequencing was performed by Advanced Identification Methods – AIM GmbH (Munich, Germany). The same PCR primers were also used for sequencing reactions.

Resulting nucleotide sequences were optimized in Jalview 2.9.0b2 software (Waterhouse et al., 2009) and translated using the Translate tool on the ExPASy server (Gasteiger et al., 2003) in order to check for open reading frames. Pairwise alignment of adult and larval sequences was done in ClustalX 2.0 (Larkin et al., 2007). Consensus sequence was archived in GenBank under the accession number MK211322 (BankIt2169206). Sequence was first compared with those available in the BOLD database using BOLD Identification Engine (accessed November 2018). Subsequently, sequence was identified based on identity and similarity indices using FASTA program (Pearson, 1994) and BLAST tool (Benson et al., 2008) on NCBI platform. From the retrieved BLAST results, the sequences that were identified to the species level, covered the majority of the barcode region and had an E-value equaling zero were selected and aligned along the consensus sequence from this study. Multiple sequence alignment (MSA) was performed using ClustalX 2.0 (Larkin et al., 2007) and edited in Bioedit v5.09 (Hall, 1999).

Dataset for phylogenetic analysis comprised 56 *Rhyacophila* species, *R. bosnica* from this study and *Adicella balcanica* (KX555470) as an outgroup. The Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees were built based on the pairwise distance matrix. The uncorrected p-distances were calculated according to the Jukes-Cantor mutation model which assumes equal rate of nucleotide substitutions. All computations were done in MEGA 7.0. software (Kumar et al., 2016).

Results and Discussion

In this research study we identified larvae of *R. bosnica* by comparing DNA barcoding sequence of *COI* gene isolated from adult males and collected larvae. The analysis of *COI* region in adult *R. bosnica* specimens resulted in a sequence of the entire barcode fragment of 658 bp. Since full barcode amplification using larval genomic DNA was hindered, mini barcodes had to be employed. Resulting 341 bp sequence corresponds to the second half of *COI* barcode. Analysis with BOLD Identification tool retrieved no match from the existing entries in the BOLD database. Both samples showed genetic distance of over 10% which indicates that there are no records of the investigated species in BOLD yet. BLAST analysis in GenBank produced the same result.

Pairwise alignment of *COI* sequences obtained from adult and larval forms in ClustalW 2.1 software showed that the first nucleotide of the larva sequence corresponds to the 344 position of the adult's sequence. The two sequences were perfectly aligned across 314 common sites indicating that analyzed sequences originate from the same species. This hypothesis was further tested through MSA analysis of consensus sequence from this study and those of *Rhyacophila* species in GenBank database. After editing, the first 649 base pairs of the barcode region were aligned over all 57 sequences. MSA revealed that more than 45% of sites within 314 bp long sequence obtained from the larval specimen are polymorphic among different *Rhyacophila* species. This finding corroborated that sequences obtained from the specimens in this study indeed belong to the same species, enabling us to link two different life stages of *R. bosnica* using DNA barcoding approach. The positive pairing sets a reliable basis for subsequent morphological description of *R. bosnica* larva (manuscript in preparation).

As expected, Neighbor-Joining analysis based on Jukes-Cantor model (Figure 2), as well as ML analysis based on Timura-Nei model (not showed), placed *R. bosnica* with other species belonging to *tristis*-group: *R. obtusa*, *R. tristis* and *R. orghidani*. This group was established by Schmid (1970), within the "invaria" branch, on the basis of morphological similarities of the adult genital appendices. It includes twelve species that inhabit Europe and are characterized by a reduced aedeagus compared to the other species in the branch (Engelhardt, 2009). This diverse group includes many endemic species of different European regions, making it ideal model for the surveys on diversification of freshwater aquatic invertebrates (Malicky, 2004; Bálint, 2008).

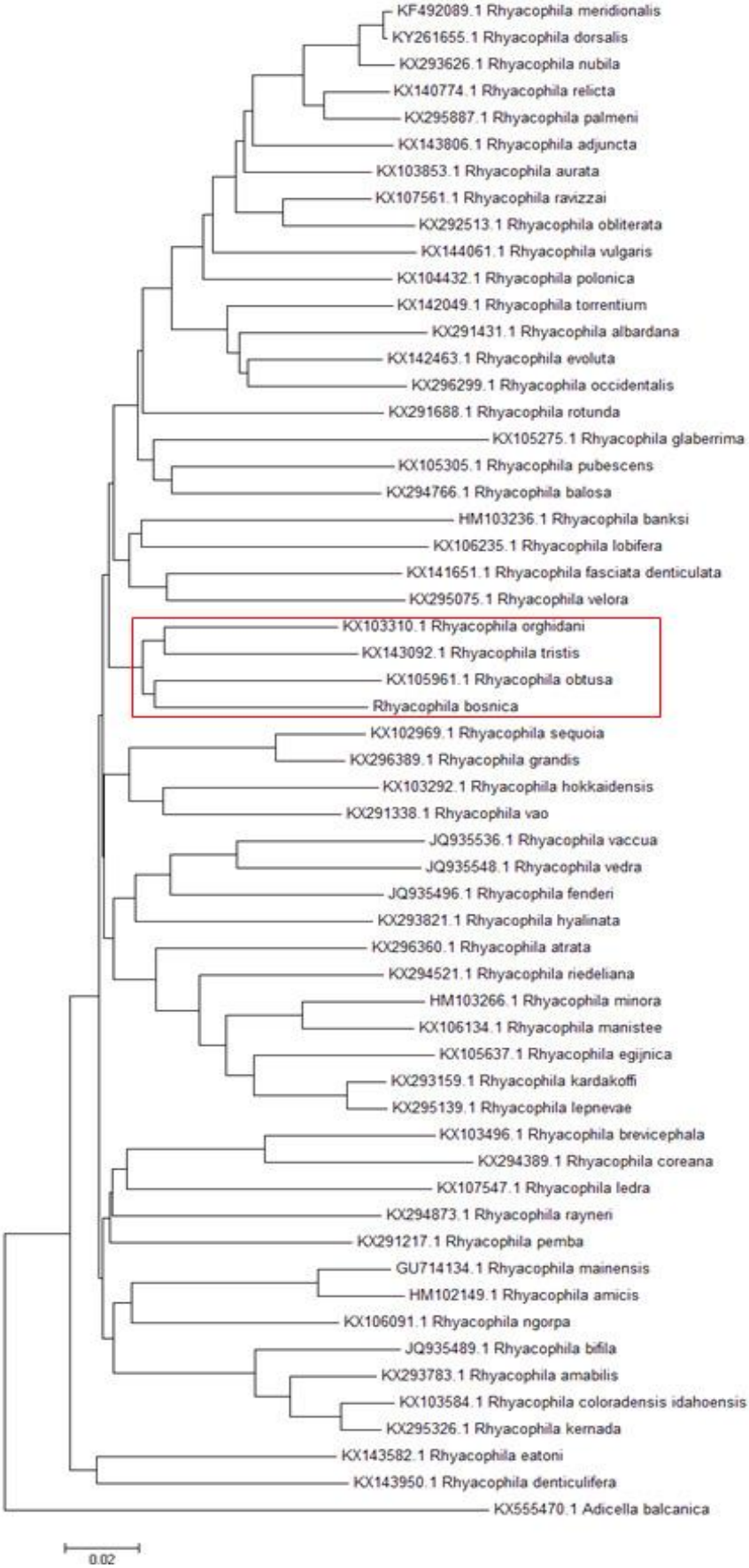

Figure 2. Neighbor-Joining (NJ) tree based on the p-distance matrix. *R.bosnica* cluster is indicated with red box

However, there are still species within this group that lack morphological description of larva as well as any molecular data, such as *R. trescavicensis* and *R. vranitzensis*, another stenoendemites of Bosnia and Herzegovina.

Based on NJ and ML models, overall p-distance values were 15% and 15.5%, respectively, which falls within reported values for interspecific variability of a barcode region in caddisflies (Ćukušić et al., 2017; Kučinić et al., 2017; Graf et al., 2015; Pauls et al., 2010). The closest to *R. bosnica* was *R. orghidani* (10.9%), endemic species of the Balkan Peninsula, followed by *R. obtusa* and *R. tristis* (12.3%). Interspecific pairwise distances ranged from 1.6% (*R. nubila*/*R. meridionalis*) to 21% (*R. glaberrima*/*R. brevicephala*). These results indicate a marked genetic differentiation among *Rhyacophila* species, although the actual presence of a barcoding gap (Čandek & Kuntner, 2015) should be inspected on a larger dataset as proposed by Astrin et al. (2016).

Due to high sensitivity to organic pollution and considerable species diversity with free-living, shelter- and case-constructing larvae, Trichoptera are widely used in bioassessment of aquatic ecosystems (Dohet, 2002; de Moor & Ivanov, 2007). Hence, the possibility of unambiguous species identification is of high importance for any survey of the community structure and habitat quality. However, since some diagnostic traits necessary for reliable identification are absent in one sex or at a certain life stage, traditional morphological species delineation within this group relies on the availability of adult males. In cases when phenotypic description of a developmental stage is unavailable or the specimen is damaged, conventional morphological identification is virtually impossible. DNA barcoding has proven to be a very helpful auxiliary tool when species discrimination based on morphology is hindered (for a general overview see Pradhan et al., 2015). In order to fully exploit the potential of this approach, establishing a comprehensive (national or international) reference DNA barcode library, based upon well-curated voucher specimens, is crucial. Although the Regional Database on Biodiversity (REBIDA) database (Kalamujić Stroil et al., 2017) has been set up, aiming to collect all known geobiological data on wild and domesticated natural resources of Bosnia and Herzegovina, it is far from completed. When the Barcode of Life Database (BOLD—www.boldsystems.org; Ratnasingham & Hebert, 2007) is considered, there are only 196 DNA barcode records with species level information out of 700 known species from the genus *Rhyacophila* (access on November 22 2018). The barcode data for third of the *Rhyacophila* species recorded in the Federation of Bosnia and Herzegovina are still missing from the BOLD database (access on November 22 2018).

Barcoding a complete fauna of one country, especially a biodiversity hotspot such as Bosnia and Herzegovina, is a daunting task both in terms of funding and labor. A rational approach would include the creation of check lists for indicator species which can be used in various assessments (such as those required by the Water Framework Directive), and gap analysis against existing international barcode databases. Efforts should then be directed on providing adequate vouchers and molecular data for the species that are missing barcode data. Once the approach is established, it can be extrapolated to other taxa and groups. Such methodology calls for close interaction among taxonomists, field biologists and molecular geneticists. Further efforts should be put into persuading the stakeholders to strategically fund field sampling, DNA analysis and comparative morphological evaluations. Ideally, marked developmental stages should be barcoded to build the existing reference libraries. Only then these data could be utilized in the assessment and monitoring of the environment and enable the application of novel genomic tools such as metabarcoding and environmental DNA (eDNA).

Conclusions

Rhyacophila bosnica Schmid, 1970 is an endemic species of the Balkans whose taxonomic identification has traditionally been based on the morphological traits of male adults. Like with many species of Trichoptera, morphology of larva is unknown, making it impossible to detect species if the only available specimens are of this life stage. In this paper we present the first DNA barcode record

for this species. Complete match of *COI* barcode sequences retrieved from an adult and larva enabled pairing of these two developmental life stages, setting a ground for morphological description of larval form.

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