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FIRST RECORD OF *CYRNUS CRENATICORNIS* (KOLENATI, 1859) (INSECTA, TRICHOPTERA, POLYCENTROPODIDAE) IN CROATIA: MORPHOLOGICAL DETERMINATION AND DNA BARCODING

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The caddisfly species *Cyrnus crenaticornis* (Kolenati, 1859) was recorded for the first time in Croatia in the Odra River during August 2015. The record refers to a larval stage which was determined according to morphological characteristics and supported by DNA barcoding.

Key words: caddisflies, larva, new record, Odra River, continental Croatia

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Tular *Cyrnus crenaticornis* (Kolenati, 1859) je po prvi puta utvrđen u Hrvatskoj u rijeci Odri u kolovozu 2015. godine. Vrsta je utvrđena na temelju morfološke determinacije ličinke, a potvrđena je primjenom metode DNA barkodiranja.

Ključne riječi: tulari, ličinka, novi nalaz, rijeka Odra, kontinentalna Hrvatska

INTRODUCTION

One of the most frequent groups of benthic macroinvertebrates in running freshwater ecosystems is Trichoptera (caddisflies). They inhabit almost every type of habitat, but their biodiversity is greatest in streams and small rivers (Wallace *et al*., 1990). The Trichoptera World Checklist counts 16,267 species in 632 genera of 63 families, including 521 fossil species, 133 fossil genera and 20 fossil families (Morse, 2021). The Western Palearctic, which includes Europe, contributes with 13.9%, or 1,888 species. In Croatia, caddisflies are among the best studied orders of insects, with a certain amount of literature; however, in some cases lack of georeferenced data or sampling data as well as of information on the depository, decreases the value of these data. Systematic studies of Trichoptera based on adults started relatively recently in Croa-

tia with fieldwork in the Plitvice Lakes National Park (e.g. Kučinić, 2002; Kučinić & Malicky, 2002; Kučinić *et al*., 2017; Marinković-Gospodnetić, 1971, 1979; Previšić *et al*., 2007a, 2010) and later in different, often restricted parts of Croatia e.g. the Krka River (Kučinić *et al*., 2011, 2019; Ridl *et al*., 2015; Valladolid *et al*., 2020), the Cetina River catchment area (Graf *et al*., 2008a; Previšić *et al*., 2014; Vučković *et al*., 2011, 2021; Waringer *et al*., 2009), the Drava River (Previšić *et al*., 2007b), Gorski kotar area (Cerjanec *et al*., 2020; Malicky *et al*., 2007; Previšić & Popijač, 2010), Banovina area (Kučinić *et al*., 2010, 2020b), Mt Papuk (Previšić *et al*., 2013), and some individual researches in the inland part of Croatia (e.g. Ćukušić *et al*., 2017; Kučinić *et al*., 2020b; Malicky & Krušnik, 1988; Malicky, 2009, 2014; Oláh, 2011; Szivák *et al*., 2017; Vrućina *et al*., 2016). On the other hand, studies on caddis larvae are much more scarce due to the lack of experts, and beside the description sometimes having to deal with previously unknown larvae (e.g. Graf *et al*., 2008a; Karaouzas *et al*., 2015; Kučinić *et al*., 2008; Previšić *et al*., 2014; Waringer *et al*., 2009, they mostly originate from studies related to the biomonitoring of surface water quality that report new records (Ćuk & Vučković, 2009, 2010, 2014; Ćuk *et al*., 2015). Sometimes they will be found as a part of the macrozoobenthic community (Habdija & Primc, 1987; Habdija *et al*., 1994, 1997, 2000, 2002, 2003, 2004; Matoničkin & Pavletić, 1961, 1965; Matoničkin *et al*., 1969, 2001; Rađa & Puljas, 2010).

DNA barcoding is a molecular method based on sequencing of the barcode region (658 bp in length) of the cytochrome c oxidase subunit I gene (*COI*) to aid species identification (Hebert *et al*., 2003a, 2003b; Ratnasingham & Hebert, 2007) and has significantly contributed to the knowledge of biodiversity, taxonomy and phylogeny of different groups of organisms (e.g. Amora *et al*., 2015; Bączkiewicz *et al*., 2017; Cárdenas *et al*., 2013; Dela Cruz *et al*., 2016; Elías-Gutiérrez *et al*., 2008, Kučinić *et al*., 2020a, Morinière *et al*., 2017, Vijayan & Tsou, 2010). This method is very useful for the determination of morphologically very similar species, and also the finding of cryptic species (e.g. Kučinić *et al*., 2013; Tyagi *et al*., 2017; Vaglia *et al*., 2008; Valladolid *et al*., 2020). According to Bączkiewicz *et al*. (2017): "DNA barcoding is a highly useful method for identifying taxonomically difficult species". In the last few years, the DNA barcoding method has been applied in the biodiversity research of Trichoptera in Croatia, including faunistic (e.g. Cerjanec *et al*., 2020; Ćukušić *et al*., 2017; Kučinić *et al*., 2020b; Vučković *et al*., 2021) and taxonomic research (Kučinić *et al*., 2013, 2020a; Valladolid *et al*., 2020).

This paper presents the first record of *Cyrnus crenaticornis* (Kolenati, 1859) in Croatia, the presence of which is confirmed with application of DNA barcoding.

MATERIAL AND METHODS

Research area. The Odra River is situated in the Hungarian lowland ecoregion (Pannonian) (ER11) (Illies, 1978) and belongs to the catchment area of the Sava River. It is an inland river and is 45.5 km long. According to national typology, the Odra River is classified among "Medium and large lowland rivers" (HR-R_4) (Official Gazette, 2013). The study site on the Odra River is located at the settlement of Čička Poljana (N45°40'26,9'', E16°10'36,8'') (Fig. 1a). The dominant substrate was psammopelal with 100% coverage of submerged and emerged macrophyte in the littoral zone where the sample was taken (HRN EN 16150, 2012) (Fig. 1b).

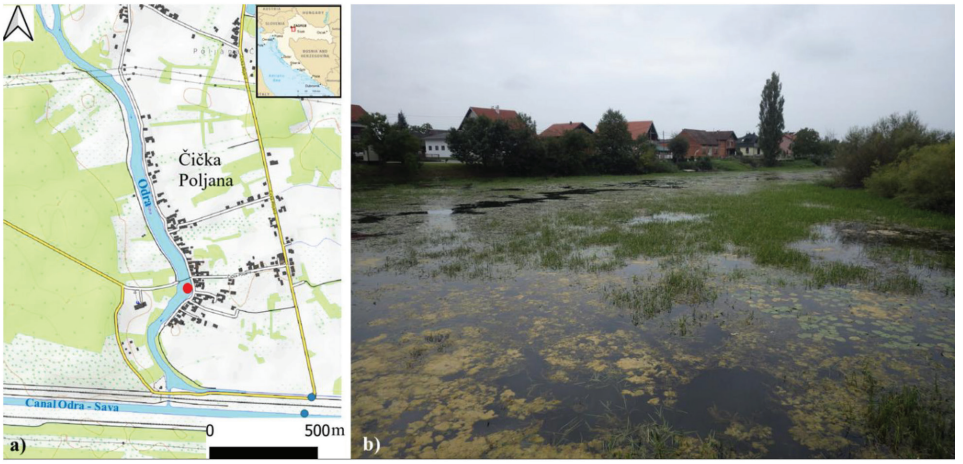

Fig. 1. Distribution of *Cyrnus crenaticornis* in Croatia: **a**) record in Croatia (red spot) with a detail of the study area; **b**) the Odra River at Čička Poljana

Sampling and laboratory work. Sampling of benthic macroinvertebrates was conducted on August 27th 2015 using a hand net with a mesh size of 500 µm according to the AQEM sampling protocol (AQEM Consortium, 2002). The collected material was preserved in ethanol in the field so the final concentration was approximately 70%. Isolation and determination of benthic macroinvertebrates were done in the laboratory using a binocular stereomicroscope (Olympus SZX10). Additional larvae (3 specimens) were collected on June 13th 2021 and stored in absolute ethanol. For species determination the keys of Waringer & Graf (2011) and Lechthaler & Stockinger (2007) were used. All specimens have been deposited in the collection of caddisflies in the Central Water Management Laboratory of Hrvatske vode.

Water samples were collected monthly in 2015 at the study site and the following physical-chemical parameters were analysed according to standard analytical methods for assessment of surface water quality (ISO norms): pH, biological oxygen demand (BOD_5) (mgO_2/l), chemical oxygen demand (COD-Mn) (mgO_2/l), ammonia (NH_4^+) (mgN/l), nitrates (NO_3^-) (mgN/l), total nitrogen (mgN/l), ortophosphates (PO_4^{3-}) (mgP/l), total phosphorus (mgP/l).

DNA extraction, amplification and sequencing. DNA barcoding was performed using one of the newly collected larvae in 2021. Total genomic DNA was extracted from two legs (1 larva) using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) following the manufacturer's protocol and eluted in 50 µL of elution buffer. The standard DNA barcode region (658 bp) of the mitochondrial cytochrome c oxidase subunit I gene (*COI*) was amplified with the use of standard PCR-protocol and universal primer pair LCO-1490/HCO-2198 (Folmer *et al*., 1994) in 20 µL reaction mixture. Polymerase chain reactions (PCRs) were carried out using: 1 x DreamTaq™ reaction buffer with 2 mM $MgCl_2$ (Thermo Fisher Scientific Inc., US), 0.2 mM dNTPs, 0.4 µM of each primer, 0.025 U/µL of DreamTaq polymerase (Thermo Fisher Scientific Inc., US) and 1 µl of eluted DNA. The PCR cycling protocol included: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C

for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Purification and sequencing were performed by Macrogen Inc. (Amsterdam, Netherlands) using the same amplification primers. Sequence obtained in this study were deposited in the Barcode of Life Database (Ratnasingham & Hebert, 2007) under the accession number CROTR362-21.

Sequence data and phylogenetic analysis. Sequence was checked, edited, assembled from both directions, and inspected manually for base pair ambiguities, as well as stop codons, indels or double peaks in chromatograms in Geneious R6 (https://www.geneious.com). All available *Cyrnus* sequences were retrieved from the GenBank and BOLD database (accessed on July 20th 2021) and aligned with the sequence from this study using MAFFT v.7 (Katoh & Standley, 2013). Sequences were collapsed into 42 unique *COI* haplotypes using the online tool FaBox v.1.5 (Villesen, 2007). The most diverse haplotypes were included in further analysis and the final data set for phylogenetic analysis comprised 22 sequences. *Limnephilus flavicornis* (CROAA008-18) was selected as outgroup. Uncorrected *p*-distances between haplotypes were calculated using MEGA-X (Kumar *et al.*, 2018). BOLD Identification Engine (accessed on July 20th 2021) was used for comparison of obtained DNA sequence with sequences available in BOLD database. BOLD IDs and accession numbers for all specimens included in final data set are given in Tab. 1. Phylogenetic analysis was performed in MEGA-X (Kumar *et al.*, 2018) and phylogenetic relationships were estimated by two different optimality criteria: neighbour joining (NJ) and maximum likelihood (ML). NJ was made using the Kimura-2-parameter (K2P) model of nucleotide substitution with pairwise deletion option and the robustness of the clades was assessed through 5000 bootstrap replicates. For ML the optimal model of nucleotide evolution (GTR+I) was selected under the Bayesian information criterion (BIC) using jModelTest 2.1.5 (Darriba *et al.*, 2012). Nearest-Neighbour-Interchange (NNI), a heuristic method using the fast bootstrap algorithm, was used in ML with 2000 replicates.

RESULTS AND DISCUSSION

Twenty-one (21) larval specimens of *C. crenaticornis* (Fig. 2a) were documented in the Odra River at Čička Poljana during August 2015 and three (3) specimens in June 2021, representing thus the first record of this species in Croatia. According to Waringer & Graf (2011) the characteristics of the larva are following: basal segment of anal proleg has numerous bristles, with short spines lacking from the ventral side of the ninth abdominal segment (Fig. 2b), inner apex of anal claw has four blunt teeth (Fig. 2c), the transverse row of dark spots is situated within the pale central frontoclypeal area, posterior angle of frontoclypeus without pale spot and pale patch at the center of frontoclypeus without dark anterior border (Fig. 2d).

Molecular analysis based on the obtained sequence of the DNA barcode region (658 bp long) confirmed the morphological identification and identified the obtained sequence as *Cyrnus crenaticornis*. Uncorrected *p*-distance to the single *C. crenaticornis* sequence (specimen with sampling site in Denmark) available in BOLD database is 0.0001.

The topology of NJ and ML trees was congruent, with only a few weakly supported nodes (Fig. 3). Sequences of *C. crenaticornis* group together in a 100% BS-supported clade, with *C. crenaticornis* being recovered as sister to *C. fennicus*.

Fig. 2. *Cyrnus crenaticornis*, a) larva; b) tip of abdomen, ventral view; c) anal claw; d) head, dorsal view

Tab. 1. Specimens and sequences used in the analysis. Newly obtained sequence is marked in bold.

Species name	Country	Sample ID	BOLD sequence ID
Cyrnus trimaculatus	Croatia	TPFLA_2	CROAA129-18
	Germany	GBOL00309	FBAQU1465-13
		BC ZSM AQU 00348	FBAQU348-09
	Belgium	UA-SG-TRICH-C46	TFLAN135-11
	Netherlands	MK093958	GBMNA42546-19
	Germany	08JPCAD-068	JPCAD068-08
		08JPCAD-069	JPCAD069-08
Cyrnus crenaticornis	Croatia	**CC1A**	**CROTR362-21**
	Denmark	JSlk-2013F077	TRIFI1002-13
Cyrnus fennicus	Japan	08JPCAD-081	JPCAD081-08
		08JPCAD-083	JPCAD083-08
Cyrnus insolutus	Sweden	JQ239776	GBMIN18566-13
	Finland	ARin-2011F193	TRIFI733-12
Cyrnus nipponicus	Japan	08JPCAD-074	JPCAD074-08
		08JPCAD-075	JPCAD075-08
Cyrnus flavidus	Finland	JSlk-20090083	TRIFI188-10
		JSlk-20090084	TRIFI189-10
	Norway	BI2019_E07	STUBA007-12
	Germany	BC ZSM AQU 00109	FBAQU109-09
	Norway	FinnCAD-003	FINNT046-12
		TRD-TRI4	ODTRI022-14

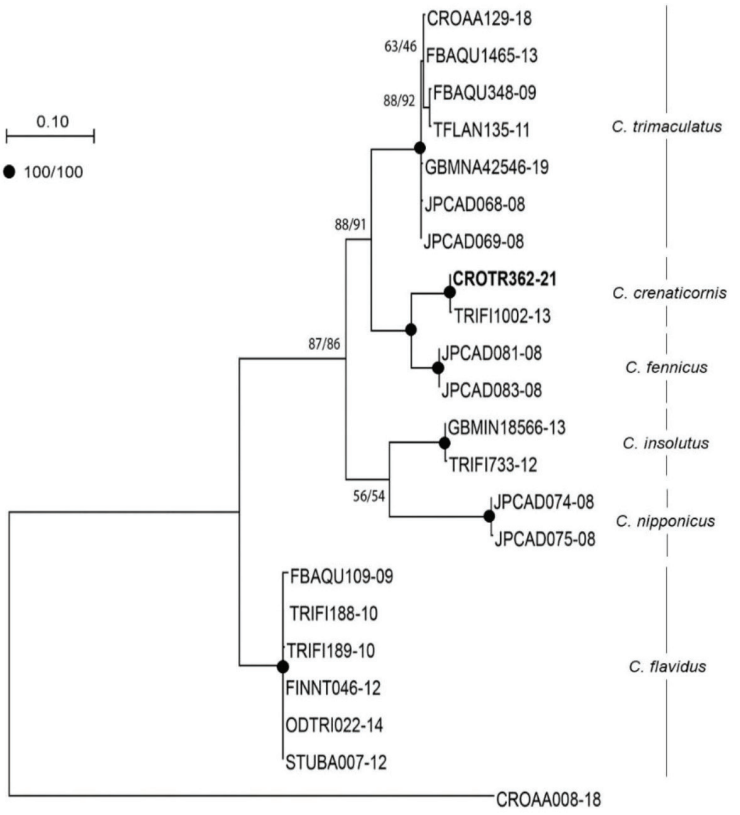

Fig. 3. Maximum likelihood phylogenetic tree based on the *COI* sequence of *Cyrnus crenaticornis* from Croatia and haplotypes of *Cyrnus* species from BOLD database. Numbers at the nodes indicate neighbour joining (NJ) and maximum likelihood (ML) ultrafast bootstrap support values (BS). Terminal codes present BOLD Process ID, as in Tab. 1.

According to Graf *et al.* (2008b), Malicky (2004, 2013) and Morse (2021) seven (7) species of the genus *Cyrnus* are present in Europe: *C. cintranus* McLachlan, 1884, *C. crenaticornis* (Kolenati, 1859), *C. fennicus* Klingstedt, 1937, C. *flavidus* McLachlan, 1864, *C. insolutus* McLachlan, 1878, *C. monserrati* Gonzalez & Otero, 1983 and *C. trimaculatus* (Curtis, 1834), the last of which has been recorded in Croatia relatively frequently, in the Pannonian-Peripannonian, Central-mountain and Mediterranean areas (e.g. Cerjanec *et al*., 2020; Kučinić *et al*., 2017, 2020b; Vučković *et al*., 2021). However, the distribution of *C. crenaticornis* in Europe (Malicky, 2013) indicates that although the species could have been expected to occur in Croatia (see Fig. 4), no previous records existed. The species has a wide range of distribution, occurring mostly in the littoral zone of standing waters (above 18°C) usually on living plants, mainly on macrophytes, very rare on algae; prefers lower altitudes mainly plains (<300m) but also 300-800 m (Graf *et al*. 2008b). The sampling site on the Odra River completely fits in the above-mentioned ecological preferences of the species, as the record refers to the littoral zone of a very slow flowing watercourse, on macrophyte vegetation.

We present the taxa list of the most common species in the benthic community found at this site: mayflies (Ephemeroptera): *Caenis* sp. and *Cloeon dipterum* (Lin-

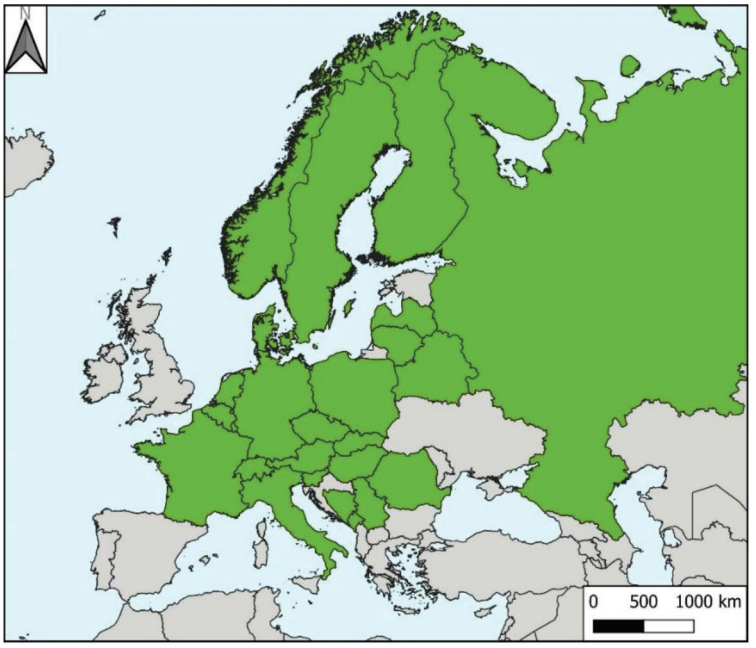

Fig. 4. Distribution of *C. crenaticornis* in Europe (Malicky, 2013)

naeus, 1761), beetle (Coleoptera) *Haliplus* sp., leach (Hirudinea) *Erpobdella octoculata* (Linnaeus, 1758), snail (Gastropoda) *Gyraulus* sp. Caddisflies recorded at the sampling site were *Athripsodes* sp. and *Leptocerus tineiformis* (Curtis, 1834). Macroinvertebrate assemblage at the study site indicates good water status regarding saprobity module, however, regarding general degradation module, the study site is classified into poor water status and therefore does not meet the Water Framework Directive (WFD) criteria.

According to the basic physical-chemical parameters at the study site (Tab. 2) the water does not meet the WFD criteria due to the increased concentrations of nitrates and total nitrogen. Other physical-chemical parameters investigated indicate high status (Official Gazette, 2013).

Tab. 2. Annual (n = 12) median value of basic physical-chemical parameters in the Odra River at Čička Poljana in 2015 (the associated colour corresponds to the water status according to national methodology; blue = high status; green = good status; yellow = below good status)

physical-chemical parameter	median value
pH	7,85
BOD_5 (mgO_2/l)	1
COD-Mn (mgO_2/l)	1,7
Ammonia (mgN/l)	0,0355
Nitrates (mgN/l)	2,105
Total N (mgN/l)	2,53
Ortophosphates (mgP/l)	0,0095
Total P (mgP/l)	0,034

The Trichoptera fauna of Croatia counts approximately 210 species (e.g. Cerjanec *et al*., 2020; Ćuk & Vučković, 2009, 2010, 2014; Ćuk *et al*. 2015; Kladarić *et al*., 2021; Kučinić *et al*., 2019, 2020a; Malicky & Krušnik, 1988; Malicky *et al*., 2007; Malicky, 2009, 2014; Marinković-Gospodnetić, 1971, 1979; Olah, 2011; Previšić *et al*., 2013, 2014; Vučković *et al*., 2021), most of which have been determined morphologically based on adult specimens, and recently sometimes additionally with the application of DNA barcoding (e.g. Cerjanec *et al*., 2020; Ćukušić *et al*., 2017; Kučinić *et al*., 2013, 2019, 2020a, 2020b; Szivák *et al*., 2017; Valladolid *et al*., 2020; Vučković *et al*., 2021). New records determined on the basis of morphological characteristics of larvae are rare (e.g. Ćuk & Vučković, 2009, 2010, 2014; Ćuk *et al*., 2015), not only due to the lack of expert knowledge and determination keys, but also due to a certain number of larvae expected to occur in Croatia based on their area of distribution not having been described. The national monitoring programme of surface water quality promises to result in new records, as caddisfly larvae are part of the benthic macroinvertebrate assemblage that are sampled regularly at a large number of sampling stations all over the country. Therefore, more attention should be given to larvae in general, as they might provide valuable information.

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SAŽETAK

Prvi nalaz tulara *Cyrnus crenaticornis* (Kolenati, 1859) (Insecta, Trichoptera, Polycentropodidae) u Hrvatskoj: morfološka determinacija i DNA barkodiranje

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Fauna tulara (Trichoptera) Hrvatske trenutno broji oko 210 vrsta, a nove vrste i nalazi se relativno često utvrđuju velikim dijelom zahvaljujući DNA barkodiranju. Ova je metoda postala odlična nadopuna standardnom morfološkom određivanju vrsta. Iako se identifikacija vrsta, kao i taksonomska istraživanja najčešće provode na odraslim jedinkama koje se smatraju pouzdanijima, ličinke tulara su također dobar izvor informacija. Ovim radom se prvi puta spominje vrsta *Cyrnus crenaticornis* (Kolenati, 1859) za Hrvatsku, pronađena u rijeci Odri u mjestu Čička Poljana u kolovozu 2015. godine s 21 utvrđenim primjerkom. Identifikacija je provedena na temelju morfoloških značajki ličinki, a potvrđena je i DNA barkodiranjem. Utvrđeni nalaz je vrijedan doprinos poznavanju faune Hrvatske.