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THE FIRST RECORD OF *TINODES ANTONIOI* BOTOSANEANU & TATICCHI-VIGANÒ, 1974 (INSECTA, TRICHOPTERA) IN CROATIA WITH DNA BARCODING AND ECOLOGICAL DATA AND NOTICE OF BIODIVERSITY AND DISTRIBUTION OF THE GENUS *TINODES* IN CROATIA

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The species *Tinodes antonioi*, a new species in the fauna of Croatia, was determined in northern Istria. The paper provides information about *T. antonioi* in Croatia (DNA barcode data, period of the finds, localities of the finds, numbers of males and females collected) as well as the biodiversity and distribution of the *Tinodes* genus in Croatia. A reference is made to the species endemic in Croatia, *Tinodes andrasi*, established to date at only one site in the south of Croatia (Konavle, Dalmacija).

Key words: caddisflies, *Tinodes*, molecular data, Istria, Dalmatia, Croatia

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Vrsta *Tinodes antonioi*, kao nova u fauni Hrvatske, zabilježena je po prvi puta u sjevernoj Istri. U radu se daju informacije o *T. antonioi* u Hrvatskoj (DNA barkod podaci, periodi nalaza, lokaliteti nalaza, broj prikupljenih mužjaka i ženki), kao i bioraznolikost i rasprostranjenost roda *Tinodes* u Hrvatskoj. Daje se osvrt i na endem Hrvatske, *Tinodes andrasi*, zabilježen samo na jednom lokalitetu u Hrvatskoj (Konavle, Dalmacija).

Ključne riječi: tulari, *Tinodes*, molekularni podaci, Istra, Dalmacija, Hrvatska

INTRODUCTION

Systematic research into Trichoptera fauna in Croatia started with the work of the Croatian Natural History Museum in Zagreb at the end of the 20th century. Before that,

research did not have a systematic character, although it had managed to record some very valuable faunistic and taxonomic results (Radovanović, 1935; Marinković-Gospodnetić, 1971; 1979) as far as the knowledge of Trichoptera fauna in Croatia is concerned. Limnological research, conducted vigorously in the streams of Croatia from the mid 20th century also provided a partial insight into the composition and structure of caddisfly fauna, although these data were limited by the very research methods, which analysed only larvae for the determination of species, and no adult forms (Matoničkin, 1959, 1987; Matoničkin & Pavletić, 1967; Matoničkin *et al.*, 1971; Habdija, 1979, 1989; Habdija *et al*., 2003).

Research initiated by staff members of the Croatian Natural History Museum (F. Perović, M. Kučinić, I. Mihoci, M. Vajdić, B. Jalžić) and later by staff of the Faculty of Science of Zagreb University and other colleagues, has been directed in the last 20 or so years to the study of faunistic (Kučinić, 2002; Previšić *et al.*, 2010; Kučinić *et al.*, 2011a; 2014; 2015a; Cerjanec, 2012; Šemnički *et al*., 2012; Ćuk & Vučković, 2014) taxonomic (Kučinić & Malicky, 2002; Malicky *et al.*, 2007; Kučinić *et al.*, 2008; 2011b; 2013; Waringer *et al.*, 2009; Oláh, 2010; 2011; Previšić *et al.*, 2014a; Vučković *et al.* 2011) and phylogeographic features of caddisflies in Croatia (Previšić *et al.*, 2009; 2014b) as well as in neighbouring countries. Fellow researchers from abroad took part in this research, which covered parts of Bosnia and Herzegovina (Kučinić *et al*., 2011b; 2015b; Previšić *et al.*, 2014a; Vitecek *et al.*, 2015c; Waringer *et al.*, 2016; Stanić-Koštroman *et al*., 2012, 2015), Kosovo (Ibrahimi *et al.*, 2012; 2014; 2015; 2016), Serbia (Waringer *et al.*, 2015) and Macedonia (Kučinić *et al.*,2016; Vitecek *et al.*, 2015a; 2015b; Waringer *et al.*, 2016).

By very similar species, identification based on morphological diagnoses requires molecular analysis to confirm results or to speed it up. Hebert *et al*. (2003a; 2003b) proposed a relatively new method named "DNA barcoding" which use a robust primer set to amplify approximately 650-base pair (bp) region of the mitochondrial (mt) cytochrome-c oxidase subunit 1 (*COI*) gene to ensure rapid and accurate identification of a broad range of biological specimens. The divergences of this *COI*-5P gene fragment (DNA barcode region) enable the discrimination of closely related species in all animal phyla except the Cnidaria (Hebert *et al*., 2003b). The Barcode of Life project (BOLD) was proposed to promote DNA barcoding as a global standard for sequence-based identification of eukaryotes (Jinbo *et al*., 2011).

This molecular-based identification is not new for study of caddisflies (e.g. Gíslason *et al*., 2015; Graf *et al.*, 2015; Waringer *et al.*, 2015; Ibrahimi *et al.*, 2015, 2016; Vitecek *et al*., 2015a), but in Croatia for this group there is so far only one study based on DNA barcoding analysis (Kučinić *et al.*, 2013).

At the beginning of 2014 the two-year implementation of the NIP project started, which covered research into 9 groups of animals, including Trichoptera. In the implementation of the NIP project, caddisflies were collected from 105 sites in various parts of Croatia and processed from a faunistic point of view. Not only were very many data collected that supplement our knowledge of the biodiversity and distribution of caddisflies in the inland, central mountain and Mediterranean areas, but a certain number of new species for the Croatian fauna were discovered (Kučinić *et al.*, 2015a; M. Kučinić unpublished data).This paper gives: 1. the finds of a new species for Croatian fauna, *Tinodes antonioi*; 2. a review of this genus in Istria; 3. the biodiversity and distribution of the *Tinodes* genus in Croatia. The first data about the genetic features of the species *Tinodes antonioi* established with mtDNA barcoding are provided, the finding sites, the number of males and females collected and photographs of the genitals of specimens collected in Croatia.

MATERIAL AND METHODS

Fieldwork

In the context of the NIP project, collection of Trichoptera was carried out at the sites Marušića Stream and Mlini Stream (Fig. 1A, B). Both streams are located in the northern parts of Istria. Collection of the material took place two times during 2014 (August and September) and one time during 2015 (May). Each of the streams had two collecting sites, the first being the spring. The second site on the Marušića was located in the upper part of the stream, on a bridge about 150 metres from the spring (Fig. 1A). The second site on Mlini Stream was 1 km downstream from the source, on a bridge alongside a road off to Mlini (Fig. 1B). This site is subject to considerable anthropological impact, for in this part the stream has been partially hydrologically engineered. At each locality, collection of adults was implemented in daytime with an entomological net, for a period of 30 minutes, and at night with the use of a 15 W UV lamp, for a period of 90 minutes. All the material collected was stored in phials containing 96% alcohol. The specimens collected now form part of the NIP Trichoptera Collection kept in the Croatian Natural History Museum in Zagreb.

Laboratory work

Determination of the genus *Tinodes* material collected was conducted after Malicky (2004), and females of the species *T. antonioi* after Cianficconi *et al.* (1999). A systematic presentation of species of the genus *Tinodes* recorded in Croatia has been given after Morse (2016) and the division of Croatia into inland (Pannonian-peripannonian), upland or mountainous and Mediterranean parts is given after Bertić *et al.* (2001) (Fig. 2).

Molecular analyses

Tinodes specimens that were DNA barcoded in this study account for one specimen undoubtedly identified as *T. antonioi*, two similar but morphologically slightly different samples refer therefore to *Tinodes* sp., and four additional *Tinodes* species occurring in Croatia: *T. braueri* McLachlan, *T. dives* (Pictet), *T. pallidulus* McLachlan and *T. waeneri* (Linnaeus) (Tab. 1).

Whole genomic DNA was extracted from single leg using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to the manufacturer's specifications and eluted in 100 μl of elution buffer. The remainder of the specimen was kept as a voucher in the Trichoptera DNA Barcode collection in the Croatian Natural History Museum in Zagreb.

For all specimens, full-length of DNA barcode region was 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 sequencing was performed by Macrogen Inc. sequencing service (Seoul, South Korea)using the same primers. Sequences were checked for errors and edited manually using the program BioEdit (Hall, 1999).

Molecular species identification

Two approaches for species identification were used, similarity analysis and phylogenetic inference. For all *Tinodes* DNA barcode sequences obtained in this study, simila-

Tab. 1. Taxonomic designation, specimen ID, BOLD Sequence ID number, mitochondrial *COI* haplotype and geographic origin of the specimens used in analysis.

No	Species name	Specimen ID	BOLD Sequence ID number	mt *COI* haplotype	Country
	Tinodes antonioi	TTANT1	NIP002-16	1	Croatia
	Tinodes sp. _F	TTIN1	NIP003-16		Croatia
	Tinodes sp._M	TTIN2	NIP004-16	2	Croatia
	Tinodes turanicus	10HMCAD-021	HMKKT021-10	3	Kyrgyzstan
	Tinodes n. sp. nr. *turanicus*	IQCAD135-09	IQCAD135-09	4	Iraq
	Tinodes n. sp. nr. *turanicus*	IQCAD144-09	IQCAD144-09	5	Iraq
	Tinodes n. sp. nr. *turanicus*	IQCAD145-09	IQCAD145-09		
	Tinodes n. sp. nr. *turanicus*	IQCAD146-09	IQCAD146-09		
	Tinodes n. sp. nr. *turanicus*	IQCAD147-09	IQCAD147-09		
	Tinodes n. sp. nr. *turanicus*	IQCAD148-09	IQCAD148-09		
	Tinodes n. sp. nr. *turanicus*	IQCAD149-09	IQCAD149-09		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0034	TRIRA034-13		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0035	TRIRA035-13		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0036	TRIRA036-13		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0037	TRIRA037-13		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0039	TRIRA039-13		
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0033	TRIRA033-13	6	Iraq
	Tinodes n. sp. nr. *turanicus*	12IQTRA-0038	TRIRA038-13	7	Iraq
	Tinodes unicolor	10HMCAD-493	HMKKT493-10	8	Austria
	Tinodes unicolor	TFLAN218-11	UA-SG-TRICH- D83		Belgium
	Tinodes unicolor	TFLAN219-11	UA-SG-TRICH- D84		
	Tinodes unicolor	TFLAN219-11	BIOUG16521-D08	9	Belgium
	Tinodes waeneri	TTWAE1	NIP001-16	10	Croatia
	Tinodes waeneri	TFLAN171-11	UA-SG-TRICH-D86	11	Belgium
	Tinodes waeneri	12HMCAD-62	KJTRI057-13	12	Austria
	Tinodes waeneri	GBMIN40244-13	FN179050	13	Belgium
	Tinodes braueri	TTBRA1	NIP005-16	14	Croatia
	Tinodes pallidulus	TTPAL1	NIP006-16	15	Croatia
	Tinodes pallidulus	12HMCAD-068	BHMKK235-12	16	Austria
	Tinodes rostocki	12HMCAD-76	KJTRI071-13	17	Austria
	Tinodes dives	TTDIV1	NIP007-16	18	Croatia
	Tinodes dives consiglioi	07HMCAD-0357	HMCAD357-08	19	Italy
	Tinodes dives consiglioi	07HMCAD-0359	HMCAD359-08	20	Italy
	Tinodes dives consiglioi	07HMCAD-0361	HMCAD361-08		
	Tinodes dives consiglioi	07HMCAD-0360	HMCAD360-08	21	Italy
	Tinodes dives consiglioi	07HMCAD-0358	HMCAD358-08	22	Italy
	Tinodes higashiyamanus	AB764093	GBMIN17824-13	23	Japan
	Tinodes provo	08OFCAD-1186	NECAD331-08	24	United States
	Tinodes provo	08OFCAD-1187	NECAD332-08	25	United States
	Tinodes provo	08OFCAD-1188	NECAD333-08	26	United States
	Psychomyia flavida	07EVCAD-0528	EVCAD528-07	27	Canada
	Psychomyia morisitai	AB764095	GBMIN17823-13	28	Japan
	Lype diversa	08DRCAD-049	DRCAD049-08	29	United States
	Lype excisa	AB764091	GBMIN17825-13	30	Japan
	Eoneureclipsis montanus	AB744044	GBMIN21069-13	31	Japan
	Eoneureclipsis yaeyamaensis	AB764090	GBMIN17793-13	32	Japan
	Eoneureclipsis okinawaensis	AB764089	GBMIN17826-13	33	Japan

rity search was performed using the BOLD Identification Engine (by April 2016) which uses all sequences uploaded to BOLD from public and private projects to locate the closest match.

Along with newly obtained *Tinodes* DNA barcodes, data set for phylogenetic analysis contained all available *Tinodes* DNA barcode sequences retrieved from the Barcode of Life Data Systems (BOLD; Ratnasingham & Hebert, 2007) and GenBank (Benson *et al*., 2009) (Tab. 1) as well as sequences of *Psychomyia flavida* Hagen, *Psychomyia morisitai* Tsuda, *Lype diversa* (Banks), *Lype excise* Mey, *Eoneureclipsis montanus* Torii & Nishimoto, *Eoneureclipsis yaeyamaensis* Torii & Nishimoto, *Eoneureclipsis okinawaensis* Torii & Nishimoto that were used as outgroups. Prior to analyses sequences were aligned using the program BioEdit (Hall, 1999) and collapsed into unique haplotypes using FaBox v.1.41 (Villesen, 2007). Phylogeny-based identification was conducted by using three different methods of tree reconstruction: Neighbor-Joining (NJ) and Maximum likelihood (ML) as implemented in MEGA 6.0. (Tamura *et al*., 2013) and Bayesian Inference (BI) in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). For ML analysis and BI the best-fitting model of DNA substitution under Bayesian information criterion, General Time Reversible model (GTR+G+I), was selected using the jModelTest 0.1.1 (Posada, 2008). The Bayesian analysis (2 independent runs of four Markov chains) was conducted for 3,000,000 generations sampling every 100th generation and discarding

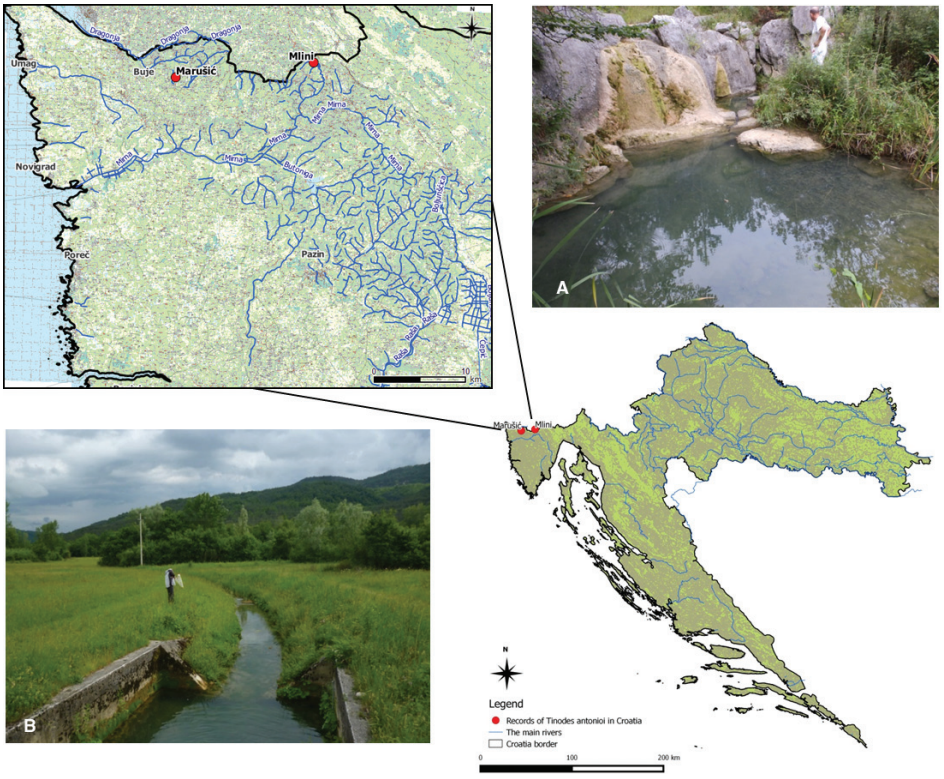

Fig. 1A-B. Map of Croatia with the study sites: a) Marušića Stream, b) Mlini Stream.

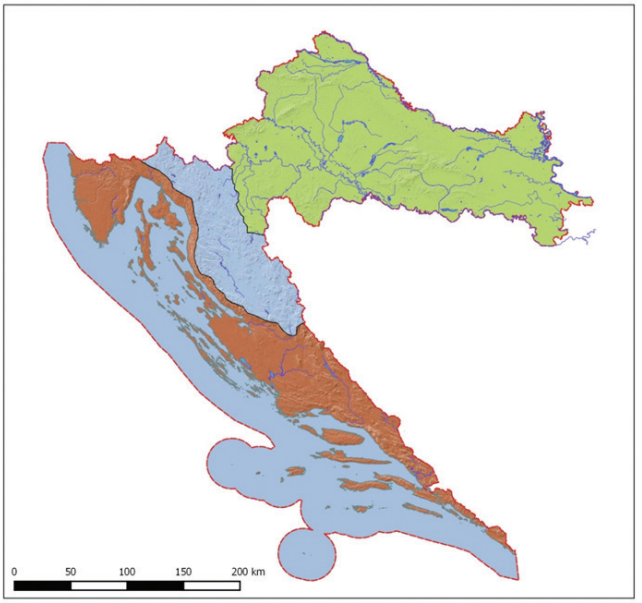

Fig. 2. Division of Croatia into: inland (Pannonian-peripannonian) (green colour), mountainous part (blue colour) and Mediterranean part (brown colour), according to Bertić *et al.* (2001).

the first 20% of trees as burn in. The nodal support for NJ and ML trees was assessed by 2000, and 1000 bootstrap replicates, respectively.

The uncorrected pairwise divergences between specimens (*p*-distance) based on the mt *COI* barcode sequences were calculated in MEGA 6.0. (Tamura *et al.*, 2013). Species delimitation method Automatic Barcode Gap Discovery, ABGD (Puillandre *et al.*, 2012) based on the barcode gap (difference between inter- and intraspecific genetic distances) was applied to estimate the number of hypothetical species within data set. Data set (containing all *Tinodes* DNA barcode sequences as well as 2 *Lype diversa* mtDNA sequences) was submitted to the ABGD online website and analysed under 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.

RESULTS AND DISCUSSION

Faunal data

While the NIP project was being conducted in the area of the Marušića Stream and Mlini Stream, three species from the *Tinodes* genus were recorded: *Tinodes antonioi* (Figs. 3A-B, 4A-B): Mlini Stream 4.09.2014, 1 ♂, 1♀, Marušići Stream 25.05.2015. 1♂; *T. waeneri*, spring of the Marušića Stream 25.05.2015 1♂, 1♀, 9.08.2014 1♂♂, 5.09.2014. 2♂♂, 2♀♀ and *T. pallidus*: spring of the Marušića Stream 25.05.2015. 2♂♂. 1♀; *T. antonioi* never having been recorded in the fauna of Croatia previously. The species was described by Botosaneanu and Taticchi-Viganò from specimens collected in Italy (Botosaneanu &

Taticchi-Viganò, 1974). The female of the species was later described in a paper of Cianficconi *et al.* (1994), also from specimens taken in Italy. *T. antonioi* has rather small dimensions, and from the data in the literature, the span of the anterior wings is 8 mm (Malicky, 2004). In the *Atlas of European Trichoptera* (Malicky, 2004) the morphological features of the genitalia of females from the genus *Tinodes* have been presented for only 10 species, for in most species they are extremely similar and the females of the different species cannot be distinguished from each other by their morphological characteristics. This is not the case with the genitalia of the females of the species *Tinodes antonioi*, for they are very specific and easily identifiable (Cianficconi *et al.*, 1999) so that unlike many other species of the genus *Tinodes* the find of a female is sufficient for certain establishment of the presence of this species in a given area.

In Europe, *T. antonioi* has been recorded in France, Italy, Switzerland, Slovenia (Cianficconi, 2002; Krušnik & Urbanič, 2002; Botosaneanu & Giudicelli, 2004) (Fig. 5) and now in Croatia. The greatest number of finds of this species have been made in Italy, and finds in Croatia are peripheral in one part of its south-east distribution area. Apart from the species *T. antonioi*, two other species of *Tinodes* have been recorded in Istria: *T. pallidulus* and *T. waeneri.*

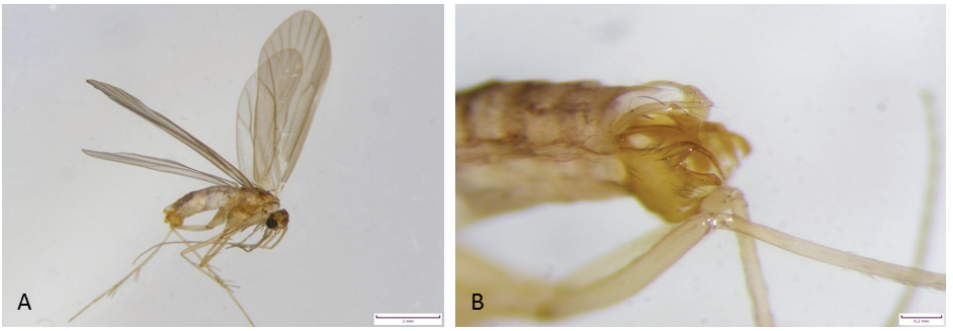

Fig. 3. A-B. Adult (A) and morphological features of male genitalia (B – lateral view) of the species *Tinodes antonioi*.

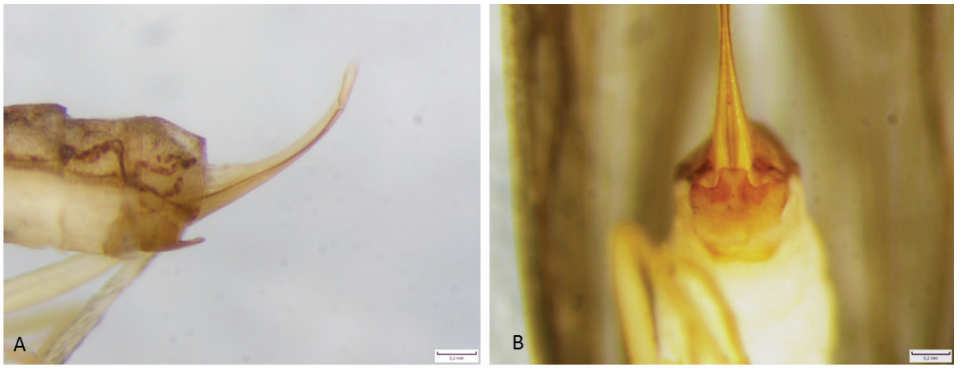

Fig. 4. A-B. Morphological feature of female genitalia of the species *Tinodes antonioi*: A – lateral view, B – ventral view.

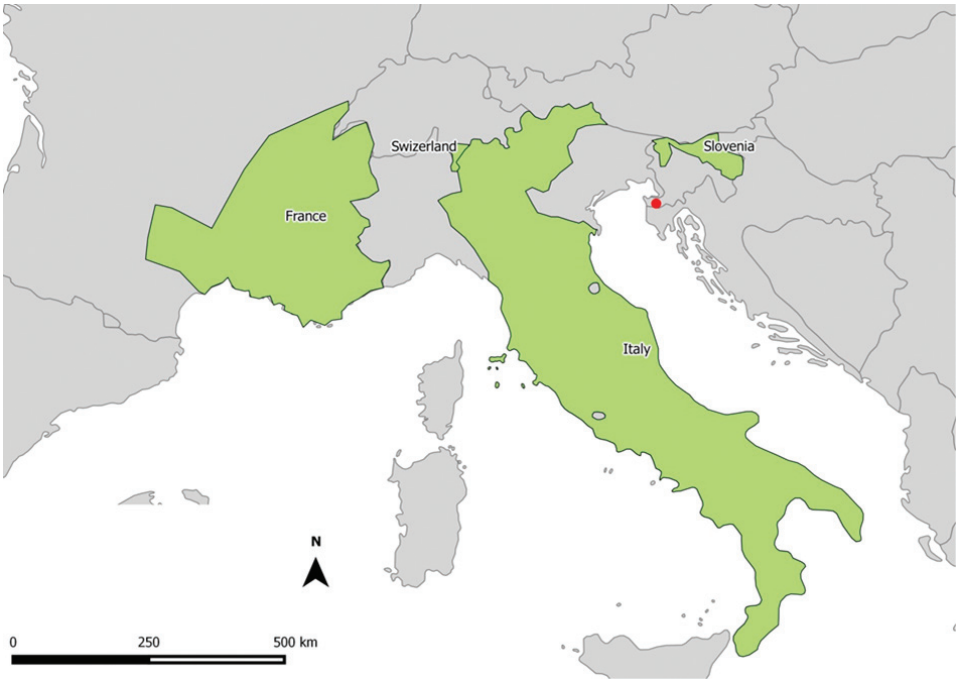

Fig. 5. Distribution of *Tinodes antonioi* in Europe (green field), compiled from Robert (2015) available at online www.freshwaterecology.info and Malicky (2013) available at web portal Fauna Europaea with new findings in Croatia (red point).

Genetic identification of **Tinodes** *species and phylogenetic analysis*

BOLD Identification Engine failed to identify *Tinodes antonioi* (TTANT1) and *Tinodes* sp. specimens (female TTIN1 and male TTIN2 having slightly different morphology) to species level since DNA barcode of this species has not yet been submitted to BOLD. That is why the molecular identification of *Tinodes* sp. could be carried out only on the basis of comparison of DNA sequences of those samples with the DNA sequence of a sample undoubtedly morphologically identified as *T. antonioi* (TTANT1) and also DNA sequences of all known species of genus *Tinodes* occuring in Croatia except *T. andrasi*. The male *T. antonioi* (TTANT1) and female *Tinodes* sp. (TTIN1) have identical *COI* haplotype, and haplotypes of *Tinodes* sp. male (TTIN2) and *T. antonioi* (TTANT1) differ by a single base substitution. In phylogenetic trees derived by all three methods (Neighbor-Joining, Maximum-Likelihood and Bayesian Inference) those three samples cluster within highly supported monophyletic clade (support 100/100/1, Fig. 6) proving *Tinodes* sp. belonging to species *T. antonioi. T. antonioi* is clearly separated from all other *Tinodes* species recorded in Croatia (Fig. 6).

The *p*-distances of the 658-bp-long fragment of the mt *COI* gene (barcoding region) between different *Tinodes* species range from 10% to 27%, while the obtained intraspecific *p*-distances are in range of 0–3% (Tab. 2). The lowest interspecific distance values are recorded between *T. antonioi* and *T.* n. sp. nr. *turanicus* (10%) and higest between *T. antonioi* and *T. higashiyamanus* (24%) (Tab. 2).

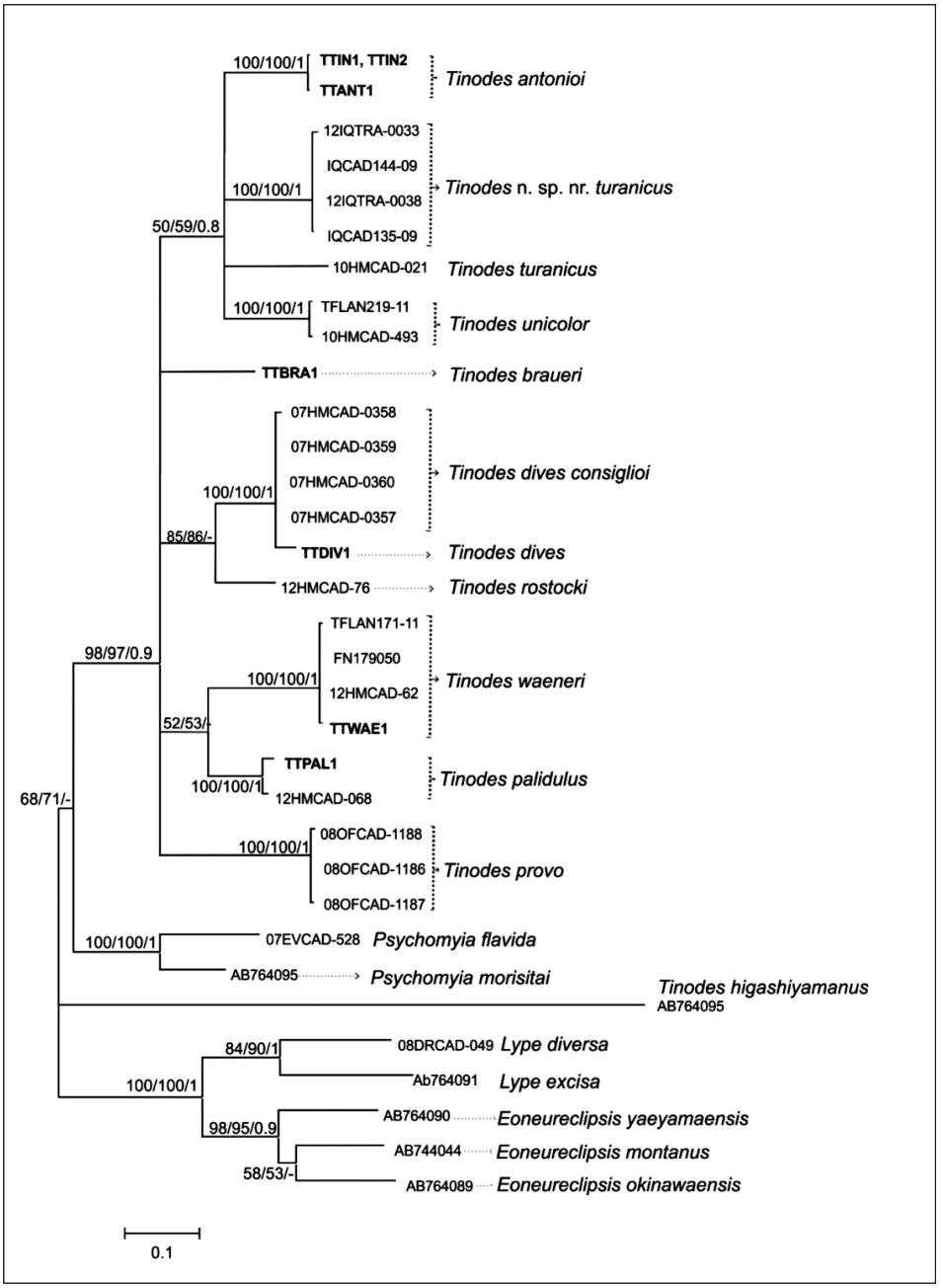

Fig. 6. Maximum likelihood phylogram based on 658 bp long fragment of the DNA barcode region showing the relationships between *Tinodes* species from this study and different species from family Psyiichomidae. Numbers above the branches represent bootstrap support (BS) and Bayesian posterior probabilities (BPP) in order NJ/ML/BA. BS values less than 50 and BPP values less than 90 are not shown. Specimen ID from sequences obtained in this study (from Croatia) are written with bold letters.

Tab. 2. Inter- and intraspecific p-distances of mt *COI* sequences for *Tinodes* species and *Psychomyia flavida*.

COI	*T. antonioi*	*T. turanicus*	*Ti*-n. sp. nr. *turanicus*	*T. unicolor*	*T. waeneri*	*T. braueri*	*T. pallidulus*	*T. rostocki*	*T. dives*	*T. dives consiglioi*	*T. higashiyamanus*	*T. provo*	*Psychomyia flavida*
T. antonioi	0-0.1												
T. turanicus	10.9	0											
T. sp. nr. *turanicus*	9.7-10.3	9.6-9.9	0.1-1										
T. unicolor	11.1	11.3-11-6	10.3-11.3	0.3									
T. waeneri	13.4-13.8	13.5-14.2	12-13.2	12.6-13.8	0.3-0.9								
T. braueri	10.5	12.2	10.6-11.1	12-12.3	12.3-13.2	0							
T. pallidulus	11.7-11.9	11.7-12.3	10.2-10.7	11.1-11.7	9.7-10.8	10.1-10.3	0-0.2						
T. rostocki	13.5	11.4	11.4-11.6	11.5-11.7	12-12.9	10.5	10.34	0					
T. dives	13.9	13.9	11.6-11.9	14.4-14.7	12.5-12.8	12.9	10.5-10.6	9.9	0				
T. dives consiglioi	13.1-13.2	13.1-13.2	10.8-11.1	13.2-13.9	11.7-12.2	11.4-11.9	9.9-10.5	8.7-9.1	2.6-2.8	0.1-0.5			
T. higashiya-manus	20.4	12.2	22-22.3	20.1-20.4	21.2-22.5	21.4	19.8-20.2	20.2	20.9	20.4	0		
T. provo	13.9-14.1	14.1-14.3	11.6-12.2	13.1-13.4	12.8-13.7	13.1-13.2	11.4-12.2	13.7-13.8	13-13.3	12.6-12.9	21.9-22.3	0	
Psychomyia flavida	18.2	17.4	16.6-16.7	18-18.3	16.1-16.8	15.5	15.8-16.1	17.8	16.8	16.1	20.8	16.1-16.3	0

The ABGD analysis clustered the sequences into 13 groups. *T. antonioi* formed one group separated from other *Tinodes.* Each of *Tinodes* species and outgrups clustered according to systematic (Fig. 7). The ABGD method shows intra- and interspecific distance variation, the barcoding gap between species and genus (Fig. 8).

Although direct molecular level conformation of morphological identification of specimens from Istria as *T. antonioi* could not been made, molecular analysis proved that those specimens do not belong to any other *Tinodes* species currently recorded in Croatia. The only exception is *T. andrasi* whose DNA barcodes was not available in this study. However, since this species is endem of Konavle region (south Croatia) it is hard to belive that Istrian specimens identified as *T. antonioi* in this study could be missidentified with this southern species.

The phenotype differences between Croatian population of *T. antonioi*, are not reflected on the molecular (mt*COI*) level. Comparison of DNA barcode sequences of morphologically different *T. antonioi* proved once again that morphological differences in caddisflies do not necessarily indicate new taxa and that inclusion of some genetic marker in the analysis have to be considered. As in many previous studies (e.g. Pauls *et al*., 2010; Jackson *et al*., 2014; Zhou *et al*., 2007; Zhou, 2009) also in this study, DNA barcoding proves as fast and reliable method for resolving morphological doubts.

The clear DNA barcode gap exists within *Tinodes.* The minimum value of uncorrected pairwise sequence divergences (*p*-distances) for DNA barcode region between *Tinodes* species analysed in this study (9%) is higher than minimum values of the interspecific variability usually observed in caddisflies for the mt*COI* barcode region (8% Pauls *et al*., 2010; 5.3% Zhou, 2009; 8.2% Graf *et al*., 2015). The maximum observed intraspecific divergence value was 3%. The unusually high maximum intraspecific divergence (3%) is recorded between *T. dives* from Croatia and subspecies *T. dives consiglioi* from Italy indicating the existence of deeply separated evolutionary lineages within species. It is interesting that lowest interspecific divergence values for DNA barcode region was found between geographically distant species *T. antonioi* from Croatia and *T.* n. sp. nr. *turanicus* from Iraq, however the sister relation between them was not supported in phylogenetic analyses. Detailed phylogenetic analysis of the genus *Tinodes* would require the implementation of additional mitochondrial and nuclear markers. At the moment, obtained DNA barcodes of rare Croatian *Tinodes* species represent valuable contribution to global DNA barcoding effort. In most papers this Alpino-Apenninic endemic, *T. antonioi*, is presented with few specimens. According to Cianficconi & Corallini (2010), 1 male, 1 female in Molise, Italy; according to Botosaneanu & Giudicelli (2004) in France on each of four locations they collected one male and on only one location one female; during six years of research on 38 sampling sites on the River Chiascio in Umbria, Italy according to Cianficconi *et al*., 1999 most specimens are present with one or two female and only one time there were 11 specimens.

Ecological data

The ecological and ethological characteristics of *T. antonioi* are not known in detail, although they do fit within some of the properties of the genus itself. We recorded the species *T. antonioi* only in the upper reaches of the two streams, the Milni and the Marušića, and did not record it in any larger watercourses (the River Raša) or in the Butoniga Reservoir in Istria during this research. The find of *T. antonioi* in the Milni Stream is interesting; it was collected only at a site located downstream from the spring, one that is subject to considerable anthropogenic influence, which means that this species

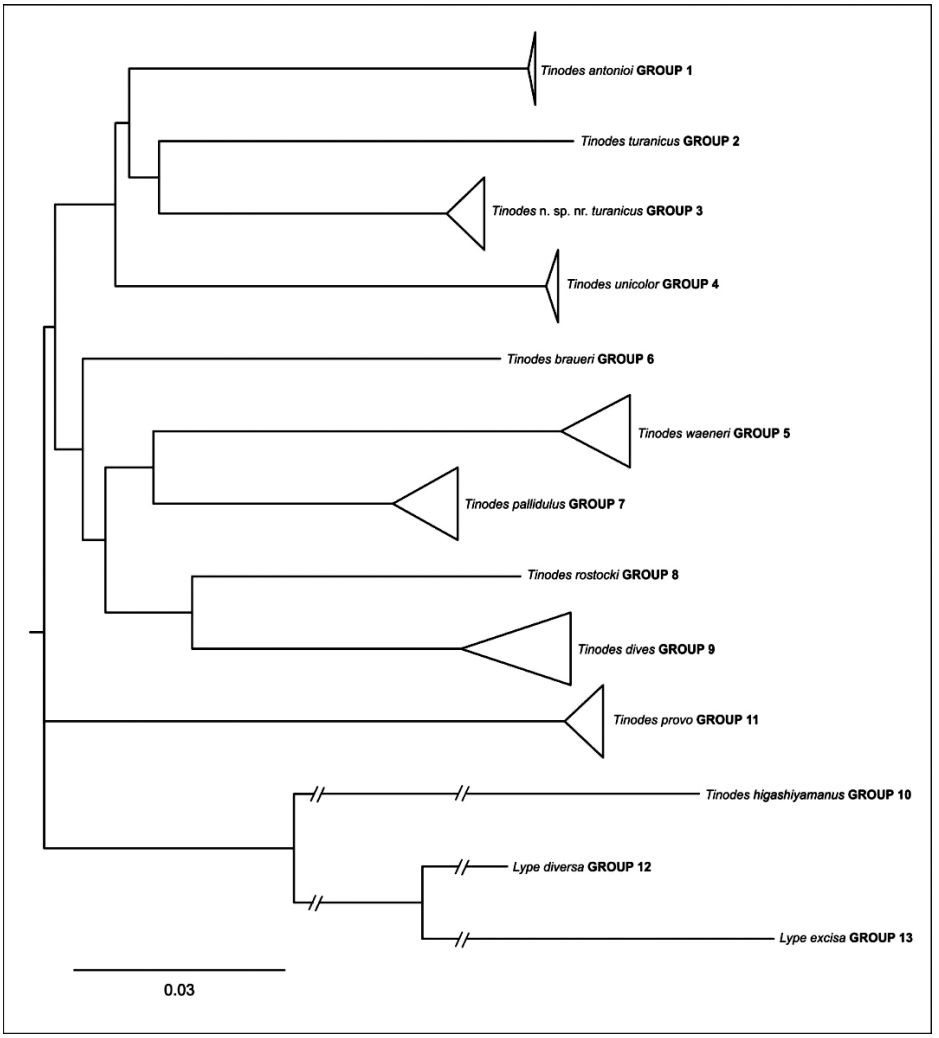

Fig. 7. Neighbor-Joining phylogenetic tree showing groups (hypothetical species) as revealed by Automatic Barcode Gap Discovery (ABGD) analysis.

may appear at this kind of habitat. We have to point out however that it seems to us the water in the Mlini at this site is of good quality (without either organic or inorganic pollution) but there has been considerable anthropogenic impact on the hydrological characteristics of the stream in his part (Fig. 1B).

This research determined adult emergence of *T. antonioi* to be in May and and September. Since collections were not made in all the spring, summer and autumn months, an earlier as well as a later emergence than the months given is also possible. Details in the literature indicate findings of *T. antonioi* in a fairly long period from May to September (Botosaneanu & Giudicelli, 2004). The strongest emergence period was recorded for Italy in August by Cianficconi *et al.* (1999). According to Botosaneanu & Giudicelli

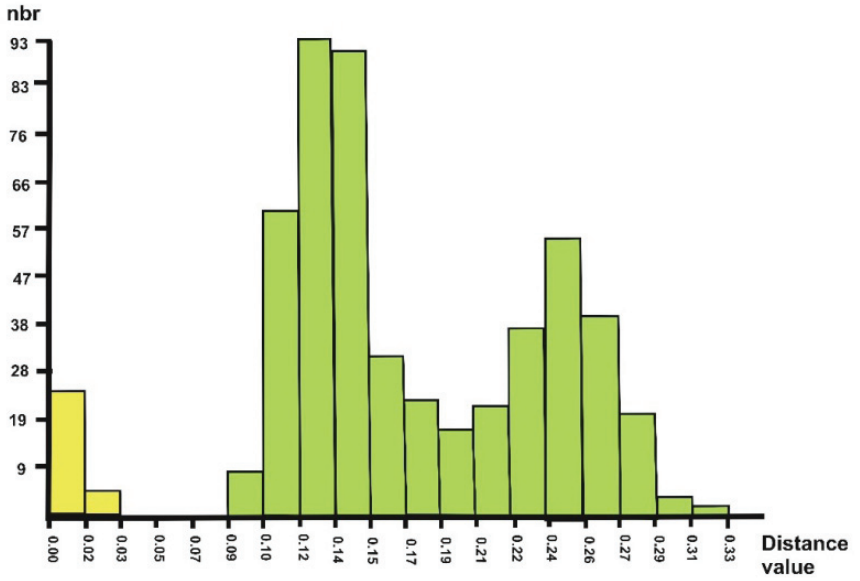

Fig. 8. Histogram depicting the frequency distribution of K2P distances within *Tinodes* as revealed by ABGD analysis. The horizontal axis shows the pairwise K2P-distance, and the vertical axis shows the number of pairwise sequence comparisons. On the left side of histogram (yellow colour) is intraspecific and on right is interspecific (green colour) distance variation.

(2004), *T. antonioi* comes in France from 120 to 1070 m above sea level, and research in Italy has found it at above sea level heights of from 360 to 870 m (Cianficconi *et al.*, 1999).

All the adults were collected at night, in UV lamp-assisted catching, and thus in this research we recorded no diurnal *T. antonioi* activity.

During caddisfly research in Croatia that has in the last 20 years included the collection of adults at more than 240 sites, *T. antonioi* has been recorded only in this research, which suggests that apart from Istria it probably does not come to any other region in Croatia. Because there are only these two finding sites in Croatia as of present, in order to preserve this species in the country's fauna, the site at the Marušića Stream, as well as the stream itself, particularly the upper part, should be protected against any anthropogenic impacts, and any such impact on Mlini Stream should be reduced to the minimum level possible.

Biodiversity of the genus **Tinodes** *in Croatia, with a note on its distribution*

The genus *Tinodes* is one of the most numerous in the fauna of Europe, with some one hundred or so species recorded (Malicky, 2004). In the fauna of Croatia, eight species of this genus have been recorded (e.g., Graf *et al*., 2008; Kučinić, 2002; Oláh, 2010; Cerjanec, 2012, M. Kučinić unpublished data): *Tinodes andrasi* Oláh,, *T. antonioi* Botosaneanui & Tattichi-Viganò, *T. braueri* McLachlan, *T. dives* Pictet, *T. pallidus* McLachlan, *T. rostocki* McLachlan, *T. unicolor* Pictet and *T. waeneri* Linnaeus. Larva of *Tinodes braueri* was described from specimens collected in the Rive Cetina in Croatia (Graf *et al*., 2008).

Every one of these species has its own particular features with respect to morphology genetics, ecology and distribution and, when the fauna of Croatia is at issue, particularly remarkable for specificity is the species *Tinodes andrasi*, which has to date been found only in Croatia. This species was described a few years back on the basis of just a single example of a male found in the area of the Ljuta River (*locus typicus*) in Konavle (Dalmatia) (Oláh, 2010).This is to date the only find of this species and still to be attempted is a more detailed investigation of its distribution in Konavle and wider and the find of a female, no collection of which has yet been made, in order to ascertain potential morphological particularities. When the NIP project was conducted in the Konavle region, the collection of caddisflies took place at four sites during 2014. At these sites, the species *T. andrasi* was not ascertained.

Tinodes andrasi, according to the author of the description (Oláh, 2010) is very similar to the species *T. rostocki*, which has also been recorded in Croatia (north), as well as more to the south, in Albania and Greece (Malicky, 2005; Oláh & Kovács, 2014). Since the morphological differences of the male genitalia in the species *T. rostocki* and *T. andrasi* are not very great (which is the case with a certain number of other species of caddisfly, (Malicky, 2004, 2014; Oláh, 2010; Previšić *et al*., 2014a) and the ranges of *T. andrasi* and *T. rostocki* are in a sense overlapping, additional molecular research into these two species is required for the sake of determining their exact taxonomic and phylogenetic relations. It can be assumed from their distributions and the similarity of the male genitalia that they have great phylogenetic affinity.

Similarly, a very nice example of speciation and the relations of two phylogenetically closely related species, one of which has a very large and the other a very small range, can be found in the genus *Ecclisopteryx*, in which the species *E. keroveci* has a large range, extending from the spring of the Čabranka to Macedonia (Previšić *et al*., 2014a; Oláh & Kovács, 2014), while the endemic species *E. ivkae* (Previšić *et al*., 2014a) makes a distinctly separate enclave in the central part of the area in the source areas of the Cetina River. The species *E. keroveci* and *E. ivkae* are fairly, or very, similar in their morphologies, although by careful analysis of the genitalia it is possible to distinguish both males and females, they are genetically different, but not in a high degree and have considerable differences in the morphology of the larvae (Previšić *et al*., 2014a). All these features are

Tab. 3. Distribution of species of the genus *Tinodes* in inland, central-mountain and Mediterranean parts of Croatia.

Species	Inland part of Croatia	Mountainous part of Croatia	Mediterranean part of Croatia
Tinodes andrasi	-	-	+
Tinodes antonioi	-	-	+
Tinodes braueri	-	+	+
Tinodes dives	+	+	+
Tinodes pallidus	+	-	+
Tinodes rostocki	+	+	-
Tinodes unicolor	+	+	-
Tinodes waeneri	-	+	+
TOTAL	4	5	6

enough for the population in the upper course of the Cetina to be defined as specific at the level of a separate species, which has been described as such (Previšić *et al*., 2014a).

In research to date in the Mediterranean region of Croatia, 6 species of the genus *Tinodes* have been recorded, with five in the upland or mountain region and four species in the inland or continental region (Tab. 3).

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SAŽETAK

Prvi nalaz *Tinodes antonioi* Botosaneanu & Taticchi-Viganò, 1974 (Insecta, Trichoptera) u Hrvatskoj s DNA barkod i ekološkim podacima i noticom na bioraznolikost i rasprostranjenost roda *Tinodes* u Hrvatskoj

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Vrsta *Tinodes antonioi*, zabilježena je po prvi puta kao nova u fauni Hrvatske u sjevernoj Istri, na dva lokaliteta (potoci Mlini i Marušića potok). U radu se daju informacije *o T. antonioi* u Hrvatskoj (DNA barkod podaci, periodi nalaza, lokaliteti nalaza, broj prikupljenih mužjaka i ženki), kao i bioraznolikost i rasprostranjenost roda *Tinodes* u Hrvatskoj. Rod *Tinodes* zastupljen je u fauni Hrvatske s 8 vrsta, od kojih 4 dolazi u panonskoj-peripanonskoj, 5 u gorskoj, a 6 u mediteranskoj Hrvatskoj. Daje se osvrt i na *Tinodes andrasi*, endemske vrste tulara, zabilježenu samo na jednom lokalitetu u Hrvatskoj (Konavle, Dalmacija).