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Article

DNA barcoding and a new taxonomic status of the *Triaenodes ochreellus lefkas* Malicky, 1974 (Insecta, Trichoptera) with new distribution data

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Abstract
In this paper new data on distribution and new taxonomic status of the caddisfly *Triaenodes ochreellus lefkas* are given. DNA barcoding data are also included into defining new status of the species *Triaenodes lefkas* stat. nov. Data from DNA barcoding analyses of 60 specimens from the genus *Triaenodes* from the BOLD database show certain taxonomic peculiarities in specimens of *T. unanimis* from Japan.

Key words: South Europe, *Triaenodes*, *T. unanimis*, molecular analyses.

Introduction

The Leptoceridae family (long-horned) is one of the largest families of Trichoptera, with more than 1800 known species (Holzenthal *et al*. 2007; Morse 2020; Oláh 2016). It is cosmopolitan although its greatest biodiversity is in Asia (Morse 2020; Holzenthal *et al*. 2007). One of the most numerous genera within the family Leptoceridae is the genus *Triaenodes* McLachlan, 1865, with more than 230 described species (Malicky 2004, 2005a; Morse 2020). This genus is widespread and diverse on all continents, including Europe. Only three species of the genus *Triaenodes* have been found in Europe (Malicky 2004): *T. bicolor* (Curtis, 1834), *T. ochreellus* McLachlan, 1877 and *T. unanimis* McLachlan 1877 (Malicky 2004; Morse 2020). Only the species *T. ochreellus* has two subspecies: *T. ochreellus ochreellus* and *T. ochrellus lefkas* Malicky, 1974 (Ibrahimi *et al*. 2017; Malicky 2004, 2005b; Morse

2020). The adult has a thin body of small to medium size, with long antennae (Holzenthal *et al*. 2007). Larvae of *Triaenodes* species live in different types of habitats, especially in lentic aquatic biotopes, usually with aquatic vegetation (Ibrahimi *et al*. 2017; Wiggins 1978). They have elongated posterior legs with long setae, known as swimming paddles (Wiggins 1978). This morphological structure helps them to make rapid movements and swim among the aquatic plants that grow in lentic waters (Wiggins 1978).

In this study we present results of the DNA barcoding of the genus *Triaenodes* in Croatia, a review of some taxonomic points of this genus, new data on the distribution and taxonomic status of the taxon *Triaenodes ochreellus lefkas*.

Material and methods

Sampling and research area

In Croatia, 7 adult specimens of *T. o. lefkas* were collected from three Mediterranean rivers: Cetina River, a locality near the town of Omiš, N 43°23´11,5″, E 16°46´15,1″, 1 m asl, 1 female, 26.07.2005, 1 female, 1 male, 27.08.2005 (leg. M. Kučinić, I. Vučković); Neretva River, near the town of Opuzen (Fig 1 A-B), 1 m asl, 1 female, 1 male, 4.09.2015 (leg. H. Plavec, M. Landeka); and Mislina River, locality Mlinište, 1 m asl, 1 male 23.05.2015, 1 female 5.09.2015 (leg. S. Žalac, M. Kučinić). The sites on the rivers Cetina and Neretva are located a few kilometers from their estuaries at the Adriatic Sea. At these localities waters have a brackish character. Three specimens collected from the Cetina river have been deposited in the Vučković Trichoptera Collection, two samples (male and female collected from the rivers Neretva and Mislina) have been deposited in the NIP Trichoptera Collection in the Croatian Natural History Museum in Zagreb, and the other two (one male from the Opuzen locality on the Neretva and one female from Mlinište on the Mislina) are kept as vouchers in the Trichoptera DNA Barcode collection in the Croatian Natural History Museum in Zagreb. One specimen of *T. o. lefkas* was collected from a locality in Montenegro: Tuz Municipality, a side spring of Skadar (Shkodër) Lake near the Vitoja Restaurant, 42.325399N, 19.362963E, 30 m asl, 1 male, 13.10.2019 (leg. H. Ibrahimi); 6 specimens were collected from two localities in Albania: 5 males on 21.05.2017 from Shkodër Lake near Shkodër town, 42.054708N, 19.476790E, 15 m asl, 21.05.2017 and 1 male from the Shkumbin River, Rogozhinë, 41.063686N, 19.681378E, 34 m asl, 19.05.2017 (leg. H. Ibrahimi). The samples collected in Montenegro and Albania have been deposited in the Ibrahimi Trichoptera Collection. The samples are stored in absolute ethyl alcohol.

Figure 1 A-B. The River Neretva in Opuzen (left bank).

Laboratory methods

Whole genomic DNA was extracted from legs using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to the manufacturer's specifications and eluted in 60 µl of elution buffer. Full-length mtCOI DNA barcode regions were amplified using LCO1490/HCO2198 (Folmer *et al*. 1994) primer sets. The 50 µl polymerase chain reactions (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) using amplification primers. Sequences were edited manually and aligned using the program BioEdit (Hall 1999). DNA sequences were submitted to Barcode of Life Data Systems (BOLD, Ratnasingham and Hebert 2007). BOLD ID and accession number of all specimens used in analyses are given in Table 1. Specimens from which DNA was extracted within this study are marked in bold in column *Specimen ID* in Table 1. To compare our mt*COI* DNA barcode region with those species from the genus *Triaenodes* that occur in Europe, selected DNA barcode sequences from BOLD database (accessed on July 2017) were employed.

Table 1. Details for specimens used in analyses with assigned taxonomic name, geographic origin, Specimen ID, BOLD Sequence ID number and haplotype.

	Taxonomic designation according to identifier	**Sex**	**Life Stage**	**Specimen morphological identification by:**	**Country**	**Location**	**Specimen ID**	**BOLD Sequence ID**	**Haplotype name**
1.	*T. ochreellus lefkas*	male	adult	Mladen Kučinić	Croatia	River Neretva, near Opuzen	**TTOCL_1**	NIPTR001-17	**1. HP**
2.	*T. ochreellus*	male	adult	Juha Salokannel	Spain	Galicia	09MNKK0409	KKUMN417-10	**2. HP**
3.	*T. ochreellus*	male	adult	Aki Rinne	Spain	Galicia	09MNKK0410	KKUMN418-10	
4.	*T. bicolor*	male	adult	Hans Malicky	Finland	Lapland	JSlk-20090076	TRIFI181-10	**3. HP**
5.	*T. bicolor*	/	larva	Stanislaw Czachorowski	Finland	Uusimaa	ARin-20100174	TRIFI319-11	**4. HP**
6.	*T. bicolor*	?	?	Sophie C. Gombeer	Norway		NIVA_TER_39	BARCO039-14	**5. HP**
7.	*T. bicolor*	?	?	Tor ERIK Eriksen	Austria		07HMCAD-0237	HMCAD237-08	
8.	*T. bicolor*	?	?	Jon K. Skei	Austria		07HMCAD-0238	HMCAD238-08	**6. HP**
9.	*T. bicolor*	male	adult	Juha Salokannel	Poland		NZCAD909-85	KKCAD701-09	
10.	*T. bicolor*	male	adult	Juha Salokannel	Poland		NZCAD909-86	KKCAD702-09	**7. HP**
11.	*T. bicolor*	male	adult	Juha Salokannel	Poland		NZCAD909-93	KKCAD709-09	
12.	*T. bicolor*	male	adult	Aki Rinne	Norway	Nord-Trondelag	TRD-TRI76	ODTRI094-14	**8. HP**
13.	*T. bicolor*	female	adult	Juha Salokannel	Norway	Nord-Trondelag	TRD-TRI77	ODTRI095-14	**9. HP**
14.	*T. bicolor*	/	larva	Stanislaw Czachorowski	Finland	Southern Savonia	ARin-20100170	TRIFI317-11	**10.HP**
15.	*T. bicolor*	/	larva	Aki Rinne	Finland	Kymenlaakso	JSlk-20090080	TRIFI185-10	**11.HP**
16.	*T. bicolor*	/	larva	Juha Salokannel	Finland	Kymenlaakso	JSlk-20090078	TRIFI183-10	**12.HP**
17.	*T. bicolor*	/	larva	Hans Malicky	Finland	Kymenlaakso	JSlk-20090079	TRIFI184-10	**13.HP**
18.	*T. bicolor*	/	larva	Jon K. Skei	Finland	Southern Savonia	ARin-20100171	TRIFI318-11	**14.HP**
19.	*T. bicolor*	/	larva	Stanislaw Czachorowski	Finland	Southern Savonia	ARin-20100168	TRIFI342-11	**15.HP**
20.	*T. bicolor*	/	larva	Aki Rinne	Finland	Kymenlaakso	JSlk-20100093	TRIFI271-10; GBMIN35596-13	**16.HP**
21.	*T. bicolor*	/	larva	Sophie C. Gombeer	Belgium	Mol, Antwerpen (Antwerp)	UA-SG-TRICH-C50	TFLAN117-11	**17.HP**
22.	*T. detruncatus*	/	larva	Aki Rinne	Finland	Nylandia, Vanhankaupunginkoski	ARin-2011F165	TRIFI705-12	**18.HP**
23.	*T. detruncatus*	/	larva	Aki Rinne	Finland	Tavastia australis, Tainionvirta	ARin-2011F170	TRIFI710-12	**19.HP**
24.	*T. detruncatus*	male	adult	Juha Salokannel	Finland	Kajaani, Nuasjaervi	JSlk-20100206	TRIFI332-11	**20.HP**
25.	*T. detruncatus*	?	?	?	?	?	FN600955	GBMIN35594-13	**21.HP**
26.	*T. pellectus*	male	adult	Ralph W. Holzenthal	Russia	Vinogradovka River, under bridge, Khasanskyi	TVTRI0036	RUSST107-12	**22.HP**
27.	*T. pellectus*	male	adult	Oliver S. Flint, Jr.	Russia	Kuchelinovo, Maksimov`s Campground, Pond, Primorye,	11TVCAD-170	KJTRI653-13	**23.HP**

	Taxonomic designation according to identifier	Sex	Life Stage	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID	Haplotype name
28.	*T. pellectus*	?	adult	Oliver S. Flint, Jr.	Japan	Shkotovsky Dist. L. Akan-panke, Hokkaido	10OFSI-0238	OFTRI237-10	**24.HP**
29.	*T. pellectus*	?	adult	Hans Malicky	hailand	Muang Pai Resort	09HMCAD-0245	HMCAD610-09	**25.HP**
30.	*T. pellectus*	?	adult	Oliver S. Flint, Jr.	Japan	Saiwai Bridge, Eniwa, Hokkaido	10OFSI-0236	OFTRI235-10	**26.HP**
31.	*T. reuteri*	female	adult	Juha Salokannel	Estonia	Austla, Saaremaa	JSlk-20110157	TRIFI505-11	**27.HP**
32.	*T. rufescens*	male	adult	Oliver S. Flint, Jr.	Russia	Primorye, Nakhodka Dist., Volchanets Lake	11TVCAD-169	KJTRI665-13	**28.HP**
33.	*T. simulans*	male	adult	Juha Salokannel	Latvia	river Gauja	JSlk-20110158	TRIFI506-11	**29.HP**
34.	*T. simulans*	?	?	Reinhard Mueller	Germany	Spree bei Doebbrick	GBOL06252	GBEPT1017-14	
35.	*T. simulans*	male	adult	Hans Malicky	Austria	Waidhofen an der Ybbs	HMCAD0111-35	HMKKT852-11	**30.HP**
36.	*T. simulans*	?	adult	Hans Malicky	Romania	Hunedoara, SW 2am, NE Salciva, am Mores, Transylvania	10HMCAD-036	HMKKT036-10	**31.HP**
37.	*T. simulans*	?	adult	Aki Rinne	Finland	Laesaekoski, Itae-Suomi, Savonia australis	ARin-2014F043	TRIFI1061-14	**32.HP**
38.	*T. simulans*	/	larva	Aki Rinne	Finland	Ilomantsi	ARin-20100180	TRIFI322-11	**33.HP**
39.	*T. simulans*	male	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05004	MGCAD266-08	**34.HP**
40.	*T. simulans*	female	adult	Suvdtsetseg Chuluunbat	Mongolia	Egiin gol 12.7 km SW of Teshig	ID-04603	MGCAD238-08	**35.HP**
41.	*T. simulans*	female	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05003	MGCAD265-08	**36.HP**
42.	*T. simulans*	male	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05005	MGCAD267-08	**37.HP**
43.	*T. simulans*	male	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05006	MGCAD268-08	**38.HP**
44.	*T. simulans*	male	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05007	MGCAD269-08	**39.HP**
45.	*T. simulans*	female	adult	Suvdtsetseg Chuluunbat	Mongolia	Delger Moron Gol, 12.0 km km W of Moron, Hovsgol	ID-05008	MGCAD270-08	**40.HP**
46.	*T. unanimis*	?	adult	Oliver S. Flint, Jr.	Sweden	10km W Bollstabruk	10OFSI-0240	OFTRI239-10	**41.HP**
47.	*T. unanimis*	male	adult	Oliver S. Flint, Jr.	Russia	Lotos lake in Khasan	11TVCAD-001	RUSST001-12	
48.	*T. unanimis*	male	adult	Ralph W. Holzenthal	Russia	Utinoye Lake at Andreevka Village	TVTRI0025	RUSST096-12	**42.HP**
49.	*T. unanimis*	male	adult	Oliver S. Flint, Jr.	Russia	Perevoznaya wet meadow & small stream	11TVCAD-014	RUSST014-12	
50.	*T. unanimis*	male	adult	T.S. Vshivkova	Russia	marshes of Perevoznaya Village, at Tyurneva`s hous	TVTRI0126	RUSST200-12	**43.HP**
51.	*T. unanimis*	?	adult	Oliver S. Flint, Jr.	Sweden	An. Forsed, Vasternorrland	10OFSI-0241	OFTRI240-10	
52.	*T. unanimis*	male	adult	Ralph W. Holzenthal	Russia	Lotos Lake	TVTRI0072	RUSST147-12	**44.HP**
53.	*T. unanimis*	male	adult	Ralph W. Holzenthal	Russia	Lotos Lake	TVTRI0088	RUSST156-12	**45.HP**
54.	*T. unanimis*	male	adult	T.S. Vshivkova	Russia	Artyomovka River, lower part	TVTRI0145	RUSST219-12	**46.HP**
55.	*T. unanimis*	female	adult	T.S. Vshivkova	Russia	eastern shore, kordon Vostochnyi	TVTRI0227	RUSST301-12	**47.HP**

	Taxonomic designation according to identifier	Sex	Life Stage	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID	Haplotype name
56.	*T. unanimis*	female	adult	Juha Salokannel	Finland	Osmankajaervi	JSlk-20090081	TRIFI186-10	**48.HP**
57.	*T. unanimis*	male	adult	Juha Salokannel	Finland	Osmankajaervi	JSlk-20090082	TRIFI187-10	**49.HP**
58.	*T. unanimis*	?	adult	Oliver S. Flint, Jr.	Japan	Chitose Lake, Hokkaido	10OFSI-0242	OFTRI241-10	**50.HP**
59.	*T. unanimis*	?	adult	Oliver S. Flint, Jr.	Japan	W Asajino, Hokkaido	10OFSI-0243	OFTRI242-10	**51.HP**
60.	*T. unanimis*	?	adult	Oliver S. Flint, Jr.	Japan	Daiichi-usakuma Bridge, Hokkaido	10OFSI-0244	OFTRI243-10	**52.HP**
61.	*Mystacides longicornis*	female	adult	Mladen Kučinić	Croatia	River Drava – Gornji Hrašćan	**TMYS_1**		outgrup

A neighbor-joining and maximum likelihood gene tree was produced in MEGA 6 (Tamura *et al.* 2013), using the Kimura 2-parameter. Intraspecific and interspecific genetic distances, as uncorrected *p*-distances, were calculated using MEGA 6 (Tamura *et al.* 2013), using pairwise deletion. The number of hypothetical species within the data set was estimated according to the barcode gap (difference between inter- and intraspecific genetic distances) with the use of Automatic Barcode Gap Discovery, ABGD (Puillandre *et al.* 2012).

Macrophotography was performed with a Leica Wild MZ8 stereomicroscope and an Olympus SP-500 UZ digital camera, and was processed with the computer programme Olympus Quick Photo Camera 2.2. For determination of the collected specimens we used Malicky (2004). Systematic presentation follows Morse (2020). Distribution map was made according to our investigation and literature data: Chvojka 1977; Karaouzas 2019; Malicky 2005b; Marinković-Gospodnetić 1981; Oláh & Kovács 2014; Stanić-Koštroman *et al.* 2015; Wallace, 2016.

Results

In figure 3 we are showing the relationships among the species of the genus *Triaenodes*, based on the 658 bp long fragment of the DNA barcode region. In this analysis we included 60 specimens from the genus *Triaenodes* from Europe and Asia (Tab. 1). Identical sequences were collapsed into unique haplotypes (Tab. 1, Fig. 3). In analysis of the genetic distance of barcode mtCOI within species, we eliminate *p*–distance value 0.0 of sequences that are not of the same length. Minimum intraspecific genetic distance between all *Triaenodes* species used in analysis is 0.002 (0.2%) recorded within *T. unanimis* (Tab. 2).

The most interesting results of our analyses relate to the species *T. unanimis* and *T. ochreellus* (Fig. 1). A specimen of *T. unanimis* (sample ID: RUSST219-12) and one of *T. rufescens* (sample ID: KJTRI665-13), both from Russia, have only 0.2% nucleotide sites different. Both of these specimens, presented as two different species in the BOLD database, group together (Fig. 1) with 100% certainty. *Triaenodes unanimis* with sample ID OFTRI242-10 and OFTRI243-10 from Japan shows 8% difference as compared to the other specimens of *T. unanimis*, forming thus a separate branch in the phylogenetic tree (Fig. 3).

In analysis of species *T. ochreellus* two barcode sequences were available in databases and they represent same haplotype, which explains the *p*–distance value of zero. DNA barcode of *T. ochreellus lefkas* from Croatia (Fig. 2 A-D) (Sample ID: TTOCL_1) was analysed using the BOLD identification engine by a comparison with the full reference database of the DNA barcodes.

The identifying DNA barcode of *T. ochreellus lefkas* from Croatia (sample ID: TTOCL_1) turns out to have 90.48% similarity with the sequence obtained from the closest available reference sequences of *T. ochreellus,* which is from Spain (sample ID 09MNKK0409). The maximum intraspecific distance among *Triaenodes* species used in the analysis is 0.017 (1,7%) (Tab. 2). The discrepancy in the value of the maximum intraspecific genetic distance appears within *T. ochreellus* and it is 0.102 (10%) (Tab. 2). According to ABGD analyses, the resulting phylogenetic tree of the species *T. ochreellus* shows two branches, traditionally placed under subspecies: one as *T. o. lefkas* and one as *T. o. ochreellus* (Fig. 1). These two taxa have a disjunct distribution, *T. ochreellus ochreellus* is distributed in the west and *T. ochreellus lefkas* in south-east part of Europe (Fig. 4).

Table 2. Values of the p-distance between groups of *Triaenodes* species and outgroup species for the barcode mtCOI region.

	T. ochreellus	***T. bicolor***	***T. detruncatus***	***T. pellectus***	***T. reuteri***	***T. rufescens***	***T. simulans***	***T. unanimis***	***Mystacides longicornis***
T. ochreellus	0-0.102								
T. bicolor	0.146-0.165	0.002-0.017							
T. detruncatus	0.154-0.181	0.147-0.167	0.002-0.017						
T. pellectus	0.142-0.159	0.127-0.149	0.137-0.161	0.002-0.017					
T. reuteri	0.157-0.174	0.145-0.158	0.106-0.121	0.147-0.153	0				
T rufescens	0.159-0.186	0.161-0.197	0.192-0.197	0.182-0.189	0.194	0			
T. simulans	0.165- 0,204	0.157- 0.184	0.110- 0,134	0.149-0,165	0.114- 0.124	0.187-0.206	0.002-0.033		
T. unanimis	0.106-0186	0.133-0.177	0.156-0.195	0.138-0.189	0.151-0.196	0.002-0.187	0.161-0.204	0.002-0.187	
Mystacides longicornis	0.0174-0.182	0.152-0.167	0.185-0.194	0.176-0.182	0-0.178	0.208	0.192-0.206	0.162-0.208	0

In addition to this, during our study we found five new localities of *T. ochreellus lefkas*, two in Albania, Shkodër (Skadar) Lake and Shkumbin River, one in Montenegro, Skadar (Shkodër) Lake, and two in Croatia, the rivers Mislina and Cetina (Fig. 4).

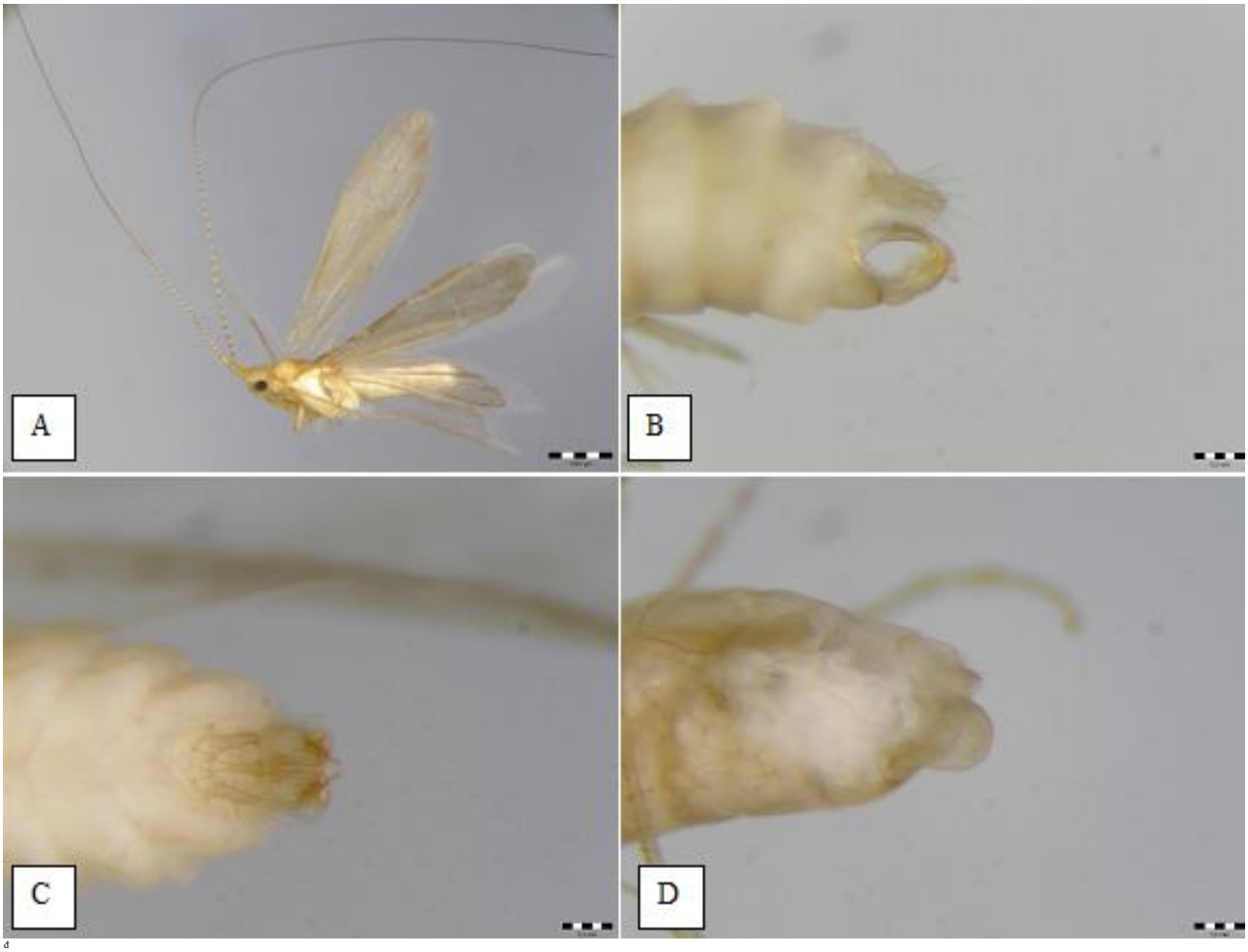

Figure 2 A-D. *Triaenodes ochreellus lefkas* Malicky, 1974. **A** Male adult from Opuzen, the River Neretva, 4.09.2015 (Croatia); **B** Male genitalia (lateral view), Mlinište, the River Mislina 23.05.2015; **C** Male genitalia (ventral view), Opuzen, the River Neretva, 4.09.2015; **D** Female genitalia (lateral view), Mlinište, the River Mislina, 5.09.2015.

Discussion

A new tool in the old practice of molecular taxonomy is DNA barcoding, proposed in 2003 (Hebert *et al*. 2003a, 2003b). This method, which in the case of animals uses a standard 648 bp long fragment of cytochrome c oxidase subunit 1 mitochondrial gene (mtCOI), along with the establishment of the Barcode of Life Data Systems (BOLD) (Ratnasingham & Hebert 2007) resulted in a new approach to species diversity. DNA barcoding is a very useful method in the study of biodiversity, taxonomy, phylogeny and phylogeography of different groups of organisms (e.g. Brehm *et al*. 2019; Cárdenas *et al*. 2013; Elías-Gutiérrez *et al*. 2008; Guo *et al*. 2016; Huemer *et al*. 2020; Léger *et al*. 2020; Tyagi *et al*. 2017; Yang *et al*. 2016), including Trichoptera (Kučinić *et al*. 2016, 2017, 2019a; Szivák *et al*. 2017; Santos *et al*. 2016; Valladolid *et al*. 2018, 2019; Vitecek *et al*. 2020). In many studies, DNA barcoding has aided morphological identification of different taxa (Ćukušić *et al*. 2017; Kučinić *et al*. 2019b, 2020), but also enabled the discovery of new species (Brehm *et al*. 2019; Dela Cruz *et al*. 2016; Graf *et al*. 2012; Kučinić et al. 2013; Léger *et al*. 2020; Tyagi *et al*. 2017; Vaglia *et al*. 2008).

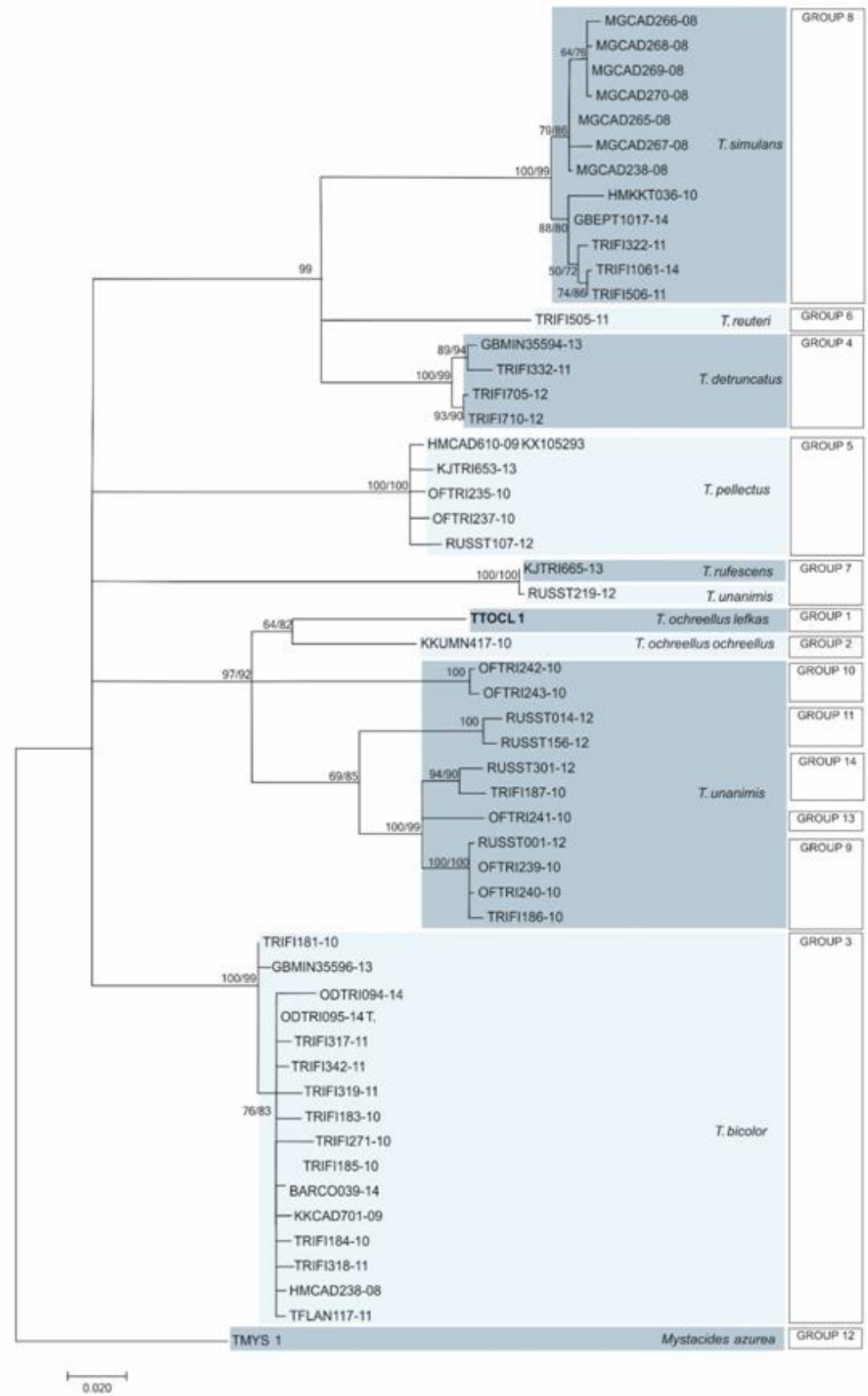

Figure 3. Maximum likelihood (Ml) phylogram based on the 658 bp long fragment of the DNA barcode region showing the relationships among the species of the genus *Triaenodes*. Numbers above the branches represent bootstrap support (bs) for Neighbor-Joining (NJ) and Ml analysis (NJ/Ml). BS values less than 70 are not shown. The groups delineated by Automatic Barcode Gap Discovery (ABGD) approach are shown on the right side of the tree.

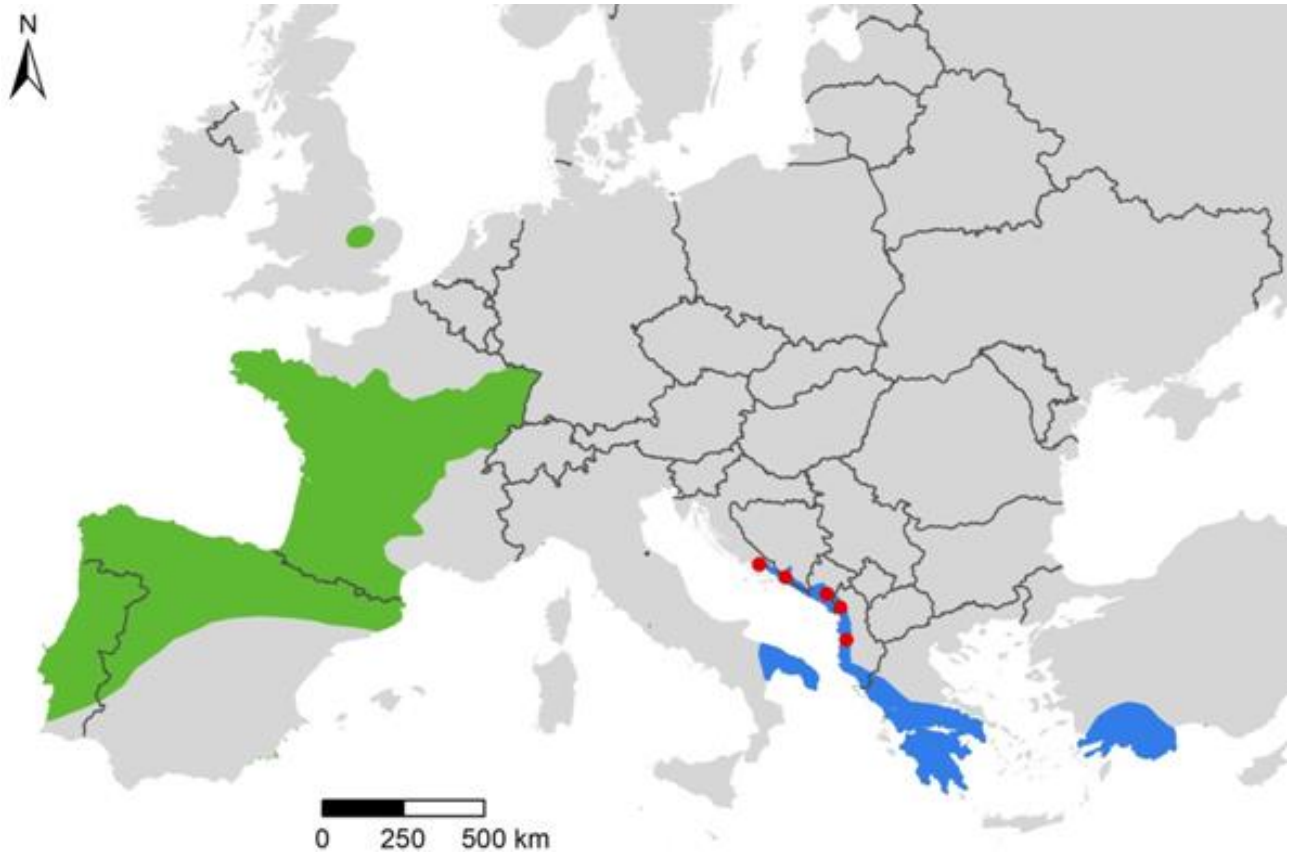

Figure 4. Distribution of *Triaenodes ochreellus ochreellus* (green field) and *Triaenodes ochreellus lefkas* (blue field) with new data in Albania, Croatia and Montenegro (red points).

Although there are no generally accepted values for genetic differences within DNA barcode region among various species, for animals it is considered that an intraspecific genetic distance of more than 2% between populations is high intraspecific variability within the same species (Hebert *et al*. 2003b). So, the genetic distance of more than 2% within Trichoptera species indicates a possibility of a different species, however, several studies on DNA barcode region show that this difference can be even over 8% (Graf *et al*. 2015; Zhou *et al*. 2007).

In this study specimen of *T. unanimis* (sample ID: RUSST219-12) and *T. rufescens* (sample ID: KJTRI665-13) both from Russia have only 0.2% nucleotide sites different, which is a value common within the same species. Also, in Fig. 3 we can see that they are grouping together with 100% certainty, indicating that they belong to the same species, *T. rufescens*.

We can assume a set of explanations: e.g. that the *T. unanimis* sequence (sample ID:RUSST219-12) is the product of contamination with the *T. rufescens* species, or that morphological identification of the specimen of *T. unanimis* is incorrect, or mislabeled.

Results of the ABGD analysis of *T. unanimis* support the separation of this species into 5 groups, possibly new species. In Fig. 3 we can notice that species *T. unanimis* shows a branching pattern with high values of nodes (minimum value is 90) indicating that this widespread species is formed of different taxa. For example, samples of *T. unanimis* shows with samples ID: OFTRI242-10 and ID: OFTRI243-10 from Japan show an 8% difference from other specimens of *T. unanimis*, forming a separate branch in the phylogenetic tree (Fig. 3). In future, taxonomic research into *T. unanimis* detailed morphological analyses of adult forms of all species potentially new to science should be included - from Russia, Sweden, Finland, Japan and other areas where *T. unanimis* is distributed.

DNA barcoded results of *T. ochreellus lefkas* from Croatia show high difference of about 10% from the *T. ochreellus* from Spain. This genetic difference between analyzed specimens and the populations to which they belong shows their interspecific relations, i.e. the state of a taxonomically separate species. This is supported by the fact that such a genetic distance is reported from various Trichoptera species (8.06-15.65,

Johanson 2007; 8.05-21.7%, Pauls *et al*. 2010; 8.2% Graf *et al.* 2015), which implies that these two taxa are also separate species.

Based on the phylogenetic species concept (PSC), developed independently by Eldredge & Cracraft (1980) and Nelson & Platnick (1981) and integrative taxonomy (e.g. Brehm *et al*. 2019; Cárdenas *et al*. 2013; Graf *et al*. 2012, Dela Cruz *et al.* 2016; Léger *et al*. 2020; Valladolid *et al.* 2018; Vitecek *et al*. 2015a, 2015b, 2017) which includes molecular data, morphology data and distribution data, we elevate subspecies *T. o. lefkas* to the species level *T. lefkas* **stat. nov**.

These two taxa have distinctly disjunct ranges with a distance more than 700 kilometers, without any contact zones between them (Fig. 4). The taxon *T. lefkas* is distributed only in the southeastern part of Europe, in the areas of southern Italy (only the province of Puglia), Greece, Albania, Montenegro, Croatia and Bosnia and Herzegovina (Karaouzas *et al*. 2019; Malicky 2005b; Oláh & Kovács 2014; Stanić-Koštroman *et al*. 2015) (Fig. 4), the finding in the River Cetina in Croatia being the north-westernmost instance of its distribution range (Fig. 4). Sipahiler for west Anatolia region in the Asian part of Turkey lists *T. ochrellus* (Sipahiler 2005) (Fig. 4), which we can assume applies to the then subspecies, and now species *T. lefkas*. In this study the distribution range of *T. lefkas* is expanded with new records from Croatia, Montenegro and Albania (Fig. 4). Before this study, in this area *T. lefkas* was found at one locality in Croatia (at the River Neretva, Kučinić *et al*. 2015; Malicky 2005b), one in Albania (Oláh & Kovács 2014) and two localities in Montenegro (Marinković-Gospodnetić 1981; Karaouzas *et al*. 2019). The nominate taxon *Triaenodes ochreellus* McLachlan, 1877 is distributed in Spain, France and Portugal (Gonzales & Menédez 2011; Malicky 2005b; Terra 1994) (Fig. 4).

It is also very interesting to find this species at one locality (Old Weston, Huntingdonshire) in Great Britain (Wallace 2016). The species was identified twice there, in 2010 and 2013, and it is considered that it was possibly introduced into Great Britain (Wallace 2016) (Fig. 4). Now *T. ochreellus* is a member of the fauna of Great Britain.

According to Malicky (2005b) *T. lefkas* has two generations, one in spring and summer and the other one in autumn which complies with our findings. The emergence of adults begins in April for first generation and the specimens of second generation were found in September and October (Malicky 2005b). This species also inhabits brackish aquatic habitats, as found in Italy (Corallini Sorcetti & Moretti 1984) and in Croatia (Kučinić *et al*. 2015; Malicky 2005b). These biological features have not been established for the species *T. ochreellus* (Malicky 2005b).

Conclusion

This study contributes to the knowledge on the distribution of rare taxa of the genus *Triaenodes* in Southeastern Europe and at the same time to the integration of molecular analyses with ecological data and morphology for the validation of the taxonomic status of caddisfly species.

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