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ELMIS RIETSCHELI STEFFAN, 1958 (INSECTA: COLEOPTERA: ELMIDAE) IN CROATIA: FIRST RECORD AND DNA BARCODING

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Elmis rietscheli Steffan, 1958 (Coleoptera: Elmidae) is a typical inhabitant of cold springs and small streams in the mountainous areas of Central Europe. Recently, three specimens of *E. rietscheli* were collected in the source area of the Šumi, a stream flowing from Mt. Ivanščica, northern Croatia. The morphological identification of the specimens was confirmed by DNA barcoding. This is the first record of *E. rietscheli* from Croatia, and it represents the southernmost record for this species known to date.

Key words: DNA sequencing, COI, faunistics, new record, species, crenal, riffle beetles

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Tipična srednjoeuropska vrsta vodenog kornjaša iz porodice slapoljuba, Rietschelijev slapoljub, *Elmis rietscheli* Steffan, 1958 (Coleoptera: Elmidae), naseljava hladne izvore i malene vodotoke u središnjoj Europi. Tri primjerka ove vrste prikupljena su na području planine Ivanščice na izvoru potoka Šumi. DNA barkodiranje potvrdilo je morfološku identifikaciju jedinki. To je prvi nalaz vrste *E. rietscheli* za faunu Hrvatske te predstavlja do sada najjužniju zabilježenu točku rasprostranjenja te vrste.

Ključne riječi: DNA sekvencioniranje, COI, fauna, novi nalaz, vrsta, izvor, slapoljubi

INTRODUCTION

Although the conservation of biodiversity is nowadays recognised as a key priority, taxonomic and faunistic research, both providing basal information for conservation issues, are still undervalued. Therefore, many insect groups, such as Elmidae, are unfortunately still very poorly studied in Europe.

Elmidae, or riffle beetles, are a water beetle family typical of springs and mountain streams (Brown, 1987; Jäch, 1998; Jäch *et al*., 2005; Elliott, 2008; Kodada *et al*., 2016).

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They are rather small-sized (adults are on average 2–3 mm long) with complex life cycles (Crowson, 1981; Elliott, 2008). They are frequently used as bioindicators for freshwater monitoring, *i.e.* assessments of ecological functionality and water quality (*e.g.* Eyre *et al.*, 1993; Richoux, 1994; Elliott, 2008; Brojer *et al.*, 2017).

In an annotated checklist of the Elmidae of Croatia, Mičetić Stanković *et al.* (2015) listed 23 species; among these there are six species of the genus *Elmis* Latreille, 1802: *E. aenea* (Müller, 1806), *E. bosnica* (Zaitzev, 1908), *E. latreillei* Bedel, 1878, *E. maugetii* Latreille, 1802, *E. obscura* (Müller, 1806), and *E. rioloides* (Kuwert, 1890).

DNA barcoding (Hebert *et al.*, 2003) is nowadays commonly used in taxonomic research as a means of species identification. This technique is based on sequencing of the standardized ~650 bp long fragment of the mitochondrial (mt) cytochrome oxidase gene subunit I (COI), which bears high interspecific and low intraspecific variability, thus enabling reliable species identifications (Ratnasingham & Hebert, 2013). DNA barcodes were found to be especially useful in cases of morphologically ambiguous or sibling species (see *e.g.* Fossen *et al.*, 2016; Bilton & Ribera, 2017; Bilton *et al.*, 2017).

In taxonomic studies on Elmidae, DNA barcoding has been used mostly for assigning immature developmental stages to adults, or in smaller phylogenetic studies, and, in a few cases, also in detecting undescribed species (Čiampor & Ribera, 2006; Čiamporová-Zatovičová *et al.*, 2007; Čiampor & Čiamporová-Zatovičová, 2008; Čiampor & Kodada, 2010; Hayashi & Sota, 2010; Freitag & Balke, 2011; Curiel & Morrone, 2012; Freitag, 2013; Laššová *et al.*, 2014; Hayashi *et al.*, 2016; Čiampor *et al.*, 2016, 2017; Freitag & Kodada, 2017). Most of these studies deal with tropical species, while for European Elmidae only a few studies based on DNA data are available (*e.g.* Hendrich *et al.*, 2015; Jović *et al.*, 2015; Múrria *et al.*, 2017). For instance, in the Barcode of Life Database (ref. webpage BOLD System v4, accessed on March 25, 2018) there are currently data on 87 species (forming distinct BIN clusters) of Elmidae, but only 23 species sampled from seven European countries are included.

Here we present the first record of *Elmis rietscheli* Steffan, 1958 from Croatia, confirmed by DNA barcoding.

MATERIAL AND METHODS

Study area

Ivanščica is the highest mountain in the north of Croatia, with a maximum elevation of 1061 m a. s. l. The mountain stretches in a west-east direction; it is about 30 km long and 9 km wide. It forms a natural barrier between the Sava and Drava rivers. It is composed of sedimentary rocks, mineral limestones, sandstones, quartz and schist, and it is considered part of the Dinaric Alps (Čaplar, 2008; Herak, 1960; Šimunić, 1983). The climate is moderate continental with some influence from the Adriatic Sea. The annual precipitation varies between 1000 and 1500 mm (Zaninović *et al.*, 2008).

Sampling and identification

The specimens were sampled with a hand net (mesh size: 500 μm), and stored in 96% ethyl alcohol. Specimens were examined with an Olympus ZX9 stereo microscope, and photographed with an Olympus Tough TG-5 camera. An inverted microscope Axio

Observer Z1 was used for genitalia photographs. All specimens are deposited in the Croatian Natural History Museum, Zagreb, Croatia.

DNA extraction, PCR amplification, sequencing and sequence analyses

Genomic DNA was extracted from whole specimens by a non-destructive method, using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to manufacturer's specifications, and eluted in 50 µl of elution buffer.

The standard DNA barcode region (650 bp of 5′ part of COI gene) was amplified with the use of universal LCO1490 / HCO2198 primers (Folmer *et al.*, 1994). PCR was conducted in a 15 µl reaction mixture, containing 1 x DreamTaq™ reaction buffer with 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM dNTP mix (Qiagen), 0.5 µM of each primer, 0.75 units of DreamTaq polymerase (Thermo Scientific) and 3 µl of eluted DNA. PCR cycling conditions were as follows: initial denaturation at 95°C for 2 minutes; 35 cycles of 95°C for 45 seconds, annealing at 52°C for 45 seconds, elongation at 72°C for 45 seconds; final elongation at 72°C for 7 minutes. PCR products were checked for quality and quantity on 1 % agarose gel stained with ethidium bromide and purified using Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase enzymatic system (ThermoFischer Scientific), according to the manufacturer's specifications.

Bidirectional sequencing of PCR products was carried out at Macrogen Inc. (Amsterdam, Netherlands), using the amplification primers LCO1490 and HCO2198. Sequences were checked, edited and assembled from both directions in programs BioEdit v. 7.2.5 (Hall, 1999) and Geneious 8.1.4 (Kearse *et al*., 2012), and submitted to BOLD (Ratnasingham & Hebert, 2007) and GenBank databases. BOLD ID and accession numbers are given in Tab. 1.

Tab. 1. Specimens and sequences used in the analyses. Newly sequenced samples are marked in bold.

Species name	Country	Sample ID	BOLD sequence ID	GenBank Acc. Nr.
Elmis rietscheli	Croatia	**ELRIO-B24/CROBB1**	**CROBF001-18**	**MH368658**
		ELRIO-B25/CROBB2	**CROBF002-18**	**MH368659**
	Germany	BC ZSM AQU 00290	FBAQU290-09	HM422035
Elmis aenea	Croatia	KJ381177	GBCL23072-15	KJ381177
		KJ381175	GBCL23073-15	KJ381175
		KJ381172	GBCL23074-15	KJ381172
		KJ381167	GBCL23078-15	KJ381167
	Austria	KJ381159	GBCL23028-15	KJ381159
		KJ381158	GBCL23029-15	KJ381158
	Germany	ZFMK-TIS-2522902	GCOL10027-16	KU910145
		BC ZSM AQU 00495	FBAQU400-10	HM401301
		ZFMK-TIS-19747	GCOL3640-16	KU910942
	Finland	ZMUO.006128	COLFF808-13	KJ965229
Elmis rioloides	Croatia	KJ381192	GBCL23067-15	KJ381192
		KJ381193	GBCL23066-15	KJ381193
		KJ381189	GBCL23068-15	KJ381189
	Austria	KJ381179	GBCL23027-15	KJ381179
		KJ381180	GBCL23026-15	KJ381180
		KJ381181	GBCL23025-15	KJ381181

Species name	Country	Sample ID	BOLD sequence ID	GenBank Acc. Nr.
	Germany	BC ZSM AQU 00154	FBAQU154-09	HM376183
		BC ZSM AQU 00500	FBAQU405-10	HM401304
	Italy	ZFMK-TIS-7959	GCOL1005-16	KU911071
Elmis obscura	Germany	BC ZSM AQU 00499	FBAQU404-10	HM401303
		BCZSMAQU00968	FBCOG778-12	KM446204
Elmis latreillei	Germany	BC ZSM AQU 00289	FBAQU289-09	HM401281
Elmis maugetii	Austria	KJ381196	GBCL23023-15	KJ381196
		KJ381194	GBCL23024-15	KJ381194
	Germany	ZFMK-TIS-2522897	GCOL10025-16	KU907029
		BC ZSM AQU 00498	FBAQU403-10	HM401302
		BC ZSM AQU 00055	FBAQU055-09	HM376142
		BC ZSM AQU 00228	FBAQU228-09	HM422008
Riolus subviolaceus	Germany	BCZSMAQU01044	FBCOG854-12	KM451547
Limnius opacus	Germany	BC ZSM AQU 00538	FBAQU443-10	HM401335

BOLD Identification Engine (accessed March 2018) was used for comparison of DNA barcodes amplified from *Elmis rietscheli* with the barcode data available in BOLD. The single sequence of *E. rietscheli* in BOLD was provided by the Barcoding Fauna Bavarica Project in Germany (Hendrich *et al.*, 2015). Sequences of *E. rietscheli* were aligned with all barcode sequences of *Elmis* species retrieved from BOLD (Tab. 1), with the addition of two outgroup sequences (Elmidae: *Limnius opacus* Müller, 1806 and *Riolus subviolaceus* (Müller, 1817)). Sequences were collapsed to haplotypes in FaBox online toolbox (Villesen, 2007); uncorrected p-distances and Kimura 2-parameter distances (K2P) between haplotypes were calculated using MEGA 7.0.25 (Kumar *et al*., 2016). Neighbour-joining (NJ) tree based on the K2P distance model was calculated in MEGA 7.0.25 (Kumar *et al*., 2016), and the robustness of the clades was assessed through 1000 bootstrap replicates. The maximum likelihood (ML) tree was constructed on PhyML 3.0 web-server (Guindon *et al*., 2010), with automatic model selection by SMS (determined through AIC selection criterion) (Lefort *et al*., 2017) and aLRT SH-like support.

RESULTS AND DISCUSSION

Elmis rietscheli was described comparatively recently. Despite its rather distinctive male genitalia, this species was overlooked for a long time, mainly because of its remarkable external variability (Jäch, 1992). For instance, Berthélemy (1979: 34) noted that *E. rietscheli* resembles *E. maugetii*, while Schulte (1989) found the specimens which he had examined more similar to *E. latreillei*; specimens recorded as *E. rietscheli* from Romania by Ieniştea (1974) turned out to belong to *E. latreillei* (Berthélemy, 1979: 26). This remarkable external changeability, known also from several other elmid species, may depend, at least to some extent, on the water temperature (see *e.g.* Knie, 1978).

Three specimens of *Elmis rietscheli* were sampled by the first author on Mt. Ivanščica (northern Croatia), in the spring area of the Šumi, a mountain stream with gravel and sand benthos (21.VI.2016, 46°11'21.7"N 16°9'16.6"E, *ca*. 410 m a.s.l.) (Figs. 1, 2).

It is the first record of *Elmis rietscheli* in Croatia. This species was so far recorded from France, Germany, Switzerland, Austria, Italy, Slovenia and Hungary (Jäch *et al.*, 2016); specimens from Hungary were collected in the Kőszeg Mountains, the eastern-

most region of the Alps, about 700 m from the Austrian border (Lőkkös, 2010). This species is typical of mountainous areas, where it prefers the eucrenal, the hypocrenal

Fig. 1. Position of Mt. Ivanščica in Croatia (beetle symbol).

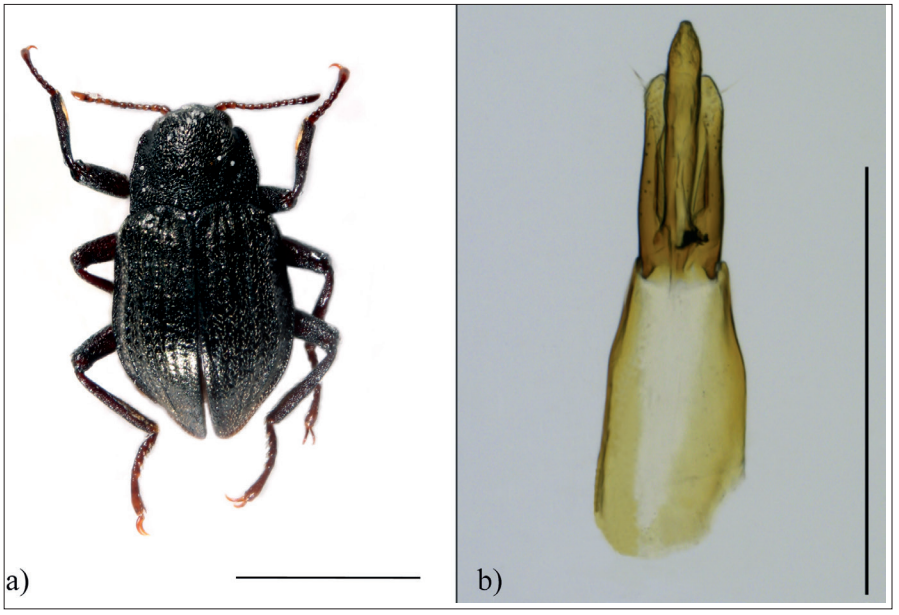

Fig. 2. *Elmis rietscheli* Steffan, 1958: a) habitus (scale = 1 mm), b) male genitalia (scale = 100 μm).

and the epirhithral (Jäch *et al*., 2005; Brojer *et al*., 2017); in some parts of the eastern Alps it is very common. Ivanščica Mountain was sometimes considered to be an extension of the Alps (Herak, 1960; Šimunić, 1983).

A molecular analysis confirmed the identification. Two COI haplotypes of the specimens sampled on Mt. Ivanščica have very low sequence divergence with respect to the single *E. rietscheli* sequence available from BOLD (0.0058 uncorrected p-distance, Tab. 2), which is within the standard intraspecific sequence divergence for COI in beetles (Pentinsaari *et al*., 2014). On the other hand, average sequence divergence between haplotypes of different species of *Elmis* is 11 % (0.043 – 0.134 uncorrected p-distance), which is also within the range of the COI interspecific divergences observed in other groups of beetles.

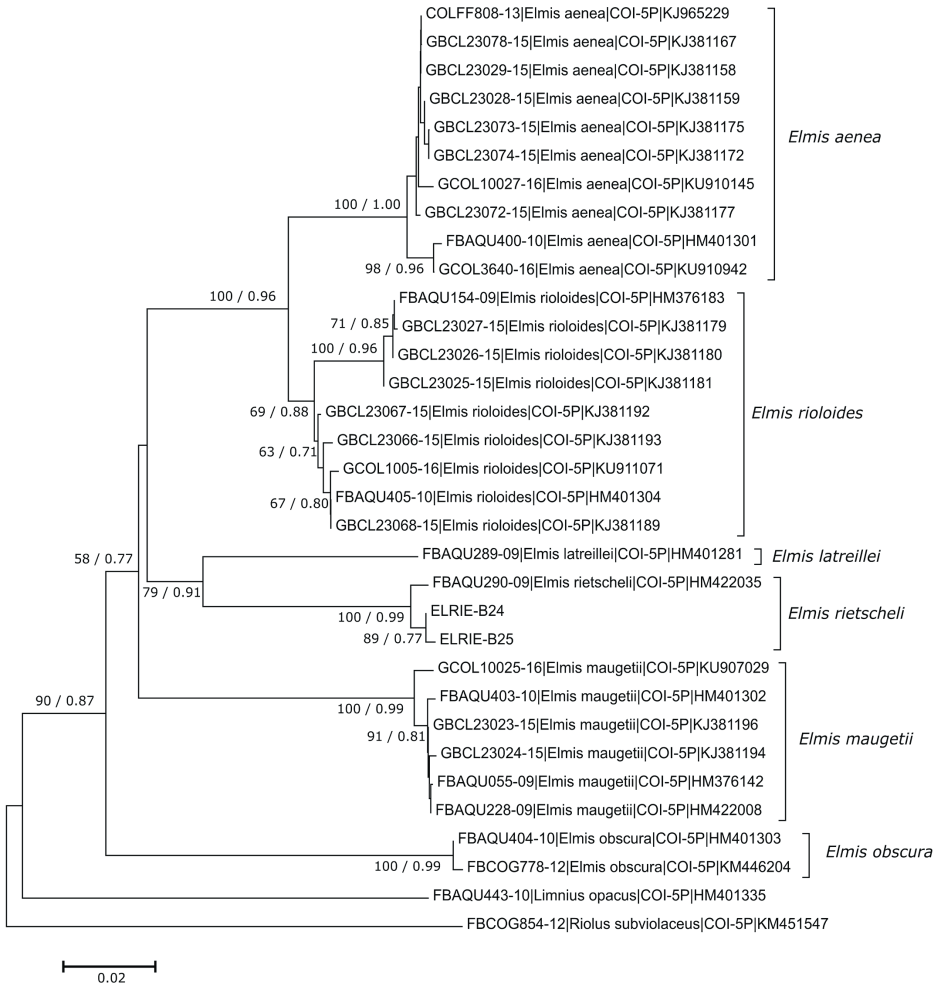

Fig. 3. Neighbour-joining phylogenetic tree constructed by COI sequences of *Elmis rietscheli* from Croatia and haplotypes of *Elmis* species from BOLD, based on Kimura-2-parameter distance model. Numbers on branches are NJ bootstrap support values calculated from 1000 bootstrap replicates (above 50 %) / aLRT-SH like ML support (above 0.7); two sequences of *E. rietscheli* from Croatia: ELRIE-B24 and ELRIE-B25.

Tab. 2. Interspecific and intraspecific (bold) uncorrected p-distances calculated for COI sequences of *Elmis* species.

Species name	*E. aenea*	*E. rioloides*	*E. latreillei*	*E. rietscheli*	*E. maugetii*	*E. obscura*
E. aenea	**0.0049**					
E. rioloides	0.0432	**0.0117**				
E. latreillei	0.1086	0.0964	–			
E. rietscheli	0.1103	0.0982	0.0888	**0.0058**		
E. maugetii	0.1148	0.1010	0.1076	0.1194	**0.0035**	
E. obscura	0.1326	0.1182	0.1254	0.1312	0.1339	**0.0017**

This result is further supported by the phylogenetic analysis. The topology of NJ and ML trees was mostly congruent, with only a few exceptions regarding the moderately or weakly supported nodes (Fig. 3). Three *E. rietscheli* haplotypes group together in a 100 % BS (0.99 aLRT-SH like) supported clade, with *E. rietscheli* being recovered as sister to *E. latreillei*.

The record of *Elmis rietscheli* in Croatia represents the southernmost record for this species. In Croatia it was found in a habitat similar to many of its habitats in Central Europe, confirming its crenal affinities. Further studies should include sampling in other parts of Croatia, with special focus on springs and mountain streams.

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