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

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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*, *Crunoecia 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 cabrakenensis*

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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 barkodirano je 70 vrsta iz 32 roda i 15 porodica. U studiji se ukazuje na neke zanimljive faunističke i taksonomske rezultate, npr. za vrste *Rhyacophila cabrankensis*, *R. balcanica*, *Crunoecia kempnyi*, *Allogmaus auricolli*s 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 a*l., 2003**)**, crustaceans (for example Glazer, 1998; Sidorov *et al*., 2012, 2018), aquatic insects (for example Graf *et al*., 2012; Ivković *et al*., 2020; Maiolini *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 divisons 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, the

central mountainous in the middle and the Mediterranean in the south (Fig. 5). Nine springs are in the Panonnian-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; Vilenica *et al*., 2015). The Black Sea Basin encompasses streams from the Panonnian-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 (TGATTYTTYGGYCACCCWGAAGTTTA), TM4 HCOI (TGATTYTTYGGRCACCCWGAAGTTTA) 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-

Fig. 1. Pašina vrela spring.

Fig. 2. Spring of the Una River.

Fig. 3. Spring of the Zrmanja River.

Fig. 4. Glavaš spring of the Cetina River.

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 included also 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 TA-VAR_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 (limnocrene spring), R (rheocrene spring) (according to Habdija & Primc, 2019); BR (biogeographical regions of Croatia): PP (Pannonian-Peripannonian part), CM (central mountainous part), ME (Mediterranean part) (according to Bertić *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	ER11	BS	45,51875	17,68664
2.	spring Škodinovac (Mt Papuk)	R	PP	ER11	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 Bjelolasica)	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 Napojište 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 Štirovača• (Mt Velebit)	R	CM	ER5	BS	44,69808	15,04992
21.	spring of the Čabranka River	R	CM	ER5	BS	45,60104	14,64079
22.	spring of the Rječina 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 Čerišnjevica	R	ME	ER5	AS	45,281389	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 River 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 Vodovađa•	R	ME	ER5	AS	42,51763	18,42215

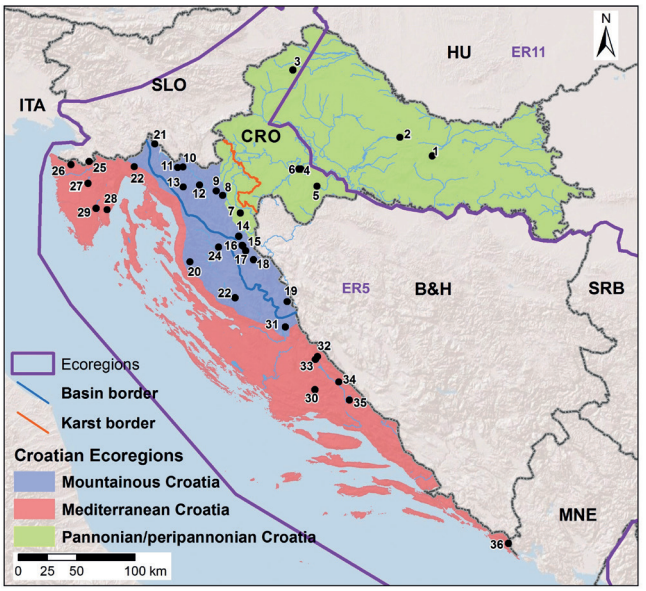

Fig. 5. Map of Croatia showing springs where caddisflies were collected; the numbers correspondns to those in Tab. 1, Ecoregions according to Illies (1978) ER5 – Dinaric Western Balkan, ER11 – Hungarian Lowlands, Basin border divides into the Black Sea Basin (eastern part) and Adriatic Sea Basin (western part).

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 Hebert *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; Ibrahimi *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,

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) (*=*Rhyacophila cabrakenensis*, **=*Glososoma discophorum*, ***=*Hydroptila phaon*, ****=*Psychomyia klapaleki*, *****=*Tinodes antonioi*, ******=*Annitella apfelbecki*, *******=*Drusus croaticus*, ********=*Micropterna wageneri)* (Ćukušić, 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				
Rhyacophila balcanica	TRBAL_1	spring of the Una River	CROTR256-19	*Rhyacophila balcanica* 96.24%
Rhyacophila cabrankensis	TRCAB_1	spring of the River Čabranka	CROAA089-18	*Rhyacophila vulgaris* (97.61%)*
Rhyacophila dorsalis	TRDOR_2	spring of the River Cabranka	CROAA060-18	*Rhyacophila dorsalis* (100%)
Rhyacophila cf. *fasciata*	TRFAS_1	spring Zeleni Vir	CROTR264-19	*Rhyacophila fasciata* (97%)
Rhaycophila laevis	TRLAE_1	spring of the Šumi stream	CROTR266-19	*Rhacophila laevis* (97.76%)
Rhyacophila torrentium	TRTOR_1	spring Zeleni Vir	CROAA018-18	*Rhyacophila torrentium* (99.54%)
Rhyacophila tristis	TRTRI_4	spring in Vodovađa village	CROAA098-18	*Rhyacophila tristis* (97.15%)
Rhyacophila tristis	TRTRI_5	spring in Vodovađa village	CROTR011-19	*Rhyacophila tristis* (99.47%)
Rhyacophila tristis	TRTRI_7	spring in Vodovađa village	CROTR031-19	*Rhyacophila 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	THPHA_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				
Philopotamus montanus	TPMON_2	spring of the Šumi stream	CROAA130-18	*Philopotamus montanus* (99.84%)
Wormaldia copiosa	TWCOP_2	spring of the River Čabranka	CROAA044-18	*Wormaldia copiosa* (99.84%)
Wormaldia occipitalis	TWOCI_4	spring of the Napojište stream	CROTR068-19	*Wormaldia occipitalis* (99.20%)
Wormaldia occipitalis	TWOC_3	spring Škodinovac	CROTR061-19	*Wormaldia occipitalis* (99.67%)
Wormaldia occipitalis	TWOCI_6	spring Bijela stijene	CROTR245-19	*Wormaldia occipitalis* (99.37%)
Wormaldia subnigra	TWSUP_2	spring Cerišnjevica	CROTR099-19	*Wormaldia subnigra* (99.52%)
Family Polycentropodidae				
Cyrnus trimaculatus	TCTRI_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)	CROTR008-19	*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				
Lype cf. *reducta*	TLRED_3	spring Cerišnjevica	CROTR081-19	*Lype reducta* (97.97%)
Psychomyia klapaleki	TPKLA_1	spring of the River Vitunjčica	CROAA038-18	*Psychomyia morisitai* 86.41, *Paduniella* 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 waeneri	TTWAE_1	spring in Marušići village	NIP001-16	*Tinodes waeneri* (99.69%)
Family Hydropsychidae				
Hydropsyche instabilis	THINS_1	spring of the River Vitunjčica	CROAA052-18	*Hydropsyche instabilis* (100%)
Hydropsyche instabilis	THINS_4	spring of the River Rječina	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 saxonica	THSAX_2	spring of the Vrba stream	CROTR149-19	*Hydropsyche saxonica* (100%)
Family Phryganeidae				
Agrypnia varia	TAVAR_2	spring of the River Grab ☼	CROTR078-19	*Agrypnia varia* (99.84%)
Trichostegia minor	TTMIN_1	spring Majerovo vrilo	CROAA133-18	*Trichostegia minor* (98.93%)
Family Goeridae				
Silo pallipes	TSPAL_1	spring Bračana (Mlini)	CROTR287-19	*Silo pallipes* (98.83%)
Silo pallipes	TSPAL_3	spring of the River Slunjčica	CROTR065-19	*Silo pallipes* (98.87%)
Family Lepidostomatidae				
Crunoecia kempnyi	TCKEM_1	spring of the Napojište stream	CROTR074-19	*Crunoecia kempeny* (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 esparraguera* 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 schmidi	TDSCH_1	spring Jankovac	CROAA021-18	*Drusus schmidi* (100%)
Drusus vespertinus	TDVES_1	spring of the River Una	CROTR275-19	*Drusus vespertinus* (97.99%)
Ecclisopteryx ivkae	TEIVK_1	spring Glavaš (Cetina river)	CROAA106-18	*Ecclisopteryx ivkae* (100%)
Glyphotaelius pellucidus	TRBAL_3	spring Nela (Cetina river)	CROTR064-19	*Glyphotaelius pellucidus* (99.36%)
Glyphotaelius pellucidus	TGPEL_4	spring of the Napojište 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	*Limnephilus marmoratus* (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)	CROTR154-19	*Stenophylax lateralis* (100%)
Micropterna nycterobia	TMIC_1	spring of the River Zrmanja	NIPM003-17	*Micropterna nycterobia* (98.89%)
Micropterna nycterobia	TMNYC_2	spring Keljevac	CROTR016-19	*Micropterna nycterobia* (100%)
Micropterna sequax	TMIC_2	spring of the River Una	NIPM004-17	*Micropterna sequax* (98.51%)
Micropterna testacea	TMTES_3	spring Majerovo vrilo (River Gacka)	CROTR028-19	*Micropterna testacea* (100%)
Micropterna wageneri	TPWAG_1	spring in the village Vodovađa	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	CROTR048-19	*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)	CROTR047-19	*Odontocerum albicorne* (97.4%)
Family Beraeidae				
Beraea pullata	TBPUL_1	spring of the Napojište stream	CROTR080-19	*Beraea pullata* (99.84%)
Family Leptoceridae				
Athripsodes bilineatus	TABIL_1	spring Pašina vrela	CROAA012-18	*Atripsodes bilineatus* (100%)
Athripsodes cinereus	TACIN_3	spring of the River Lika (Mt Velebit)	CROTR049-19	*Athripsodes cinereus* (99.63%)
Oecetis notata	TONOT_2	spring Majerovo vrilo (River Gacka)	CROTR072-19	*Oecetis notata* (99.84%)
Oecetis testacea	TOTES_4	spring Zeleni vir	CROTR165-19	*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, *Psychmia 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 (Ćukušić, 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; Graf *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.

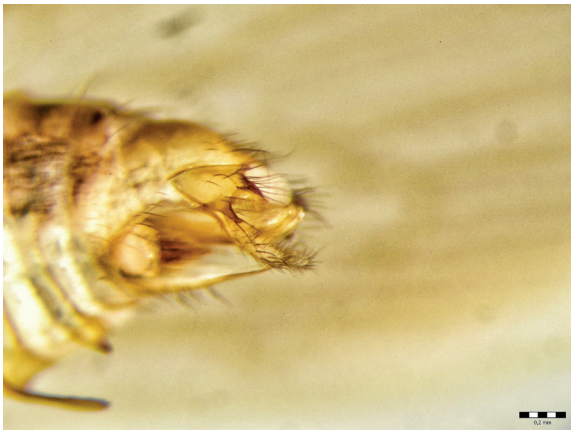

Fig. 6. *Glossosoma discophorum* Klapálek, 1902. Male genitals, lateral view, collected at the spring of the Una River.

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. cabrankensis* 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 *Rhycophila cabrankensis* Malicky, Previšić & Kučinić 2007, collected in the spring of the Čabranka River (photo M. Kučinić).

Fig. 8. *Rhyacophila cabrankens*is Malicky, Previšić & Kučinić, 2007, male genitalia, lateral view, left side (photo M. Kučinić).

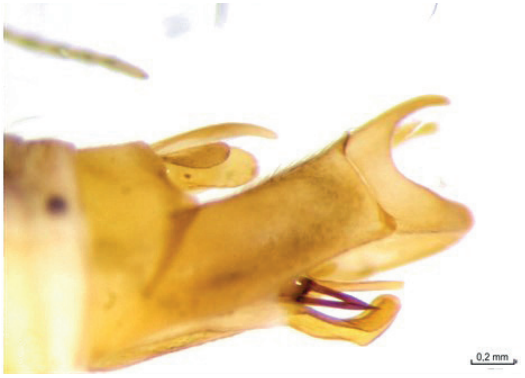

Fig. 9. *Rhyacophila vulgaris* Pictet, 1834, male genitalia, lateral view, left side (photo A. Čukušić).

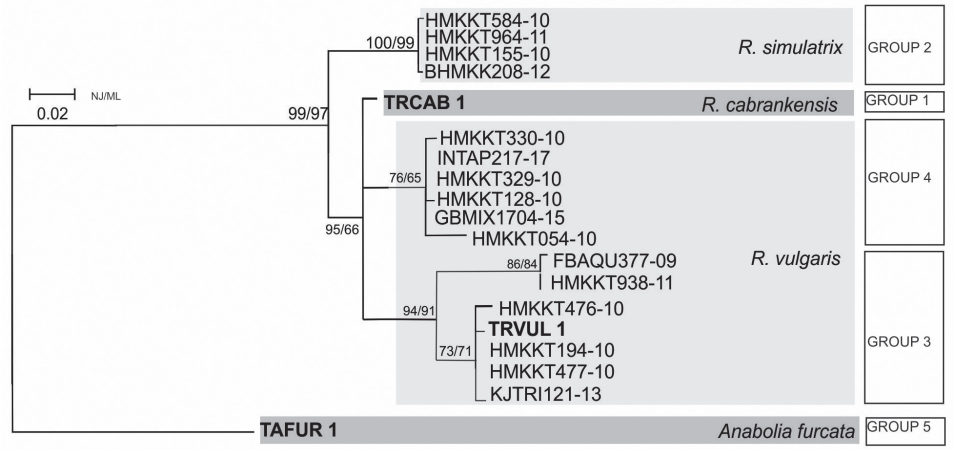

Fig. 10. Maximum likelihood (ML) phylogram based on a 658 bp long fragment of the DNA barcode region showing the relationships between *Rhyacophila* species. Numbers above the branches represent bootstrap support (BS) for Neighbor-Joining (NJ) and ML analysis (NJ/ML). The groups delineated by the Automatic 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).

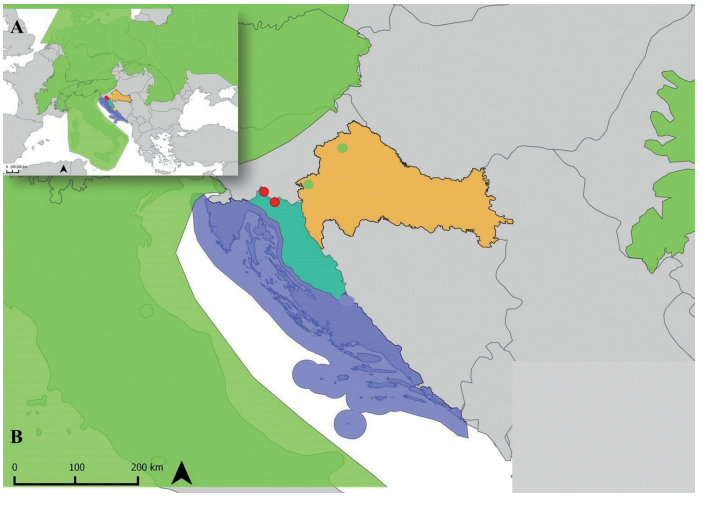

Fig. 11. Records of *R. cabrankensis* (red dots) and *R. vulgaris* (green dots) in Croatia with regions according to Bertić *et al*. (2001) (dark green – mountains, orange – Panonnian-peripanonnian and blue – Mediterranean region) and distribution of *R. vulgaris* in Europe (green field) according to 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	0-0.3	-
R. vulgaris	1.8	6.4	0-2.3
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, 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 (Balint *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 (Ćuk & 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 Lepidostomatidae, Limnephilidae and Odontoceridae. For instance, a specimen of *Crunoecia kempnyi* Morton, 1901 from the family Lepitostoatidae collected at the spring of the Napojište 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, amore 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 auricolli*s (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. auricolis 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. auricoliis 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 (Cerjanec, 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 variabilities 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) (Malicky, 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; Malicky *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 t he 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 Cerjanec, 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 specimes, 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; Ratnasingham & 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 cabrakensisi* 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
*Rhyacophila cabrankens*is Malicky, Previšić & Kučinić, 2007				
Croatia	spring of the River Čabranka	TRCAB_1	CROAA089-18	A
Rhyacophila simulatrix McLachlan, 1879				
Austria	St. Konrad -Hausern	HMKKT584-10	10HMCAD-584	-
Austria	St. Konrad -Hausern	HMKKT964-11	HMCAD0111-147	A
Austria	Rohrwiesteich	BHMKK208-12	12HMCAD-042	A
France	Mercantour NP, Saorge E	HMKKT155-10	10HMCAD-155	A
Rhyacophila vulgaris Pictet, 1834				
Austria	Rankweil: Weitried/ Landesforst-garten	HMKKT054-10	10HMCAD-054	A
Austria	Klostertal, Nenzigast Alpe	HMKKT128-10	10HMCAD-128	A
Austria	Seeausrinn bei Lunz	HMKKT194-10	10HMCAD-194	A
Austria	Rankweil: Weitried/ Landesforst-garten	HMKKT329-10	10HMCAD-329	-
Austria	Rankweil: Weitried/ Landesforst-garten	HMKKT330-10	10HMCAD-330	-
Austria	Seeausrinn bei Lunz	HMKKT476-10	10HMCAD-476	-
Austria	Seeausrinn bei Lunz	HMKKT477-10	10HMCAD-477	-
Austria	St. Konrad-Hausern	HMKKT938-11	HMCAD0111-121	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
Germany	Oberallgaeu: Baeche oh Gras-gehren-Azw. Balderschw.	GBMIX1704-15	GBOL12189	A
Germany	Isar km 247, Hoehe Wallgau	FBAQU377-09	BC ZSM AQU 00377	A
Anabolia furcata Brauer, 1857				
Croatia	creek Jankovac	TAFUR_1	CROAA002-18	A