SPRINGS: DNA BARCODING OF CADDISFLIES (INSECTA, TRICHOPTERA) IN CROATIA WITH NOTES ON TAXONOMY AND CONSERVATION BIOLOGY

Mladen Kučinić1*, Andela Ćukušić2, Sanja Žalac3, Antun Delić4, Darko Cerjanec5, Martina Podnar6, Renata Ćuk7, Ivan Vučković8, Ana Previšić1, Marijana Vuković6, Svetlana Stanić Koštroman9, Višnja Bukvić10, Ana Šalinović11 & Mladen Plantak8

1Department of Biology (*Laboratory for Entomology), Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia
2Ministry of Environment and Energy, Radnička cesta 80/7, 10000 Zagreb, Croatia
3ZSC „Dr. Ivo Pevalek”, Plitvice Lakes National Park, Josipa Jovića 19, 53231 Plitvička jezera, Croatia
4Nikole Šubića Zrinskog 3, 43290 Grubišno Polje, Croatia
5Barilović Primary school, Barilović 96, 47252 Barilović, Croatia
6Croatian Natural History Museum, Demetrova 1, 10000 Zagreb, Croatia
7Hrvatske vode, Central Water Management Laboratory, Ulica grada Vukovara 220, 10000 Zagreb, Croatia
8Elektroprojekt d.d., Civil and Architectural Engineering Department, Water Resources, Nature and Environmental Protection, Alexandera von Humboldt 4, 10000 Zagreb, Croatia
9Department of Biology, Faculty of Science and Education, University of Mostar, Matice hrvatske, 88000 Mostar, Bosnia and Herzegovina
10University of Hercegovina, Blajburških žrtava 100, 88000 Mostar, Bosnia and Herzegovina
11Postelska ulica 10, 2000 Maribor, Slovenia

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The paper provides the results of DNA barcoding based on the cytochrome c oxidase subunit 1 mitochondrial gene (mtCOI) of 110 Trichoptera specimens collected in 36 springs in the Pannonian-Peripannonian, central mountainous and Mediterranean part of Croatia. We barcoded 70 species from 32 genera and 15 families. The data obtained show interesting faunistic and taxonomic results, for, for example, the species Rhyacophila cabrankensis, R. balcanica, Crunoezia kempnyi, Allogmaus auricollis and emphasize the need for further faunistic research into springs, in their role as habitats with a specific and very interesting fauna. The mtCOI DNA barcoding should be included in such research, because it would enable better presentation of the results, especially regarding biodiversity, taxonomy, phylogeny and conservation biology, not just as a segment of a local but also of a global process of understanding biodiversity in a different way. The results of this study show a global need for the protection of springs, because they are specific not only as habitats, but also as localities with an interesting fauna and often endemic species of very limited distribution (for example Rhyacophila cabrankensis).

Key words: upper stream reaches, caddisflies, biodiversity, molecular methods, Rhyacophila cabrankensis
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U radu se prikazuju rezultati DNA barkodiranja temeljenog na mitohondrijskom genu za podjedinicu 1 citokrom c oksidaze (mtCOI), za 110 primjeraka Trichoptera prikupljenih u 36 izvora u panonsko-peripanonskom, središnje-planinskom i mediteranskom području Hrvatske. DNA barkodiranje je 70 vrsta iz 32 roda i 15 porodica. U studiji se ukazuje na neke zanimljive faunističke i taksonomsko-biološke rezultate, npr. za vrste Rhyacophila cabrankensis, R. balcanica, Crunoecia kempnyi, Allogmaus auricolli te potrebu daljnjih faunističkih istraživanja izvora kao staništa sa specifičnom i vrlo zanimljivom faunom. U ta istraživanja zbog kvalitetnije prezentacije rezultata, posebno u područjima bioraznolikosti, taksonomiji, filogeniji i konzervacijskoj biologiji, potrebno je uključiti i metodu DNA barkodiranja mtCOI, kao segment ne samo lokalnog, nego i globalnog procesa u spoznavanju bioraznolikosti na jedan drugačiji način. Navedeni rezultati ovog rada ukazuju na globalnu potrebu veće zaštite izvora jer su specifični ne samo kao staništa, nego vrlo često i kao područja nalaza endemskih vrsta s vrlo malim područjem rasprostranjenja (npr. Rhyacophila cabrankensis).

Ključne riječi: gornji dijelovi tekućica, tulari, biološka raznolikost, molekularne metode, Rhyacophila cabrankensis

INTRODUCTION

Springs comprise a particularly interesting type of aquatic habitats characterized by specific hydrological, geological and geomorphological features. They are considered biodiversity hotspots, and also among the most endangered freshwater habitats (Kučinić et al., 2015a, 2015b; Pešić et al., 2019; Vitecek et al., 2015, 2017). Along with biological characteristics of various animal groups, certain spring features are dominant in affecting the composition and structure of their fauna. Type of benthic substrate, spring morphology, water temperature and location of springs (for example springs in forests, springs in open areas) are very important for composition of fauna (Govoni et al. 2018; Ilmonen & Paasivirta 2005; Ivković et al., 2013; Kreiling et al., 2020; Matić et al., 2016; Myers & Resh, 2002). Springs are, in hydrological terms, ‘places where subterranean water emerges to the surface’ (Habdija & Primc, 2019) (Figs 1-4). There are many classifications of springs, and one of them is based on their geomorphological and hydrological characteristics, which have a major effect on spring hydrology, and divides them into limnocrene and rheocrene springs (Habdija & Primc, 2019; Steinmann, 1907). Limnocrene springs are shaped like lakes of various depths and sizes (Figs 1-2, 4). In contrast, rheocrene springs (Fig. 3) emerge as water flowing to the surface mostly on rocks, thereby creating a waterfall as the initial part of the stream (Habdija & Primc, 2019).

The faunistic uniqueness of springs is also a consequence of their spatial isolation, which can be bigger or smaller, leading to disjunct distributions of populations, which can in time cause allopatric speciation and produce new taxa (subspecies, species) (for example Erman & Erman, 1995; Marinković Gospodnetić, 1971, 1976, Malicky, 2020, Previšić et al., 2014; Vitecek et al., 2017), by geographic isolation (Nei, 1975). Those characteristics favour many endemic, rare and interesting species belonging to various animal groups, e.g. water mites (for example Pešić et al., 2019; Pozojević et al., 2020; Di Sabatino et al., 2003), crustaceans (for example Glazer, 1998; Sidorov et al., 2012, 2018), aquatic insects (for example Grae et al., 2012; Ivković et al., 2020; Majolini et al., 2011; Pollet & Ivković, 2018; Waringer et al., 2009) and others. There is a great level of endemism in Trichoptera as well, and there are genera and species which can be found only in springs or in upper stream reaches (Cianficconi et al., 1998; Hinić et al. 2020; Kučinić
et al. 2015a; MALICKY, 2020; MARINKOVIĆ-GOSPODNETIĆ 1971, 1976, 1979; OLÁH, 2010; PREVIŠIĆ et al. 2014a, 2014b; VITECEK et al. 2015, 2020; WARINGER et al., 2009, 2013, 2015, 2016).

The study of the Earth’s biodiversity attained scientific dimensions with the establishment of binomial nomenclature, the taxonomic and basic evolutionary model for the depiction of this diversity (Linnaeus, 1758). Since that period, a large number of organisms have been described, with more than a million known species, which is considered as just a part of total existing biodiversity. Each year thousands of new species within various groups of organisms are described, and the introduction of DNA barcoding based on the cytochrome c oxidase subunit 1 mitochondrial gene (mtCOI), along with the establishment of the Barcode of Life Data Systems (BOLD) (HEBERT et al., 2003a, 2003b; RATNASINGHAM & HEBERT, 2007) resulted in new aspects of global biodiversity on Earth. DNA barcoding has proved to be a useful method in studies of the taxonomy, phylogenesis, phylogeography and biodiversity of different groups of organisms (for example AMORA et al. 2015; BREHM et al., 2019; CÁRDENAS et al., 2013; DE BARROS MACHADO et al., 2017; DELA CRUZ et al., 2016; ELÍAS-GUTIÉRREZ et al., 2008; GUO et al., 2016; HUEMER et al., 2020; KUČINIĆ et al., 2019a, 2019b; LÉGER et al., 2020; PAULS et al., 2009; SANTOS et al. 2016; TYAGI et al., 2017; VAGLIA et al. 2008; VIJAYAN & TSOU, 2010; YANG et al., 2015).

Regarding Trichoptera, DNA barcoding has been used in numerous studies in different regions (for example GERACI et al. 2011; HJALMARSSON et al., 2018; MORINIÈRE et al., 2017; PAULS et al. 2010; VALLADOLID et al., 2018, 2019; ZHOU et al., 2016) and that approach has been also applied in Croatia (for example ĆUKUŠIĆ, 2019; ĆUKUŠIĆ et al., 2017; KUČINIĆ et al., 2013, 2019a, 2019b; SZIVÁK et al., 2017).

In this paper we provide (1) an overview of DNA barcoded species of Trichoptera collected in springs in different parts of Croatia, including some literature data (KUČINIĆ et al., 2016, 2017, 2019a, Tab. 2); (2) a review of some preliminary taxonomic features; (3) some aspects of threats to the caddisfly spring fauna and their conservation.

This study does not encompass certain genera and species that were found in Croatian springs and are DNA barcoded (for examples Rhyacophila hirticornis McLachlan, 1879, Agapetus sp., Diplectrona sp., Potamophylax sp.), and also does not provide detailed information about trichopteran spring fauna, which are the subject of other scientific studies in progress.

MATERIAL AND METHODS

Field work

Collecting of Trichoptera was performed at 36 springs presented in Tab. 1 containing a checklist of all springs with data on spring type (limnocrene- or rheocrene), geocoordinates, biogeographical region, basin and ecoregion. Caddisflies were collected during the night, with small portable batteries and 12 W UV lamps and during the day by entomological nets. All collected specimens were stored in absolute ethanol.

Biogeographical presentation

There are three biogeographical divisions of Croatia relevant for this study, and the results are presented according to each of them. BERTIĆ et al. (2001) divide Croatia into three biogeographical regions: the Pannonian-Peripannonian in the north and east,
central mountainous in the middle and the Mediterranean in the south (Fig. 5). Nine springs are in the Panonnnian-Peripannonian part, fifteen in the central mountainous part and twelve are in the Mediterranean part (Tab. 1, Fig. 5).

All streams in Croatia belong to one of two basins: the Black Sea and the Adriatic Sea Basin (Tab. 1, PRIMC & HABDIJA, 2019; VILENCA et al., 2015). The Black Sea Basin encompasses streams from the Panonnnian-Peripannonian and central mountainous parts (21 springs in this paper), and the Adriatic Sea Basin those in the Mediterranean region (15 springs in this paper) (Tab. 1).

In the 1970-ies Illies divided Europe, regarding hydrology and biological freshwater data, into 25 biocenotic ecoregions (ILLIES, 1978), with Croatia lying in two of them, Dinaric Western Balkans - Ecoregion 5 (ER5) and Hungarian (Pannonian) Lowland - Ecoregion 11 (ER11) (ILLIES, 1978; GRAF et al., 2020 - www.freshwater.info). In this study, 34 springs are in Ecoregion 5, and 2 springs in Ecoregion 11 (Tab. 1, Fig. 5).

Karst boundaries are given according to BIONDIĆ et al. (2009). There are 30 springs from this study in the karst area (Fig. 5).

Laboratory work

In order for us to be able to use DNA-based methods of specimen identification along with morphological features, all collected material was preserved in absolute ethanol. The DNA vouchers of the barcoded samples are stored in the Croatian Natural History Museum.

Species identification was done according to MALICKY (2004) and KUMANSKI (1985, 1988). Systematics follows MORSE (2020). In Tab. 2 there are data concerning determination according to morphological features (first column), specimens ID, Locality/Family, BOLD Sequence ID and species identification after DNA barcoding analyses (last column).

Macrophotographing of Trichoptera adults was carried out using a Leica Wild MZ8 stereomicroscope and Olympus SP-500 UZ digital camera, processed with the computer program Olympus Quick Photo Camera 2.2 at the tree pathology laboratory, Department of Forest Protection and Wildlife Management at the Faculty of Forestry, University of Zagreb.

DNA extraction and PCR amplification. Genomic DNA was extracted from legs of 110 specimens listed in Tab. 2. Genomic DNA was extracted from legs or part of body for small specimens using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to the manufacturer’s specifications and eluted in 50 µl of elution buffer. For the amplification of the COI-5P barcode region primers: LCO1490 and HCO2198 (FOLMER et al., 1994) were used. For specimens that could not be amplified with Folmer primers, specific primers were designed: TM3 HCOI (TGATTYTTYGGYACCCCWGAAGTITA), TM4 HCOI (TGATTYTTYGGRACCCCWGAAGTITA) or a mix of primers C_LepFolF and C_LepFolR was used (HERNÁNDEZ-TRIANA et al., 2014). The volume of mixture for polymerase chain reactions (PCR) was 50 µl. The PCR mixture contained 1 x Go Taq® Reaction Buffer (containing 1.5 mM MgCl2, Promega), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 units of Go Taq® DNA Polymerase (Promega) and 5 µl of DNA eluate. PCR cycling conditions comprised an initial denaturation step (94°C for 2 min) followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 90 s and a final extension step of 72°C for 7 min. Product purification and bidirectional sequencing was performed by
Macrogen Inc. Sequencing Service (Seoul, South Korea and Macrogen Europe) using the amplification primers. Sequences were edited manually and aligned using the program BioEdit (Hall, 1999). DNA sequences obtained in this study were submitted for phylogenetic analysis of *Rhyacophila* species to Barcode of Life Data Systems (BOLD, Ratnasingham & Hebert 2007, Tab. 2). For the 110 DNA barcode sequences obtained in this study, a similarity search was performed using the BOLD Identification Engine (available on http://boldsystems.org/) which uses all sequences uploaded to BOLD from public and private projects to locate the closest match.

**DNA data analysis – phylogenetic reconstruction and species delimitation methods.** For phylogenetic analysis of *Rhyacophila* species two different methods of tree reconstruction were used: Neighbor-Joining (NJ) and Maximum likelihood (ML) as implemented in MEGA 7.0. (Kumar et al., 2016) to infer phylogeny-based specimen identifications. Inter- and intraspecific genetic uncorrected pairwise divergences (\(p\) - distances) were calculated in MEGA 7.0. (Kumar et al., 2016). The number of hypothetical species within the data set was estimated based on barcode gap (difference between inter- and intraspecific genetic distances) with the use of Automatic Barcode Gap Discovery, ABGD (Puillandre et al., 2012) (Fig. 10, Appendix 1). DNA barcode sequences were submitted to the ABGD online website and analysed under the following settings: P (prior intraspecific divergence) set from 0.001 (Pmin) to 0.08 (Pmax) and Steps set to 10; X (minimum relative gap width) set to 1; Nb bins (for distance distribution) set to 20; we selected the Kimura (K80) model and set TS/TV to 2.0. The data set for phylo-
genetic analysis comprised the DNA barcodes amplified from *Rhyacophila cabrankensis* Malicky, Previšić & Kučinić, 2007 (TRCAB_1), *R. vulgaris* Pictet, 1834 (TRVUL) and the outgroup species *Anabolia furcata* Brauer, 1857 (TAFUR_1), along with all available *Rhyacophila* barcode sequences retrieved from the Barcode of Life Data Systems (BOLD; Ratnasingham & Hebert, 2007)

Due to the more detailed presentation of DNA barcoded caddisflies in the springs in this study we also included the DNA barcoding data presented in previous studies (Kučinić et al., 2016, 2017, 2019a, Tab. 2). Additionally, there are some corrections of previous data; *Agrypnia varia* (Fabricius, 1793) for the Ruda spring (specimen ID TAVAR_2; BOLD Sequence ID CROTR078-19) given in Kučinić et al. (2019a) actually relates to the Grab spring, which is corrected in this paper (Tab. 2), and *M. wageneri* Malicky, 1971 was not found at the spring Palje in Konavle (Kučinić et al., 2017) but at the spring in Vodovađa village (Tab. 2).

**Tab. 1.** List of the 36 study springs where caddisflies were collected with basic characteristics: TS (type of spring): L (limnicrene spring), R (rheocrene spring) (according to Habjina & Primc, 2019); BR (biogeographical regions of Croatia): PP (Pannonian-Peripannonian part), CM (central mountainous part), ME (Mediterranean part) (according to Bertíć et al., 2001); EC (ecoregions): EC5 (ecoregion 5), EC 11 (ecoregion 11) (according to Illies, 1978); BA (basin): BS (Black Sea Basin), AS (Adriatic Sea Basin), * - closed karstic system, * – anthropogenic influence.

| Localities | TS | BR | EC | BA | Long | Lat |
|------------|----|----|----|----|------|-----|
| 1. spring Jankovac (Mt Papuk) | R | PP | ER1 | BS | 45.51875 | 17.68664 |
| 2. spring Škodinovac (Mt Papuk) | R | PP | ER1 | BS | 45.66388 | 17.33289 |
| 3. spring of the Šumi stream (Mt Ivanšćica) | R | PP | ER5 | BS | 46.18884 | 16.15777 |
| 4. spring of the Križ stream* | R | PP | ER5 | BS | 45.4225 | 16.248 |
| 5. spring Pašina vrela | L | PP | ER5 | BS | 45.28936 | 16.42339 |
| 6. spring Bijele stijene* | R | PP | ER5 | BS | 45.42317 | 16.22337 |
| 7. spring of the Slunjčica River | L | PP | ER5 | BS | 45.07964 | 15.58925 |
| 8. spring of the Rudnica River (Ožanići) | R | PP | ER5 | BS | 45.21457 | 15.39262 |
| 9. spring of the Tounjčica River | R | PP | ER5 | BS | 45.24844 | 15.32317 |
| 10. spring of the Dobra River* | R | CM | ER5 | BS | 45.42795 | 14.95681 |
| 11. spring Zeleni Vir* | L | CM | ER5 | BS | 45.42289 | 14.89573 |
| 12. spring of the Vitunjčica River | R | CM | ER5 | BS | 45.29117 | 15.14049 |
| 13. spring Izvor (Mt Bijelasica) | R | CM | ER5 | BS | 45.2731 | 14.96323 |
| 14. spring of the Plitvica stream | R | CM | ER5 | BS | 44.90137 | 15.57379 |
| 15. spring of the Napojiště stream | R | CM | ER5 | BS | 44.82661 | 15.61666 |
| 16. spring of the Crna Rijeka River | R | CM | ER5 | BS | 44.83086 | 15.61343 |
| 17. spring of the Drakulić River | R | CM | ER | BS | 44.78892 | 15.65101 |
| 18. spring Keljevac | L | CM | ER5 | BS | 44.72094 | 15.7376 |
| 19. spring of the Una River | L | CM | ER5 | BS | 44.39934 | 16.10382 |
| 20. spring in the Sturovača* (Mt Velebit) | R | CM | ER5 | BS | 44.69808 | 15.04992 |
| 21. spring of the Cabranka River | R | CM | ER5 | BS | 45.60104 | 14.64079 |
| 22. spring of the Bječina River | R | CM | ER5 | AS | 45.42199 | 14.42127 |
| 23. spring of the Lika River (Mt Velebit) | R | CM | ER5 | AS | 44.42618 | 15.541 |
| 24. spring Majerovo vrilo (Gacka River) | L | CM | ER5 | AS | 44.81471 | 15.3588 |
| 25. spring Bračana (village Mlini)* | R | ME | ER5 | AS | 45.45257 | 13.92448 |
| 26. spring in the village of Marušići | L | ME | ER5 | AS | 45.42331 | 13.72946 |
| 27. spring Cerinjevica | R | ME | ER5 | AS | 45.261389 | 13.926111 |
| 28. spring Špilja (Rabac) | R | ME | ER5 | AS | 45.08494 | 14.13915 |
| 29. spring Grdak (Raša River) | L | ME | ER5 | AS | 45.0926 | 14.01831 |
| 30. spring of the Vrba stream* | R | ME | ER5 | AS | 43.72087 | 16.40175 |
| 31. spring of the Zrmanja River* | R | ME | ER5 | AS | 44.20484 | 16.08444 |
| 32. spring Glavaš (Cetina River) | L | ME | ER5 | AS | 43.97648 | 16.4302 |
| 33. spring Nela (Cetina River)* | R | ME | ER5 | AS | 43.95345 | 16.40573 |
| 34. spring of the Rumin | L | ME | ER5 | AS | 43.77979 | 16.6566 |
| 35. spring of the Grab River* | L | ME | ER5 | AS | 43.64099 | 16.76997 |
| 36. spring in the village of Vodovoda* | R | ME | ER5 | AS | 42.51763 | 18.42215 |
RESULTS AND DISCUSSION

A great number of species and specimens were collected in 36 springs in Croatia (Tab. 1) during the last 12 years, and 110 specimens belonging to 70 species, 32 genera and 15 families have been successfully DNA barcoded (Kučinić et al., 2016, 2017, 2019a, Tab. 2). A few of the specimens/species shows genetic variability when compared with data previously entered in the BOLD database (Tab. 2). There is a tendency to establish the smallest value between two different species based on the DNA barcode region (=2% in Hébert et al., 2003b), but there are no generally accepted values. Within the order Trichoptera intraspecific values range from 0.2% (Graf et al., 2015), to 9.4% (Zhou et al., 2007). For this type of taxonomical research, in addition to the use of DNA barcoding, it is necessary to make detailed analyses of morphological traits, which generally refers to adults’ genitalia for Trichoptera. If possible it is also useful to make analyses of other genes, including nuclear, which generally have a slower evolutionary rate than mitochondrial genes and show less intra- and interspecific genetic divergence values than mitochondrial genes (Geraci et al., 2010; Ibrahimí et al. 2015; Johanson & Keijsner, 2008; Saito et al. 2018; Waringer et al., 2015). The employment of species delimitation bioinformatic tools like ABGD (Puillandre et al., 2012) may also aid in taxonomic decisions (in this study for R. cabrakensis, Fig. 10). Integrative taxonomy represents the basic framework of today’s studies of taxonomic features of certain species and groups of organisms (Bilandžija et al., 2013, Previšić et al., 2014; Valladolid et al., 2018, 2019; Vitecek et al., 2017; Yánez-Muñoz et al., 2018).

In Tab. 2 we provide a short review of the DNA barcoding results according to the families and species registered in this study and literature data (Kučinić et al., 2016, 2017, 2019a, Tab. 2).
Tab. 2. List of caddisfly species discussed in this study: first column - identification according to morphological features; followed by specimens’ ID; Locality/Family; BOLD Sequence ID; last column - DNA species identification with percentage similarity to existing DNA sequences in the BOLD database (identification according to BOLD Identification Engine) (*=Rhacophila cabrakensis, **=Glossosoma discophorum, ***=Hydroptila phaon, ****=Psychomyia klapaleki, *****=Tinodes antonii, ******=Anitella apfelbecki, *******=Drusus croaticus, ********=Micropterna wageneri) (Čukić, 2019; Kučinić et al., 2016, 2017); ☼ data for the spring Rude (Kučinić et al., 2019a), here corrected as the accurate locality of spring Grab.

| Species (morphologically) | Specimen ID | Locality | BOLD Sequence ID | DNA species identification (BOLD) |
|---------------------------|-------------|----------|------------------|----------------------------------|
| **Family Rhyacophilidae**  |             |          |                  |                                  |
| Rhacophila balcanica      | TRBAL_1     | spring of the Una River | CROTR256-19 | Rhacophila balcanica 96.24%     |
| Rhacophila cabrakensis    | TRCAB_1     | spring of the River Čabranka | CROAA089-18 | Rhacophila vulgaris (97.61%)*   |
| Rhacophila dorsalis       | TRDOR_2     | spring of the River Cabranka | CROAA060-18 | Rhacophila dorsalis (100%)      |
| Rhacophila cf. fasciata   | TRFAS_1     | Zeleni Vir | CROTR264-19 | Rhacophila fasciata (97%)       |
| Rhacophila laevis         | TRLAE_1     | spring of the Šumi stream | CROTR266-19 | Rhacophila laevis (97.76%)      |
| Rhacophila torrentium     | TRTOR_1     | Zeleni Vir | CROAA018-18 | Rhacophila torrentium (99.54%)  |
| Rhacophila tristis        | TRTRI_4     | spring in Vodovada village | CROAA098-18 | Rhacophila tristis (97.15%)     |
| Rhacophila tristis        | TRTRI_5     | spring in Vodovada village | CROTR011-19 | Rhacophila tristis (99.47%)     |
| Rhacophila tristis        | TRTRI_7     | spring in Vodovada village | CROTR031-19 | Rhacophila tristis (97.71%)     |
| **Family Glossostomatidae** |           |          |                  |                                  |
| Glossosoma discophorum    | TGDIS_1     | spring of the River Tounjčica | CROAA004-18 | Glossosoma neretvae** (99.08%) |
| Glossosoma discophorum    | TGDIS_2     | spring of the River Vitunjčica | CROAA035-18 | Glossosoma neretvae** (98.88%) |
| Glossosoma discophorum    | TGDIS_3     | spring of the River Slunjčica | CROAA036-18 | Glossosoma neretvae** (98.88%) |
| Glossosoma discophorum    | TGDIS_4     | spring of the River Una | CROAA037-18 | Glossosoma neretvae** (98.49%) |
| Glossosoma discophorum    | TGDIS_5     | spring of the River Rumin | CROAA064-18 | Glossosoma neretvae** (99.54%) |
| Glossosoma discophorum    | TGDIS_6     | spring of the Plitvica stream | CROTR057-19 | Glossosoma neretvae** (99.67%) |
| Glossosoma discophorum    | TGDIS_7     | spring of the River Rumin | CROTR063-19 | Glossosoma neretvae** (99.84%) |
| Glossosoma discophorum    | TGDIS_8     | spring of the River Grab | CROTR090-19 | Glossosoma neretvae** (100%)    |
| **Family Hydroptiliidae**  |             |          |                  |                                  |
| Hydroptila phaon          | TPHA_1      | spring Marušići | CROTR232-19 | Hydroptila occulta** (85.51%)  |
| Hydroptila sp.            | THYD_5      | spring of the River Rudnica (Ožanići) | CROTR087-19 | Hydroptila martini (100%)     |
| Hydroptila sp.            | THYD_7      | spring of the River Rudnica (Ožanići) | CROTR088-19 | Hydroptila martini (100%)     |
| Hydroptila sp.            | THYD_8      | spring of the River Rudnica (Ožanići) | CROTR141-19 | Hydroptila martini (100%)     |
| Species (morphologically) | Specimen ID | Locality | BOLD Sequence ID | DNA species identification (BOLD) |
|---------------------------|-------------|----------|-----------------|-----------------------------------|
| Hydroptilidae             | THYD_6      | spring of the River Rudnica (Ožanići) | CROTR139-19 | Hydroptila tineoides (100 %) |
| Hydroptilidae             | THYD_14     | spring Pecki | CROTR251-19 | Hydroptila lotensis (99.84%) |
| Hydroptilidae             | THTIN_3     | spring of the River Rudnica (Ožanići) | CROTR102-19 | Hydroptila tineoides (98.54%) |

**Family Philopotamidae**

| Species                  | Specimen ID | Locality                  | BOLD Sequence ID | DNA species identification (BOLD) |
|--------------------------|-------------|----------------------------|-----------------|-----------------------------------|
| Philopotamus montanus    | TPMON_2     | spring of the Šumi stream  | CROAA130-18     | Philopotamus montanus (99.84%)    |
| Wormaldia copiosa        | TW COP_2    | spring of the River Čabranka | CROAA044-18 | Wormaldia copiosa (99.84%) |
| Wormaldia occipitalis    | TWOCL_4     | spring of the Napožište stream | CROTR068-19 | Wormaldia occipitalis (99.20%) |
| Wormaldia occipitalis    | TWOC_3      | spring Škodinovac          | CROTR061-19     | Wormaldia occipitalis (99.67%) |
| Wormaldia occipitalis    | TWOCL_6     | spring Bijela stijene      | CROTR245-19     | Wormaldia occipitalis (99.37%) |
| Wormaldia subnigra       | TWSUP_2     | spring Cerišnjevica        | CROTR099-19     | Wormaldia subnigra (99.52%) |

**Family Polycentropodidae**

| Species                  | Specimen ID | Locality                  | BOLD Sequence ID | DNA species identification (BOLD) |
|--------------------------|-------------|----------------------------|-----------------|-----------------------------------|
| Cyrnus trimaculatus      | TCTRIL_6    | spring Cerišnjevica        | CROTR217-19     | Cyrnus trimaculatus (99.84%)     |
| Plectrocnemia brevis     | TPBRE_1     | spring of the River Dobra  | CROAA071-18     | Plectrocnemia brevis (98.39%)    |
| Plectrocnemia conspersa  | TPCON_2     | spring Izvor (Bjelolasica Mt) | CROTR088-19 | Plectrocnemia conspersa (100%) |
| Plectrocnemia conspersa  | TPCON_4     | spring of the Drakulić River | CROTR076-19 | Plectrocnemia conspersa (100%)  |
| Plectrocnemia conspersa  | TPCON_5     | spring of the stream Plitvica | CROTR192-19 | Plectrocnemia conspersa (99.75%) |
| Plectrocnemia conspersa  | TPCON_6     | spring of the River Dobra  | CROTR144-19     | Plectrocnemia conspersa (99.83%) |
| Polycentropus flavomaculatus | TPCON_1 | spring of the River Zrmanja | CROTR272-19 | Polycentropus flavomaculatus (99.67%) |
| Polycentropus sp.         | TPLE_1      | spring Bračana (Mlini)     | CROTR273-19     | Polycentropus flavomaculatus (99.83%) |
| Polycentropus irroratus   | TPIRR_2     | spring of the River Rudnica (Ožanići) | CROTR046-19 | Polycentropus irroratus (99.84%) |

**Family Psychomyiidae**

| Species                  | Specimen ID | Locality                  | BOLD Sequence ID | DNA species identification (BOLD) |
|--------------------------|-------------|----------------------------|-----------------|-----------------------------------|
| Lype cf. reducta         | TLRED_3     | spring Cerišnjevica        | CROTR081-19     | Lype reducta (97.97%)             |
| Psychomyia klapalekii    | TPKLA_1     | spring of the River Vitunjčica | CROAA038-18 | Psychomyia morisitai 86.41, Pahuniella sp. 86.41 **** |
| Tinodes antonioi         | TTANT_1     | spring in Marušići village | NIP002-16       | Tinodes n. sp. nr. turanicus 89.1**** |
| Tinodes sp., female      | TTIN_1      | spring in Marušići village | NIP003-16       | Tinodes n. sp. nr. turanicus 89.1**** |
| Tinodes sp., female      | TTIN_2      | spring in Marušići village | NIP004-16       | Tinodes n. sp. nr. turanicus 88.75 **** |
| Tinodes dives            | TTDIV_1     | spring of the River Una    | NIP007-16       | Tinodes dives (98.37%)            |
| Species (morphologically) | Specimen ID | Locality | BOLD Sequence ID | DNA species identification (BOLD) |
|--------------------------|-------------|----------|------------------|----------------------------------|
| Tinodes pallidulus       | TTPAL_1     | spring in Marušići village | CROTR158-19 | Tinodes pallidulus (97.82%) |
| Tinodes unicolor         | TTUNI_1a    | spring Šumi | CROTR204-19 | Tinodes unicolor (100%) |
| Tinodes unicolor         | TTUNI_2     | spring of the Vrba stream | CROTR205-19 | Tinodes unicolor (98.94%) |
| Tinodes unicolor         | TTUNI_3     | spring Cerišnjevica | CROTR206-19 | Tinodes unicolor (99.82%) |
| Tinodes unicolor         | TTUNI_4     | spring Rabac | CROTR089-19 | Tinodes unicolor (99.52%) |
| Tinodes unicolor         | TTUNI_5     | spring Cerišnjevica | CROTR207-19 | Tinodes unicolor (99.82%) |
| Tinodes waeneri          | TTWAEC_1    | spring in Marušići village | NIP001-16 | Tinodes waeneri (99.69%) |

**Family Hydropsychidae**

| Hydropsyche instabilis | THINS_5 | spring of the River Vitunjčica | CROAA052-18 | Hydropsyche instabilis (100%) |
| Hydropsyche instabilis | THINS_4 | spring of the River Kječina | CROTR201-19 | Hydropsyche instabilis (100%) |
| Hydropsyche instabilis | THINS_5 | spring of the Plitvica stream | CROTR270-19 | Hydropsyche instabilis (99.84%) |
| Hydropsyche instabilis | THINS_6 | spring of the River Grab | CROTR091-19 | Hydropsyche instabilis (100%) |
| Hydropsyche saxonia     | THSAX_2 | spring of the Vrba stream | CROTR149-19 | Hydropsyche saxonia (100%) |

**Family Phryganeidae**

| Agrypnia varia          | TAVAR_2 | spring of the River Grab | CROTR078-19 | Agrypnia varia (99.84%) |
| Trichostega minor       | TTMIN_1 | spring Majerovo vrilo | CROAA133-18 | Trichostega minor (98.93%) |

**Family Goeridae**

| Silo pallipes           | TSPAL_1 | spring Braćana (Mlini) | CROTR287-19 | Silo pallipes (98.83%) |
| Silo pallipes           | TSPAL_3 | spring of the River Slunjčica | CROTR065-19 | Silo pallipes (98.87%) |

**Family Leptidostomatidae**

| Crunoea kempnyi         | TCKEM_1 | spring of the Napožije stream | CROTR074-19 | Crunoea kempnyi (96.67%) |
| Lepidostoma basale      | TLBAS_1 | spring Pašina vrela | CROAA024-18 | Lepidostoma basale (99.84%) |
| Lepidostoma basale      | TLBAS_2 | spring Pašina vrela | CROAA025-18 | Lepidostoma basale (99.66%) |
| Lepidostoma basale      | TLBAS_3 | spring of the River Grab | CROTR122-19 | Lepidostoma basale (99.22%) |
| Lepidostoma hirtum      | TLHIT_2 | spring of the River Rudnica | CROTR053-19 | Lepidostoma hirtum (100%) |

**Family Limnephilidae**

| Allogamus auricollis    | TAAUR_1 | spring of the River Una | CROAA040-18 | Allogamus auricollis (96.83%) |
| Annitella apfelbecki    | TAAPF_1 | spring of the River Zrmanja | CROTR290-19 | Annitella eparargaeura (95.69%) |
| Drusus croaticus        | TDCRO_1 | spring of the River Vitunjčica | CROAA041-18 | Drusus monticola (92.9%) |
| Drusus croaticus        | TDCRO_2 | spring Izvor (Bjelolasica Mt) | CROTR017-19 | Drusus monticola (93.69%) |
| Species (morphologically) | Specimen ID | Locality | BOLD Sequence ID | DNA species identification (BOLD) |
|---------------------------|-------------|----------|------------------|----------------------------------|
| Drusus croaticus          | TDCRO_3     | spring Majerovo vrilo (River Gacka) | CROTR019-19 | Drusus monticola (93.63%) ******* |
| Drusus croaticus          | TDCRO_4     | spring Majerovo vrilo (River Gacka) | CROTR043-19 | Drusus monticola (93.43%) ******* |
| Drusus discolor           | TDDIS_1     | spring of the River Čabranka | CROTR020-19 | Drusus discolor (98.87%) |
| Drusus schmidt            | TDSCH_1     | spring Jankovac | CROAA021-18 | Drusus schmidt (100%) |
| Drusus vespertinus        | TDVES_1     | spring of the River Una | CROTR275-19 | Drusus vespertinus (97.99%) |
| Ecclisopteryx irvae       | TEIVK_1     | spring Glavaš (Cetina river) | CROAA106-18 | Ecclisopteryx irvae (100%) |
| Glyphotaelius pellucidus  | TRBAL_3     | spring Nela (Cetina river) | CROTR064-19 | Glyphotaelius pellucidus (99.36%) |
| Glyphotaelius pellucidus  | TGPEL_4     | spring of the Napožište stream | CROTR069-19 | Glyphotaelius pellucidus (100%) |
| Glyphotaelius pellucidus  | TGPEL_5     | spring Bijela stijena | CROTR227-19 | Glyphotaelius pellucidus (99.22%) |
| Halesus digitatus         | THDIG_1     | spring of the River Zrmanja | NIPM009-17 | Halesus digitatus (100%) |
| Halesus digitatus         | THDIG_2     | spring of the River Rječina | CROTR038-19 | Halesus digitatus (99.68%) |
| Halesus digitatus         | THDIG_4     | spring of the River Crna rijeka | CROTR221-19 | Halesus digitatus (99.84%) |
| Limnephilus flavicornis   | TLFLA_1     | spring Majerovo vrilo | CROTR073-19 | Limnephilus flavicornis (99.19%) |
| Limnephilus ignavus       | TLING_2     | spring Keljevac | CROTR040-19 | Limnephilus ignavus (99.21%) |
| Limnephilus hirsutus      | TLHIR_1     | spring Keljevac | CROTR029-19 | Limnephilus hirsutus (99.68%) |
| Limnephilus lunatus       | TLLUN_1     | spring Keljevac | CROTR009-19 | Limnephilus lunatus (99.51%) |
| Limnephilus lunatus       | TLLUN_2     | spring of the stream Plitvica | CROTR071-19 | Limnephilus lunatus (100%) |
| Limnephilus lunatus       | TLLUN_3     | spring of the River Grab | CROTR233-19 | Limnephilus lunatus (99.84%) |
| Grammotaulius nigropunctatus | TGNIG_2  | spring Grdak (Raša river) | CROTR276-19 | Grammotaulius nigropunctatus (98.90%) |
| Limnephilus rhombicus     | TLRHO_2     | spring in the Štirovača (Mt Velebit) | CROTR023-19 | Limnephilus rhombicus (99.84%) |
| Limnephilus rhombicus     | TLRHO_5     | spring Majerovo vrilo (Gacka river) | CROTR188-19 | Limnephilus rhombicus (99.36%) |
| Limnephilus sparsus       | TLSPA_1     | spring of the River Lika (Mt Velebit) | CROTR001-19 | Limnephilus sparsus (100%) |
| Limnephilus vittatus      | TLVIT_1     | spring Keljevac | CROTR006-19 | Limnephilus vittatus (99.84%) |
| Mesophylax aspersus       | TMASP_3     | spring Špilja (Rabac) | CROTR083-19 | Mesophylax aspersus (99.38%) |
| Mesophylax aspersus       | TMASP_4     | spring Špila (Rabac) | CROTR281-19 | Mesophylax aspersus (100%) |
| Stenophylax lateralis     | TMLAT_1     | spring of the River Lika (Mt Velebit) | CROTR002-19 | Stenophylax lateralis (98.46%) |
| Species (morphologically) | Specimen ID | Locality | BOLD Sequence ID | DNA species identification (BOLD) |
|---------------------------|-------------|----------|------------------|----------------------------------|
| Stenophylax lateralis    | TMLAT_1f    | spring of the River Lika (Mt Velebit) | CROT154-19 | Stenophylax lateralis (100%) |
| Micropterna nycterobia   | TMIC_1      | spring of the River Zrmanja          | NIPM003-17 | Micropterna nycterobia (98.89%) |
| Micropterna nycterobia   | TMNYC_2     | spring Keljevac                      | CROT016-19 | Micropterna nycterobia (100%) |
| Micropterna sequax       | TMIC_2      | spring of the River Una              | NIPM004-17 | Micropterna sequax (98.51%) |
| Micropterna testacea     | MTES_3      | spring Majerovo vrilo (River Gacka) | CROT028-19 | Micropterna testacea (100%) |
| Micropterna wageneri     | TPWAG_1     | spring in the village Vodovada       | NIPM002-17 | Micropterna sequax (90.38%) **
| Stenophylax permistus    | TSPER_1     | spring of the River Una              | CROAA065-18 | Stenophylax permistus (99.85%) |
| Stenophylax permistus    | TSPER_2     | spring Keljevac                      | CROT048-19 | Stenophylax permistus (100%) |
| Family Sericostomatidae  |             |          |                  |                                  |
| Sericostoma flavicorne   | TSFLA_1     | spring of the River Tounjčica        | CROAA062-18 | Sericostoma flavicorne (99.72%) |
| Family Odontoceridae     |             |          |                  |                                  |
| Odontocerum albicorne    | TOALB_3     | spring of the River Rudnica (Ožanići) | CROT047-19 | Odontocerum albicorne (97.4%) |
| Family Beraeidae         |             |          |                  |                                  |
| Beraea pullata           | TBPUL_1     | spring of the Napožište stream      | CROT080-19 | Beraea pullata (99.84%) |
| Family Leptoceridae      |             |          |                  |                                  |
| Atripsodes bilineatus    | TABIL_1     | spring Pašina vrela                  | CROAA012-18 | Atripsodes bilineatus (100%) |
| Atripsodes cinereus      | TACIN_3     | spring of the River Lika (Mt Velebit) | CROT049-19 | Atripsodes cinereus (99.63%) |
| Oecetis notata           | TONOT_2     | spring Majerovo vrilo (River Gacka) | CROT072-19 | Oecetis notata (99.84%) |
| Oecetis testacea         | TOTES_4     | spring Zeleni vir                    | CROT165-19 | Oecetis testacea (99.37%) |

2017, 2019a). We should emphasize that data from the last column (“species identification”) in Tab. 2 are not ‘stable’ and ‘constant’ and will change when new DNA barcoding data become available, both regarding new localities and species not previously DNA barcoded will be available. For example, five species included in the current study, *Rhyacophila cabrankensis* Malicky, Previšić & Kučinić, 2007, *Glossosoma discophorum* Klapálek, 1902, *Hydroptila phaon* Malicky, 1976, *Psychia klapaleki* Malicky, 1995 and *Annitella apfelbecki* Klapálek, 1898 were not present in the BOLD database and therefore species identification showed great differences in relation to the nearest species (Tab. 2). The first entries of DNA barcodes of these species into the BOLD database provided the references for reliable species identification for all subsequent specimens belonging to those species (Čukušić, 2019; Tab. 2). For example, no data existed previously in the BOLD database for *Hydroptila phaon*, and our identification was closest to *Hydroptila occulta* Eaton, 1873 (Tab. 2). Every new entry will therefore ensure a high percentage of identity with *H. phaon* originating from this study (Tab. 2). The same applies to the other four species not present in the BOLD database so far (Tab. 2).
On the other hand, there are some interesting novelties from the DNA barcoding for eight specimens of *Glossosoma discophorum* (Fig. 6) found at seven study springs (Tab. 2). This species is distributed in part of SE Europe, i.e. the limnoecoregions ER5, ER6 (Hellenic Western Balkan), ER7 (Eastern Balkan) and ER10 (the Carpathians; Grač et al., 2020). From the ER5 it was recorded in Bosnia and Herzegovina (Stanić-Koštroman et al., 2015), Montenegro (Krušnik, 1987) and Serbia (Živić et al., 2006), being described at the beginning of the 20th century from central Bosnia (Klapálek, 1902). However, no data for this species existed in the BOLD database. All our data were grouped together with a high similarity of up to 98.49% - 100% (Tab. 2) with *Glossosoma neretvae* Marinković-Gospodnetić, 1988 which is present in the BOLD database with one, probably misidentified, specimen. According to the research so far, *G. neretvae* is a microendemic specis of Bosnia and Herzegovina, distributed only in the lower part of the Neretva River (Marinković-Gospodnetić, 1988; Stanić-Koštroman et al., 2015, M. Kučinić unpublished data). The ongoing study, which includes these two species and DNA barcoded data, shows significant differences in the DNA barcode between *G. discophorum* and *G. neretvae* at the level of ‘true’ species (unpublished data A. Ćukušić, M. Kučinić). Thus all our data in Tab. 2 are related only to *Glossosoma discophorum*, and not to *G. neretvae* as matched by the BOLD identification engine (species identification, Tab. 2). This is a very good example of potential consequences of misidentified samples in the BOLD database.

Within the family Rhyacophilidae some species included in the current study show considerable variability of DNA barcoded specimens (Tab. 2). Especially interesting is the endemic species *Rhyacophila cabrankensis* (Figs 7-8), described on specimens collected from the spring of the Čabranka River (Malicky et al., 2007). Results of the phylogenetic analysis based on COI show an unresolved pattern of divergence between this species and *R. vulgaris* Pictet, 1834 (Fig. 9), i.e. they resolved the *R. cabrankensis* and two lineages of *R. vulgaris* trichotomy (Fig. 10). According to the same phylogenetic tree (Fig. 10), *R. simulatrix* McLachlan, 1879 is highly supported as a sister taxon to *R. cabrankensis* and *R. vulgaris*. P-distance values supported the presumed close relationship of two species, *R. cabrankensis* and *R. vulgaris*, based on morphology. The value of uncorrected pairwise distance (p-distance) between *R. vulgaris* and *R. cabrankensis* (1.8%) is lower than the maximum intraspecific value of *R. vulgaris* (2.3%) (Tab. 3). In addition, the interspecific genetic distance between *R. vulgaris* and *R. cabrankensis* is
lower than the intraspecific distance reported in Morinière et al. (2017) within R. fasciata (3.86%), R. obliterata (3.64%) and R. vulgaris (3.15%), which indicates a possibility that R. cabrakenensis has subspecies status. Nevertheless, in the ABGD analysis (Fig. 10), R. cabrakenensis formed one group (Group 1), separated from group R. vulgaris (Group 3 and 4), which would indicate that R. cabrakenensis is a true species.

Fig. 7. Adult male of Rhycophilidae cabrakenensis Malicky, Previšić & Kučinić 2007, collected in the spring of the Čabranka River (photo M. Kučinić).

Fig. 8. Rhycophilidae cabrakenensis Malicky, Previšić & Kučinić, 2007, male genitalia, lateral view, left side (photo M. Kučinić).

Fig. 9. Rhycophilidae vulgaris Pictet, 1834, male genitalia, lateral view, left side (photo A. Ćukušić).
Fig. 10. Maximum likelihood (ML) phylogram based on a 658 bp long fragment of the DNA barcode region showing the relationships between *Rhyacophila* species. Numbers above the branches represent bootstrap support (BS) for Neighbor-Joining (NJ) and ML analysis (NJ/ML). The groups delineated by the Automatic Barcode Gap Discovery (ABGD) approach are shown on the right side of the tree. Specimen ID from sequences obtained in this study written in bold.

However, two lineages of *R. vulgaris* were also delineated in separate groups by ABGD analysis, which indicates the possibility of there being two species (Fig. 10), even though this is not supported by the morphology (Malicky, 2004). In order to resolve phylogenetic relationships of these species it is necessary to include additional markers, such as nuclear genes and more specimens. *Rhyacophila vulgaris* and *R. cabrankensis* are allopatric species. *Rhyacophila cabrakenensis* is endemic to the central-mountainous part of Croatia (the Gorski kotar region) while *R. vulgaris* is widespread in Europe (Fig. 11). In Croatia, *R. vulgaris* was recorded in two localities on Mt Žumberak in the northwest part of the Pannonian-peripannonian region of Croatia (Kučinić et al., 2015a, Fig. 11).

Fig. 11. Records of *R. cabrankensis* (red dots) and *R. vulgaris* (green dots) in Croatia with regions according to Bertić et al. (2001) (dark green – mountains, orange – Pannonian-peripannonian and blue – Mediterranean region) and distribution of *R. vulgaris* in Europe (green field) according to Graf et al. (2020). Fig. B represents the magnified part of Fig. A in the upper left corner.
Tab. 3. P-distance between *R. cabrankensis*, *R. simulatrix*, *R. vulgaris* and an outgroup species for the barcode COI region.

| Species          | *R. cabrankensis* | *R. simulatrix* | *R. vulgaris* |
|------------------|-------------------|-----------------|---------------|
| *R. cabrankensis* | -                 | -               | -             |
| *R. simulatrix*  | 5.9               | 0.3             | -             |
| *R. vulgaris*    | 1.8               | 6.4             | 0.2-2.3       |
| *Anabolia furcata* | 28.5             | 28.9            | 29.4          |

Within the family Rhyacophilidae there are further examples of relatively high intraspecific p-distances observed within DNA barcoded specimens in the current study, i.e. in *R. balcanica* Radovanović, 1953 (3.78%), *R. laevis* Pictet, 1834 (2.4%) and *R. fasciata* Hagen, 1859 (3%). *Rhyacophila balcanica* can be found mainly in springs and the upper parts of streams and rivers in southeastern Europe (ecoregions ER5, ER6, ER7; Malicky, 2005; Kučinić et al., 2011; Karaouzas et al., 2015; Krušnik, 1987; Radovanović, 1953), and because of its disjunct distribution between different populations we could well expect even higher intraspecific genetic variabilities between various populations. However, no regular or constant morphological differences among adults collected from various localities and populations have been determined. Similar data were obtained from analyses of the larvae collected from the Krka River in Croatia (Karaouzas et al., 2015).

In *R. fasciata* Hagen, 1859, unlike in *R. cabrankensis*, a significant morphological variability of the male genitalia was noted by Malicky & Sipahiler (1993) and Malicky (2004) for nominal species and 5 forms (subspecies) distributed in various parts of Europe and Asia (Malicky, 2004). In more recent research the subspecies (forma) kykladica Malicky & Sipahiler 1993 from Greece was given species rank (Valladolid et al., 2019), and similar taxonomic research has been conducted analysing populations from other parts of its distribution range, including Croatia (Valladolid et al., 2020, in press)). *Rhyacophila tristis* Pictet, 1834 was the extensively studied including morphological and genetic analyses; the results showed significant genetic differences between eastern (Carpathians) and western populations (Alps), but with no clear morphological differences (Bálint et al., 2011). Three specimens of *R. tristis* collected in the Konavle area in the south-easternmost part of Croatia exhibit intraspecific genetic distances in the range of 0.53% - 2.85% (Tab. 2).

In this study, two other interesting species from the family Rhyacophilidae were noted. One is *Rhyacophila laevis* Pictet, 1834 reported with one DNA barcoded specimen from the spring of Šumi in northwestern Croatia, which is 2.24% different from the specimen in the BOLD database (Tab. 2). The obtained values from just one DNA barcoded specimen are not enough for any conclusions to be made, but additional genetic and more extensive morphological analyses can be planned; however, we can assume that this is the case of intraspecific genetic variability of the COI genes in *R. laevis*. The record from the spring of Šumi on Mt Ivanščica is the second finding of *R. laevis* in the Pannonian-Peripannonian part of Croatia. So far, this species was reported from the Žumberačka Reka River in the western part of the Pannonian-Peripannonian region of Croatia (Čuk & Vučković, 2009) and in the spring of the Dobra River in the central-mountainous part of Croatia (Cerjanec, 2012 Previšić et al., 2012). Another species is *Rhyacophila torrentium* Pictet, 1834 recorded at the Zeleni Vir spring in the central mountainous part of Croatia. So far, this species was recorded only at the spring
of the River Kupa in the central-mountainous part of Croatia (Vučković et al., 2011). The specimen from Zeleni Vir spring matches data for R. torrentium from other parts of Europe in the BOLD database with high compatibility (99.54%) (Tab. 2).

Unlike in the mentioned families, higher degrees of genetic variability of the DNA barcoded region of the COI gene were noted in some species from the families Lepadostomatidae, Limnephilidae and Odontoceridae. For instance, a specimen of Crunoeinia kempnyi Morton, 1901 from the family Lepadostomatidae collected at the spring of the Napojsite stream in Plitvice Lakes National Park (central mountainous part of Croatia) differs considerably from the data contained in the BOLD database by 3.33% (Tab. 2) however, still indicating the intraspecific variability. Since this is a spring species with disjunct distribution, more detailed morphological analysis of the population from that locality in Plitvice Lakes National Park and comparison with other populations will be needed in the future. Plitvice Lakes is the only area in Croatia with records of this species, and the closest populations in Bosnia and Herzegovina are located more than 200 km away (Stanić-Koštroman et al. 2015).

During this research one interesting species, Allogamus auricollis (Pictet, 1834) from the family Limnephilidae was recorded with a higher level of genetic variability (Tab. 2), probably within intraspecific variability. The specimen of this species was collected at the spring of the Una River (Tab. 2) with a compatibility in the COI region of 96.83% with data from the BOLD database. This species is morphologically very variable (Malicky, 2004, 2016), and DNA barcoding confirmed its taxonomic affiliation. In this case DNA barcoding once again proved to be a useful tool for the identification of the taxonomic status of morphologically variable or similar species and confirmed the data of the Malicky study from 2016 (Malicky, 2016). In it, Malicky showed the morphology and distribution of two taxa: A. auricollis auricollis and A. auricollis braueri Kolenati, 1859. The nominal taxa were distributed in Central Europe (western and central Alps) while subspecies braueri is widespread in Europe including the Carpathians, Balkan Peninsula and British Isles (Malicky 2016). According to these data and DNA barcoding data from the current study, A. auricollis braueri is probably distributed in Croatia, which should be confirmed in future research. Allogamus auricollis is a rare species of the Croatian fauna and has been found so far only at the springs of the Una and the Dobra rivers in the central-mountainous part of Croatia (Cerjanc, 2012; Previšić et al. 2012).

A faunistically very interesting finding from the family Limnephilidae is Mesophylax aspersus (Rambur, 1842) in the Špilja spring, near the town of Rabac, in the Mediterranean part of Croatia, the second finding for this region (Malicky, 1979). M. aspersus was recorded for the first time in Croatia at the beginning of the 20th century on the island of Hvar with two collected specimens, deposited in the collection of Pater Gabriel Strobl in the Admont museum in Austria (Kučinić et al., 2019b; Malicky, 1979). Two DNA barcoded specimens of M. aspersus from the Špilja spring are compatible with the data of this species in the BOLD database with the high percentages of 99.38% and 100%, respectively (Tab. 2). The family Odontoceridae is represented in our fauna with one very common species, Odontocerum albicorne (Scopoli, 1763). One specimen of O. albicorne was collected from the spring of the Rudnica River, showing differences of 2.6% in the DNA barcoded region, which makes this finding interesting, although we can assume that it is the intraspecific genetic variability of O. albicorne.

All three mentioned species, C. kempnyi, A. auricollis and O. albicorne, should be studied further because of the differences obtained by DNA barcoding, having in mind...
the distribution, morphological and genetic characteristics of various populations of them in Europe.

Specimens within the families Glossosomatidae, Hydroptilidae, Philopotamidae, Polycentropodidae, Psychomyiidae, Hydropsychidae, Phryganeidae, Goeridae, Beraeidae and Leptoceridae that were DNA barcoded in this study indicate no large variations in comparison with corresponding species represented in the BOLD database (Tab. 2). In these families, including also the families Rhyacophilidae and Hydroptilidae, the DNA barcoding method has proved to be useful in confirming identifications of similar species (for example Hydropsyche), of small-sized specimens (for example family Hydroptilidae) or of females which could not be identified by morphology (for example genera in the families Hydrosychidae, Hydroptilidae, Psychomyiidae) (Málický, 2004) (Tab. 2).

Results from this study, in line with the results from previous faunistic research of Trichoptera in springs, proved to be interesting faunistically, taxonomically, phylogenetically and phylogeographically (for example Cianficconi et al., 1998; Ibrahimi et al., 2015; Kreiling et al., 2020; Kučinić et al., 2011, 2015; Málický et al., 2007; Pauls et al., 2006, 2009; Pauls et al., 2006; Previšić et al., 2009, 2014; Vitecek et al. 2015, 2017; Waringer et al., 2013, 2016), and it is to be expected that the research will continue and result in new valuable results.

Springs are globally, and not only in Croatia, subjected to a great deal of anthropogenic influence (for example Kučinić et al., 2015b; Vitecek et al. 2015, 2017) which ranges from low-impact to completely destructive. From the 36 springs included in this research, anthropogenic influence is visible in 13 of them, i.e. in 34% (Tab. 2). Water protection today is very important but also it is one of the key segments in protecting Earth’s biodiversity, with springs having an essential role on the global level. By protecting springs, we protect the best resources of drinking water, and their biodiversity, which is unique in most of its characteristics (e.g. endemic, rare species etc.).

CONCLUSION

DNA barcoding shows its value in its ability to reveal the species sets of certain areas or habitat types, in this case of springs, by an approach different from the morphological methodology (for example Čerjanec, 2012; Kučinić et al., 2011; Previšić et al. 2012; Waringer et al., 2009), analysing genetic characteristics of each analysed specimen, or species (for example Kučinić et al., 2019a; Szivák et al., 2017). Results obtained by this approach are very interesting either because they differ at the species level in different populations, or because they are a 100% match with analyzed specimens from different populations. Both examples in their own way show characteristics of the analyzed specimens, populations and species, i.e. the fauna of the particular area. This is the very reason that DNA barcoding of the Croatian fauna will continue in the future. On one hand, a better scientific presentation of the biodiversity is needed, and on the other, we need it to be efficiently protected. DNA barcoding of organisms – here Trichoptera from Croatian springs – is an additional contribution to the knowledge on this aspect of biodiversity, not only locally, but also as a part of global processes (for example Brehm et al., 2019; Dela Cruz et al., 2016; Hebert et al., 2003a, 2003b; Huemer et al., 2020; Léger et al., 2020; Kučinić et al., 2013; Morinière et al., 2017; Ratnavickingham & Hebert, 2007; Santos et al. 2016; Tyagi et al., 2017; Vaglia et al., 2008; Yang et al., 2015; Zhou et al., 2016).
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Appendix 1. List of specimens used in the phylogenetic analysis of *Rhyacophila cabrankensis* and *R. vulgaris* in this study, showing life stage, origin, BOLD Sequence ID number, specimen ID, number of unique haplotypes. Specimens which genomic DNA extracted in this study are written in bold letters. Abbreviation used: ID = Identification number, BOLD = Barcode of Life data system, A = adult, M = male, F = female, No. = number.

| Country       | Location                                      | Specimen ID | BOLD Sequence ID | Life stage |
|---------------|-----------------------------------------------|-------------|------------------|------------|
| Croatia       | spring of the River Čabranka                  | TRCAB_1     | CROAA089-18      | A          |
| *Rhyacophila cabrankensis* Malicky, Previšić & Kučinić, 2007 |
| Austria       | St. Konrad - Hausern                         | HMKKT584-10 | 10HMCAD-584      | -          |
| Austria       | St. Konrad - Hausern                         | HMKKT964-11 | HMCAD0111-147    | A          |
| Austria       | Rohrwiesteich                                | BHMKK208-12 | 12HMCAD-042      | A          |
| Austria       | St. Konrad - Hausern                         | HMKKT938-11 | HMCAD0111-121    | A          |
| Croatia       | creek Jankovac                               | TAFUR_1     | CROAA002-18      | A          |
| Germany       | Oberallgaeu: Baeche oh Grasgehren-Azw. Balderschw. | GBMIX1704-15 | GBOL12189       | A          |
| Germany       | Isar km 247, Hoehe Wallgau                   | FBAQU377-09 | BC ZSM AQU 00377 | A          |
| *Anabolia furcata* Brauer, 1857 |
| Austria       | Seeausrinn bei Lunz                          | HMKKT329-10 | 10HMCAD-329      | A          |
| Austria       | Rankweil: Weitried/ Landesforstgarten        | HMKKT330-10 | 10HMCAD-330      | A          |
| Austria       | Flexenpass                                    | INTAP217-17 | PE256            | A          |
| Austria       | Salzburg City, Thumegger Bezirk              | KJTRI121-13 | 12HMCAD-131      | A          |
| Croatia       | Kupčina, upper part, Vrabac                  | TRVUL_1     | CROAA031-18      | A          |