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FIRST DNA BARCODING AND NEW RECORDS OF THE MEDITERRANEAN CADDISFLY SPECIES *MICROPTERNA WAGENERI* MAL. (TRICHOPTERA, LIMNEPHILIDAE) IN CROATIA WITH NOTE ON DNA BARCODING AND DIVERSITY OF GENUS *MICROPTERNA* IN CROATIA

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The paper brings data about the new findings of the rare Mediterranean caddisfly species *Micropterna wageneri* Mal. in Croatia with first data of DNA barcoding for this species. The species is recorded in Konavle region and on the Mt. Biokovo. We also present the diversity of the genus *Micropterna* in Croatia and discuss issues related to the DNA barcoding for this genus in Croatia and Europe.

Key words: Trichoptera, *Micropterna*, DNA barcoding, museum collections, Croatia, Biokovo, Konavle

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Rad donosi podatke o novim nalazima rijetke mediteranske vrste tulara *Micropterna wageneri* Mal. u Hrvatskoj, s prvim podacima o DNA barkodiranju te vrste. Vrsta je zabilježena u Konavlima i na Biokovu. Također se predstavlja raznolikost roda *Micropterna* u Hrvatskoj i raspravlja o DNA barkodiranju tog roda u Hrvatskoj i Europi.

Ključne riječi: Trichoptera, *Micropterna*, DNA barkodiranje, muzejske zbirke, Hrvatska, Biokovo, Konavle

INTRODUCTION

Identification of various animal species is a starting point of research into the morphology, histology, genetics, physiology, distribution, ecology, phylogenetics and other biological characteristics of individual species. Such approach in biology is based on the rules of binomial nomenclature set up by Carl Linnaeus in the 18th century (Linneaus, 1758). Morphological characteristics have for a long time been used as the basis for determination of organisms and this practice is being used until today. Determination of populations to the level of species enables us to understand the characteristics of fauna, its diversity and biogeography within a certain area. Due to a high complexity of diversity of numerous groups of animals, in particular invertebrates, many of which are similar, sibling or cryptic species, other methods are used to support a more exact determination of species and finding those that cannot be determined by conventional morphological methods (Ross, 1974; Bickford *et al.*, 2007: Dincă *et al.*, 2013, 2016; Previšić *et al.*, 2014). One of the recent and most frequently used methods in analysis of biodiversity and determination of organisms within a particular area is the DNA barcoding method (Hebert *et al.*, 2003a, 2003b). This method is used to identify animal species and is based on sequencing of the standardized segment of the mitochondrial (mt) cytochrome c oxidase subunit 1 (*COI*) gene (Hebert *et al.*, 2003a, 2003b).

Research of caddisflies in Croatia became more intensive in the second half of the 20th century through a larger number of limnological studies (Matoničkin *et al.*, 1971; Matoničkin, 1987; Matoničkin & Pavletić, 1967; Habdija, 1989) and continued with increased intensity during the last two decades with numerous taxonomic, faunal and ecological studies (e.g. Krulik, 1979; Kučinić & Ilić, 1993; Kučinić, 2002; Kučinić & Malicky, 2002; Habdija *et al.*, 2004; Previšić *et al.*, 2007, 2009, 2014; Kučinić *et al.*, 2008, 2011; Vučković *et al.*, 2011; Szivák *et al.*, 2013; Matić *et al.*, 2016). Application of the DNA barcoding method in these studies has started recently in taxonomic and faunistic studies of Croatian Trichoptera (Kučinić *et al.*, 2013, 2016).

This paper presents the new finding of the rare Mediterranean caddisfly species *Micropterna wageneri* Malicky 1971 in Croatia with first data of DNA barcoding for this species. We also present the diversity of the genus *Micropterna* Stein in Croatia and discuss issues related to the DNA barcoding for this genus in Croatia and Europe.

MATERIAL AND METHODS

Fieldwork and research areas

Our research took place in the Konavle region and in the Nature Park Biokovo. During our investigation in the Konavle region (valley) we collected caddisfly specimens on several locations on springs, streams and the River Ljuta. Konavle region is located in the southern part of Croatia (Dalmatia region). In this part of Croatia there are only few permanent streams and rivers and some temporary streams. Very interesting aquatic habitat in Konavle valley are small manmade canals. We visited Konavle valley three times during 2015 and one time during 2016.

Nature Park Biokovo is situated in central Dalmatia. It is very impressive mountain area with lot of endemic species of animals and plants (e.g. Casale & Jalžić, 1988). The highest peak is Sv. Jure with 1762 m above sea level. Except several small springs and streams on south expositions on lower elevation, in the highest area of Biokovo there

are no natural aquatic habitats. During 2015 we visited Nature Park Biokovo three times and one time during May of 2017.

Adults of caddisflies were collected using UV light traps during night and with entomological net during day. The specimens were stored in 96% alcohol. After determination all caddisfly specimens collected during 2015 were deposited in the Trichoptera collection in the Croatian Natural History Museum in Zagreb. NIP Trichoptera collection is one of the results of the NATURA 2000 Integration Project (NIP). Specimens collected in 2016 were deposited in the Trichoptera collection Vučković and in the Trichoptera collection Kučinić.

For determination of species from the genus *Micropterna* we used standard literature Kumanski (1988) and Malicky (2004). Systematic presentation follows Malicky (2004) and Morse (2017).

DNA extraction, PCR amplification and phylogenetic analysis

Genomic DNA was extracted from legs of nine specimens, two *M. wageneri* and other caddisflies listed in Tab. 1 with specimen ID marked with bold letters. All specimens used in this study are kept as voucher specimens in the Trichoptera DNA Barcode Collection in the Croatian Natural History Museum in Zagreb. Genomic DNA was extracted using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Germany) according to the manufacturer's specifications and eluted in 100 μl of elution buffer. For the amplification of the *COI*-5P barcode region LCO1490 and HCO2198 (Folmer *et al*., 1994) primers were used. The volume of mixture for polymerase chain reactions (PCR) was 50 μl. The PCR mixture contained 1 x Go Taq®Reaction Buffer (containing 1.5 mM MgCl2, Promega), 0.2 mM of each dNTP, 0.4 μM of each primer, 1.25 units of Go Taq®DNA Polymerase (Promega) and 5 μl of DNA eluate. PCR cycling conditions comprised an initial denaturation step (94°C for 2 min) followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 90 s, and a final extension step of 72°C for 7 min. Product purification and bidirectional sequencing was performed by Macrogen Inc. sequencing service (Seoul, South Korea) using the amplification primers. Sequences were edited manually and aligned using the program BioEdit (Hall, 1999).

DNA sequences obtained in this study were submitted to Barcode of Life Data Systems (BOLD, Ratnasingham & Hebert, 2007; Tab. 1). For phylogenetic analysis all available *Micropterna* DNA barcode sequences were retrived from the BOLD System database (BOLD IDs are given in Tab. 1). As outgroups we selected four species belonging to different genera of the family Limnephilidae, such as genus *Mesophylax* McLachlan; *Potamophylax* Wallengren; *Allogamus* Schmid; *Halesus* Stephens (Tab. 1). Identical sequences were collapsed into unique haplotypes using FaBox v.1.41 (Villesen, 2007). All haplotypes are listed in Tab. 1. Two different methods of tree reconstruction were used: Neighbor-Joining (NJ) and Maximum likelihood (ML) as implemented in MEGA 7.0. (Kumar *et al*., 2016) to infer phylogeny-based specimens identification. Details of phylogenetic analyses were the same as in Kučinić *et al*. (2016).

Inter- and intraspecific genetic uncorrected pairwise divergences (*p* - distances) were calculated in MEGA 7.0. (Kumar *et al*., 2016). The number of hypothetical species within data set was estimated based on barcode gap (difference between inter- and intraspecific genetic distances) by using Automatic Barcode Gap Discovery, ABGD (Puillandre *et al*., 2012). The mt*COI* data set was submitted to the ABGD online website using the same settings as described in Kučinić *et al*. (2016).

Tab. 1. List of specimens used in the phylogenetic analysis. Specimen ID of the specimens DNA barcoded in this study is indicated in bold.

*Morse, J.C. (ed.) 2017: Trichoptera World Checklist. http://entweb.clemson.edu/database/trichopt/index.htm [Accessed 7 June 2017.

mt *COI* haplotype	**Taxonomic designation according to identifier**	Synonim according to Morse*	Life Stage	Sex	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID
HP 1	*Micropterna wageneri* Malicky		Adult	Female	Mladen Kučinić	Croatia	The creek in village Palje, Konavle region near Dubrovnik	TMWAG_1	NIPM001-17
HP 2	*Micropterna wageneri* Malicky		Adult	Female	Mladen Kučinić	Croatia	Spring of the creek in village Palje, Konavle region near Dubrovnik	TPWAG_1	NIPM002-17
HP 3	*Micropterna nycterobia* McLachlan		Adult	Male	Anđela Ćukušić	Croatia	Sprig of the river Zrmanja near Knin	TMIC_1	NIPM003-17
HP 4	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	Adult	Male	Mladen Kučinić	Croatia	The river Krupa, Krupa monastery, Knin	TMNYC_1	NIPM006-17
HP 5	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	?	?	Kate Perez	Germany	Landskrone, Kreis Ahrweiler	BIOUG17228-F01	GMGML065-14
HP 6	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	Adult	Male	Hans Malicky	Italy	Valle della Rovina, Entraque, Piedmont	HMCAD0111-107	HMKKT924-11
HP 7	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	Adult	Male	Hans Malicky	Greece	Passhoehe NE Karies Lakonien Parnon, Peloponnese	07HMCAD-0173	HMCAD173-08
HP 8	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	Adult	Male	Hans Malicky	Greece	Passhoehe NE Karies Lakonien Parnon, Peloponnese	07HMCAD-0175	HMCAD175-08
HP 9	*Stenophylax nycterobius* McLachlan	*Micropterna nycterobia*	Larva	?	Monika Hess	Germany	Einsiedelbach oberhalb Einmuendung Eichelbach, am¸ Bavaria	BCZSMAQU00923	FBAQU1208-12
HP 10	*Stenophylax testaceus* Gmelin	*Micropterna testacea*	Adult	?	Hans Malicky	Austria	St. Konrad - Hausern	10HMCAD-261	10HMCAD-261
HP 11	*Micropterna testacea* Gmelin		Adult	Male	Hans Malicky	Italy	Abruzzi, Rieti, Mte Terminillo	12HMCAD-052	BHMKK233-12

mt *COI* haplotype	Taxonomic designation according to identifier	Synonim according to Morse *	Life Stage	Sex	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID
HP 12	*Stenophylax testaceus* Gmelin	*Micropterna testacea*	Adult	Male	Hans Malicky	Italy	Valle della Rovina, Entraque, Cuneo, Piedmont	HMCAD0111-108	HMKKT925-11
HP 13	*Stenophylax testaceus* Gmelin	*Micropterna testacea*	Adult	Male	Carmen Zamora Munoz	Spain	Nacimiento, Rio Guadalquivir, Jaen, Andalusia	10ESCAD-011	ESCAD011-10
	Stenophylax testaceus Gmelin	*Micropterna testacea*	Adult	Male	Carmen Zamora Munoz	Spain	Nacimiento, Rio Guadalquivir, Jaen, Andalusia	10ESCAD-012	ESCAD012-10
	Stenophylax testaceus Gmelin	*Micropterna testacea*	Adult	Male	Carmen Zamora Munoz	Spain	Nacimiento, Rio Guadalquivir, Jaen, Andalusia	10ESCAD-013	ESCAD013-10
	Stenophylax testaceus Gmelin	*Micropterna testacea*	Adult	Male	Carmen Zamora Munoz	Spain	Nacimiento, Rio Segura, Jaen, Andalusia	10ESCAD-015	ESCAD015-10
HP 14	*Micropterna testacea* Gmelin		Adult	Female	Mladen Kučinić	Croatia	The river Lika, Bilaj, Gospić	TMTES_1	NIPM008-17
	Stenophylax testaceus Gmelin	*Micropterna testacea*	Adult	?	Hans Malicky	Czech Republic	Sumava, Horni Plana Olsina village (Langebrucke)	10HMCAD-077	HMKKT077-10
	Stenophylax testaceus Gmelin	*Micropterna testacea*	Adult	Male	Carmen Zamora Munoz	Spain	Nacimiento, Rio Segura, Jaen, Andalusia	10ESCAD-014	ESCAD014-10
HP 15	*Micropterna testacea* Gmelin		Adult	Male	Hans Malicky	Switzerland	Chalet de la Dole, Cheserex	12HMCAD-143	KJTRI133-13
HP 16	*Micropterna lateralis* Stephens		Larva	?	Reinhard Mueller	Germany	Lower Saxony, Schierpkebach	GBEPT617-14	KX294231
HP 17	*Stenophylax lateralis* Stephens	*Micropterna lateralis*	Adult	Male	Juha Salokannel	Finland	Poksaselkae, Sodankylae, Lapponia kemensis pars orientalis	JSlk-2011F120	RIFI660-12

mt *COI* haplotype	Taxonomic designation according to identifier	Synonim according to Morse *	Life Stage	Sex	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID
	Limniphilidae		Adult	Male	Mladen Kučinić	Croatia	River Dretulja, location Plaški, near Ogulin	TLIM_2	NIPM005-17
	Micropterna sequax McLachlan, syn. *Micropterna lateralis*		Adult	?	Hans Malicky	Croatia	Exit from motorway, Ogulin	10HMCAD-035	HMKKT035-10
	Stenophylax sequax, syn. *Micropterna lateralis*		Adult	?	Hans Malicky	Croatia	Exit from motorway, Ogulin	10HMCAD-306	HMKKT306-10
HP 18	*Stenophylax sequax*, syn. *Micropterna lateralis*		Adult	?	Hans Malicky	Croatia	Exit from motorway, Ogulin	10HMCAD-307	HMKKT307-10
	Micropterna lateralis Stephens		Larva	?	Reinhard Mueller	Germany	Schierpkebach, Lower Saxony	GBOL03947	GBEPT618-14
	Micropterna lateralis Stephens		Adult	?	Arne Beermann	Germany	Breitenbach (first order stream), Hesse	M1_BBT130724_01	GST398-15
	Micropterna lateralis Stephens		Adult	Male	Hans Malicky	Austria	Lunz Pressireith	12HMCAD-77	KJTRI072-13
HP 19	*Micropterna* sp.		Adult	Female	Anđela Ćukušić	Croatia	Spring of river Una, near Knin	TMIC_2	NIPM004-17
HP 20	*Micropterna sequax* McLachlan		Adult	Male	Mladen Kučinić	Croatia	Spring of the creek Marušić, Roč, Istra	TMSEQ_1	NIPM007-17
	Micropterna sequax McLachlan		Adult	Male	Lars Hendrich	Germany	Emse bei Winterstein, Thuringia	BCZSM_EPH_0171	FBAQU1406-13
	Micropterna sequax McLachlan		Adult	Male	Lars Hendrich	Germany	Emse bei Winterstein, Thuringia	BCZSM_EPH_0175	FBAQU1410-13
HP 21	*Micropterna sequax* McLachlan		Adult	Male	Lars Hendrich	Germany	Emse bei Winterstein, Thuringia	BCZSM_EPH_0188	FBAQU1423-13
	Micropterna sequax McLachlan		Adult	Male	Lars Hendrich	Germany	Breitenbach, Hesse	Ms_BBT130828_02	GST400-15
	Micropterna sequax McLachlan		?	?	Arne Beermann	Germany	Breitenbach (first order stream), Hesse	Ms_BBT130828_01	GST399-15

mt *COI* haplotype	Taxonomic designation according to identifier	Synonim according to Morse *	Life Stage	Sex	Specimen morphological identification by:	Country	Location	Specimen ID	BOLD Sequence ID
HP 22	*Micropterna sequax* McLachlan		Adult	Male	Lars Hendrich	Germany	Emse bei Winterstein, Thuringia	BCZSM_EPH_0185	FBAQU1420-13
HP 23	*Stenophylax sequax* McLachlan	*Micropterna sequax*	Adult	Female	Hans Malicky	Greece	Passhoehe NE Karies, Lakonien Parnon, Peloponnese	07HMCAD–0123	HMCAD123-08
HP 24	*Stenophylax sequax* McLachlan	*Micropterna sequax*	Adult	?	Hans Malicky	Czech Republic	Maly Polec, Churanov, Sumava	HMCAD1211-164	BHMKK164-12
HP 25	*Stenophylax sequax* McLachlan	*Micropterna sequax*	Adult	Male	Hans Malicky	San Marino	Rio delta Fratta, La Venezlat	HMCAD0111-97	HMKKT914-11
HP 26	*Micropterna solotarevi* Martynov		Adult	Male	Hans Malicky	Iran	Golestan Deraznu	HMCAD0810-173	HMKKT800-10
HP 27	*Micropterna fissa* McLachlan		Adult	?	Hans Malicky	Italy	NW, MP Maremma, Grosseto, Orbetello	12HMCAD-049	BHMKK197-12
	Mesophylax aspersus Rambur		Adult	?	Hans Malicky	Italy	Sicily, NE, Mandanici	08HMCAD-111	HMTRI111-08
	Potamophylax cingulatus Stephens		Adult	Female	Juha Salokannel	Finland	Melkoniemi, Parikkala, South Karelia	JSlk-2011F026	TRIFI566-11
	Allogamus auricollis Pictet		Adult	?	Hans Malicky	Austria	St. Konrad - Hausern	10HMCAD-262	HMKKT262-10
	Halesus tesselatus Rambur		Adult	Male	Mladen Kučinić	Croatia	Spring of the river Zrmanja near Knin	THDIG_1	NIPM009-17

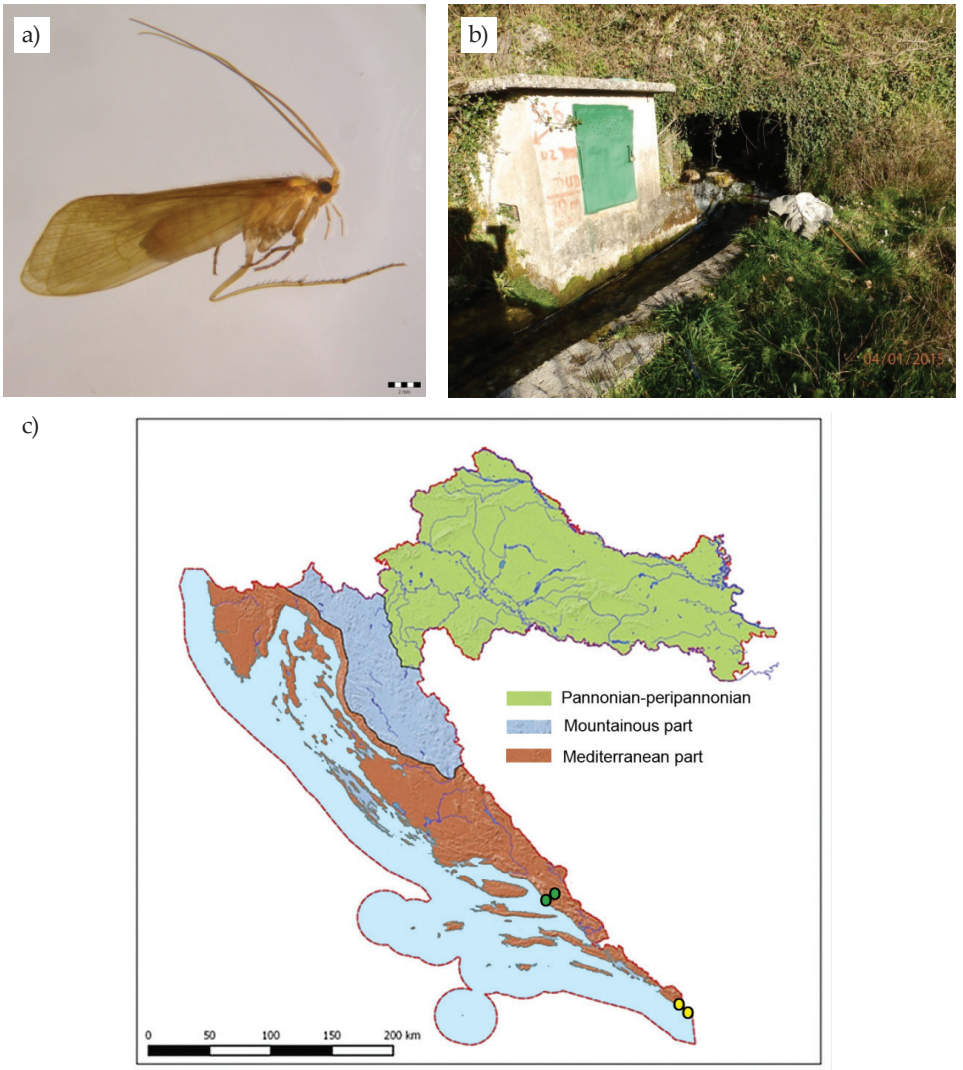


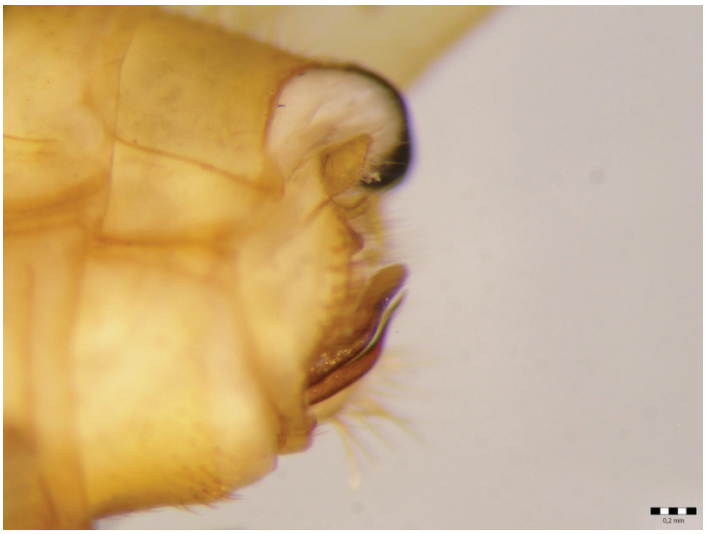
Fig. 1 A-C. *Micropterna wageneri*: A) adult (collected in Konavle region and deposited in the NIP-Trichoptera collection in the Croatian Natural History Museum in Zagreb); B) spring in the village Vodovađa (Konavle valley); C) geographical regions in Croatia according to Bertić *et al.* (2001), with locations where *M. wageneri* was found: Mt. Biokovo (green points) and Konavle region (yellow points).

RESULTS AND DISCUSSION

Faunal data

Micropterna wageneri (Fig. 1 A) was described by male adults (Fig. 2A) from Italy (Malicky, 1971). Female (Fig. 2B) was described by Moretti also from this region (Moretti, 1981). According to the literature data this species is distributed in southern Europe

a)



b)

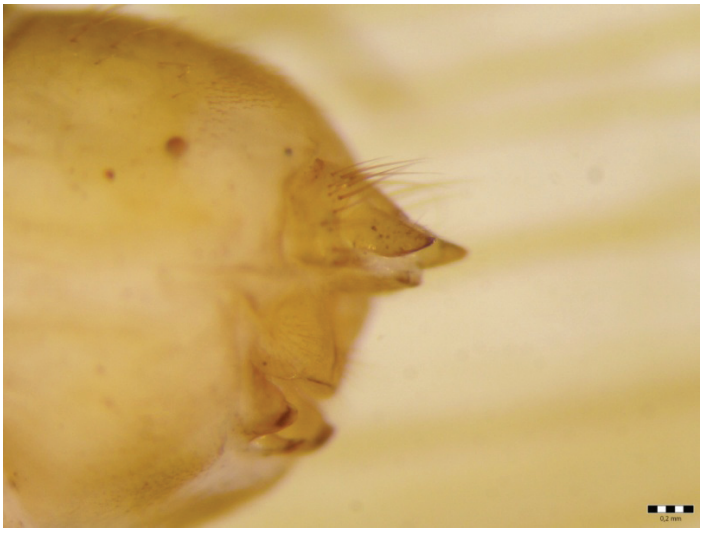


Fig. 2 A-B. *Micropterna wageneri*: A) male genitalia (left lateral view; B) female genitalia (left lateral view); specimens were collected in Konavle region and deposited in the NIP-Trichoptera collection in the Croatian Natural History Museum in Zagreb.

(Italy, Croatia, Albania…) (e.g. Malicky, 1971; Moretti, 1981, 1988; Cianficconi, 2002; Oláh, 2010). Fauna Europaea reports this species from Serbia/Montenegro, however literature data for these two countries do not support these data. In the last 25 years we have visited Mt. Biokovo many times and collected a lot of species of butterflies and moths (e.g. Mladinov & Kučinić, 1993; Kučinić *et al*., 1998; Mihoci *et al*., 2011) and several species of caddisflies (M. Kučinić, unpublished data). Until our investigation in Konavle region in southern Croatia (region Dalmatia) *M. wageneri* was only known from two localities on Mt. Biokovo: first locality is at an altitude of 550 m (28 May 1995, 1

male) and the second locality named Lađena at an altitude of 1270 m above sea level (28 May 1995, 2 males and 27 September 1995, 1 male). These specimens were collected 22 years ago (leg. M. Vajdić, F. Perović, M. Kučinić, det. H. Malicky) and are deposited in the Croatian Natural History Museum in Zagreb. During our investigation in Konavle region we collected *M. wageneri* at two locations, first one in a spring in the village Vodovađa (250 m a.s.l.) and the second one in the middle section of a stream near village Palje (150 m a.s.l.). Thus currently *M. wageneri* is known from four localities in Croatia, two on Biokovo Mt. and two in the Konavle region. Distance between these two areas is approximately 140 kilometres (Fig. 1 C).

Molecular data

The sequences obtained from *M. wageneri* samples submitted to the BOLD Identification Engine as well as to the GenBank did not find any match. Analysis of the genetic distance of DNA barcode region (mt*COI*) between species of the genus *Micropterna* is shown in Tab. 2. Genetic difference among two specimens of *M. wageneri* (MW) is only 0.0019 (0.2%). Intraspecific genetic difference within *M. wageneri* is identical to intraspecific difference (0.2%) within *M. testacea* Gmelin. For comparison: Zhou (2009) reported the mean value of 0.48 for the intraspecific differences based on mt*COI* barcode region (1481 sequence of 53 caddisfly taxa). This observed low value is in line with the observed variability of *Drusus* Stephens species (0.2% in Kučinić *et al*., 2015).

The minimum interspecific difference between *M. wageneri* and other *Micropterna* species and outgroup species is 0.096 (9.6%) (Tab. 2), which is above minimum difference between caddisfly species noted in literature (8.05% in *Smicridea* species, Pauls *et al*., 2010; 8.2% in *Anisogamus* species Graf *et al*., 2015). Most similar species to *M. wageneri* in this analysis are *M. sequax* McLachlan (TLIM_2) from Croatia and *M. lateralis* Stephens (GBEPT617-14) from Germany (0.096/9.6%). Relatively same value of minimum interspecific difference for DNA barcode region is noted for *Tinodes* Curtis (10% in Kučinić *et al*., 2016) and other Limnephilidae (8.2% and 9.6% in Graf *et al*., 2015).

Micropterna wageneri specimens clustered within the well supported clade which is distinct from other *Micropterna* species (100% BS support in both, ML and NJ analyses). The clustering of *M. sequax* sequences in three independent, well-supported clades in both NJ and ML analyses as well as in three distinct groups identified in ABGD analysis (group 2, group 3 and group 4 in Fig. 1) point to the existence of potentially new taxa within *M. sequax.* Often this kind of intraspecific divergence indicates the presence of cryptic species (Hebert *et al*., 2004). Clustering of *M. lateralis* with several *M. sequax* specimens as well as the sharing of the same *COI* haplotype between those two species (HP 18 haplotypes in Tab. 1) also prompts the need for further investigation of those taxa.

Thereby, population of these species from different parts of Europe should be analysed by means of morphological and more comprehensive phylogenetic analysis by employing additional molecular markers (mitochondrial as well as nuclear) to determine their exact taxonomic and phylogenetic status. In this case, DNA barcoding flagged possibly still unrecognised cryptic species complex. The use of morphological and molecular methods as a basis for integrative taxonomy (Vitecek *et al*., 2017), as well as an additional analysis of ecological and biogeographic data, are very important aspect of modern taxonomic and phylogenetic research of such taxa whose status cannot be efficiently resolved without employing all mentioned approaches.

Tab. 2. Values of the *p* distance between groups of *Micropterna* and outgroup species for barcode mt*COI* region. Partition mt*COI* haplotype into distinct groups was based on phylogenetic similarity.

	M.wageneri TMWAG_1	M.wageneri TPWAG_1	*M.nycterobia* TMNYC_1	*M.nycterobia* TMIC_1	*M.nycterobia* BIOUG17228-F01	*M.nycterobia* HMCAD0111-107	*M.nycterobia* 07HMCAD-0173	*M.nycterobia* 07HMCAD-0175	*M.nycterobia* BCZSMAQU00923	*M.testacea* TMTES_1	*M.testacea* KJTRI133N13	*M.testacea* BHMKK233N12	*M.fissa* BHMKK197N12	*M.sequax* TLIM_2	*M.sequax* TMIC_2	*M.sequax* FBAQU1406N13	*M.sequax* FBAQU1420N13	*M.sequax* 07HMCAD-0123	*M.lateralis* GBEPT617N14	*M.lateralis* JSIk-2011F1120	*M.sequax* HMCAD0111-97	*M.sequax* HMCAD1211-164	*M.testacea* 10ESCAD-011	*M.testacea* 10HMCAD-261	*M.testacea* HMCAD0111-109	*M.solotarewi* HMKKT920-10	*Halesus digitatus* THDIG_1	*Mesophylax aspersus* 08HMCAD-111	*Potamophylax cingulatus* JSIk-2011F026	*Allogamus auricollis* 10HMCADN262
M.wageneri TMWAG_1																														
M.wageneri TPWAG_1	0.0019																													
M.nycterobia TMNYC_1	0.1566	0.1590																												
M.nycterobia TMIC_1	0.1590	0.1566	0.0075																											
M.nycterobia BIOUG17228-F01	0.1590	0.1614	0.0094	0.0133																										
M.nycterobia HMCAD0111-107	0.1590	0.1614	0.0094	0.0133	0.0038																									
M.nycterobia 07HMCAD-0173	0.1616	0.1591	0.0171	0.0171	0.0229	0.0229																								
M.nycterobia 07HMCAD-0175	0.1616	0.1591	0.0171	0.0171	0.0229	0.0229	0.0000																							
M.nycterobia BCZSMAQU00923	0.1542	0.1566	0.0094	0.0133	0.0038	0.0038	0.0229	0.0229																						
M.testacea TMTES_1	0.1224	0.1201	0.1414	0.1414	0.1438	0.1438	0.1460	0.1460	0.1438																					
M.testacea KJTRI133N13	0.1247	0.1224	0.1438	0.1438	0.1462	0.1462	0.1484	0.1484	0.1462	0.0019																				
M.testacea BHMKK233N12	0.1316	0.1293	0.1391	0.1391	0.1414	0.1414	0.1437	0.1437	0.1414	0.0190	0.0210																			
M.fissa BHMKK197N12	0.1288	0.1311	0.1221	0.1243	0.1289	0.1289	0.1311	0.1311	0.1243	0.1222	0.1244	0.1244																		
M.sequax TLIM_2	0.0990	0.0967	0.1472	0.1448	0.1448	0.1401	0.1447	0.1447	0.1401	0.1253	0.1277	0.1277	0.1271																	
M.sequax TMIC_2	0.1189	0.1166	0.1503	0.1503	0.1503	0.1503	0.1550	0.1550	0.1455	0.1416	0.1440	0.1345	0.1546	0.1057																
M.sequax FBAQU1406N13	0.1032	0.1009	0.1404	0.1380	0.1475	0.1475	0.1473	0.1473	0.1427	0.1122	0.1145	0.1213	0.1289	0.0793	0.0841															
M.sequax FBAQU1420N13	0.1076	0.1054	0.1451	0.1427	0.1522	0.1522	0.1521	0.1521	0.1475	0.1122	0.1145	0.1213	0.1289	0.0836	0.0884	0.0075														
M.sequax 07HMCAD-0123	0.1120	0.1098	0.1479	0.1479	0.1479	0.1479	0.1526	0.1526	0.1432	0.1345	0.1369	0.1275	0.1475	0.0990	0.0056	0.0776	0.0819													
M.lateralis GBEPT617N14	0.0990	0.0967	0.1472	0.1448	0.1448	0.1401	0.1447	0.1447	0.1401	0.1253	0.1277	0.1277	0.1271	0.0000	0.1057	0.0793	0.0836	0.0990												
M.lateralis JSIk-2011F120	0.0990	0.0967	0.1472	0.1448	0.1448	0.1401	0.1447	0.1447	0.1401	0.1253	0.1277	0.1277	0.1271	0.0000	0.1057	0.0793	0.0836	0.0990	0.0000											
M.sequax HMCAD0111-97	0.1054	0.1032	0.1427	0.1404	0.1498	0.1498	0.1497	0.1497	0.1451	0.1145	0.1167	0.1237	0.1312	0.0814	0.0863	0.0019	0.0094	0.0797	0.0814	0.0814										
M.sequax HMCAD1211-164	0.1012	0.0990	0.1496	0.1472	0.1425	0.1378	0.1471	0.1471	0.1378	0.1277	0.1300	0.1300	0.1294	0.0019	0.1034	0.0814	0.0857	0.0967	0.0019	0.0019	0.0836									
M.testacea 10ESCAD-011	0.1270	0.1247	0.1344	0.1344	0.1367	0.1367	0.1389	0.1389	0.1367	0.0210	0.0229	0.0058	0.1222	0.1230	0.1298	0.1167	0.1167	0.1229	0.1230	0.1230	0.1190	0.1253								
M.testacea 10HMCAD-261	0.1268	0.1246	0.1460	0.1460	0.1484	0.1484	0.1507	0.1507	0.1484	0.0037	0.0019	0.0229	0.1268	0.1298	0.1482	0.1166	0.1166	0.1391	0.1298	0.1298	0.1189	0.1322	0.0249							
M.testacea HMCAD0111-109	0.1316	0.1293	0.1391	0.1391	0.1414	0.1414	0.1437	0.1437	0.1414	0.0210	0.0229	0.0019	0.1267	0.1277	0.1345	0.1213	0.1213	0.1275	0.1277	0.1277	0.1237	0.1300	0.0038	0.0249						
M.solotarewi HMKKT920-10	0.1322	0.1298	0.1411	0.1387	0.1387	0.1387	0.1365	0.1365	0.1435	0.1345	0.1369	0.1275	0.1339	0.1027	0.1006	0.1096	0.1119	0.0984	0.1027	0.1027	0.1119	0.1049	0.1275	0.1391	0.1275					
Halesus digitatus THDIG_1	0.1629	0.1605	0.1539	0.1515	0.1515	0.1515	0.1541	0.1541	0.1515	0.1680	0.1704	0.1631	0.1605	0.1513	0.1680	0.1630	0.1630	0.1607	0.1513	0.1513	0.1654	0.1490	0.1583	0.1727	0.1631	0.1444				
Mesophylax aspersus 08HMCAD-111	0.1513	0.1490	0.1595	0.1571	0.1644	0.1644	0.1569	0.1569	0.1595	0.1670	0.1695	0.1670	0.1468	0.1378	0.1617	0.1517	0.1517	0.1545	0.1378	0.1378	0.1541	0.1378	0.1621	0.1718	0.1670	0.1587	0.1746			
Potamophylax cingulatus JSIk-2011F026	0.1800	0.1775	0.1557	0.1533	0.1582	0.1582	0.1584	0.1584	0.1533	0.1691	0.1716	0.1691	0.1635	0.1743	0.1762	0.1658	0.1707	0.1687	0.1743	0.1743	0.1658	0.1768	0.1691	0.1739	0.1691	0.1679	0.1910	0.1912		
Allogamus auricollis 10HMCADN262	0.1749	0.1725	0.1610	0.1610	0.1683	0.1634	0.1681	0.1681	0.1634	0.1452	0.1476	0.1335	0.1537	0.1536	0.1752	0.1562	0.1562	0.1678	0.1536	0.1536	0.1562	0.1560	0.1335	0.1498	0.1335	0.1403	0.1660	0.1922	0.1739	

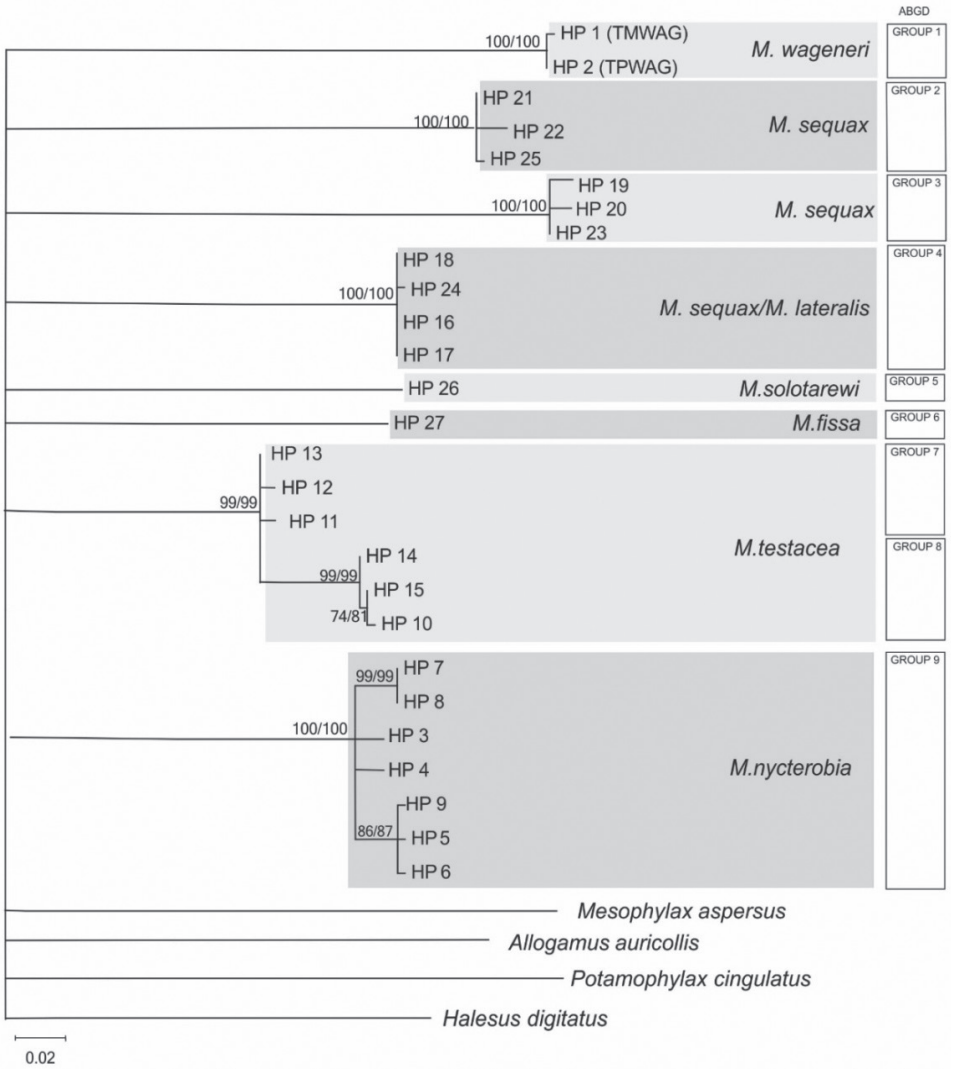


Fig. 3. Maximum likelihood (ML) phylogram based on 658 *bp* long fragment of the DNA barcode region showing the relationships between species of the genus *Micropterna*. Numbers above the branches represent bootstrap support (BS) for Neighbor-Joining (NJ) and ML analysis (NJ/ML). BS values less than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

The ABGD analysis revealed 9 genetic groups (Fig. 3). Interspecific distances of *M. wageneri,* among other specimen groups, did not overlap with intraspecific divergences as the ABGD analysis shows (Fig. 4). *M. wageneri* formed one group separated from other group of *Micropterna* and strongly indicates that *M. wageneri* is independent species. For 5 species from the genus *Micropterna* DNA barcoding analyses is done (Tab. 1, 3).

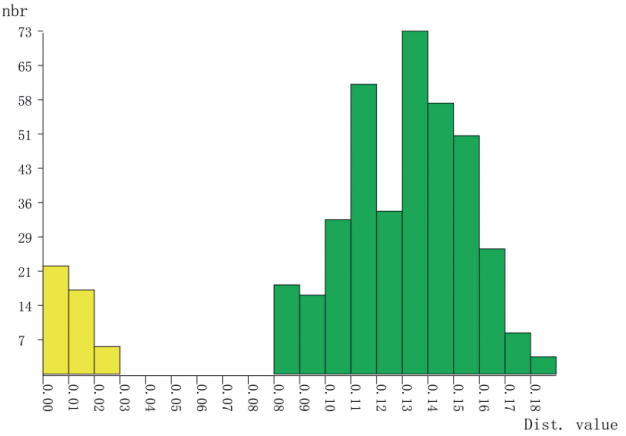


Fig. 4. Histogram depicting the frequency distribution of K2P distances for species of Liniphilidae family used in this study, calculated by ABGD. The horizontal axis shows the pairwise K2P-distance, and the vertical axis shows the number of pairwise sequence comparisons. On the left side of histogram (yellow colour) is intraspecific and on right is interspecific (green colour) distance variation.

Biodiversity of the genus *Micropterna*

Thirty four species genus *Micropterna* are recorded in Europe, North Africa and West Asia (Malicky, 2004). In southeastern Europe (Balkan Peninsula) ten species from this genus are present: *M. caesareica* Schmid, *M. coiffaiti* Décamps, *M. fissa* McLachlan, *M. lateralis* Stephens, *M. malaspina* Schmid, *M. nycterobia*, *M. sequax*, *M. testacea*, *M. taurica* Martynov and *M. wageneri* (Kumanski, 1988; Živić *et al*., 2002; Malicky, 2004, 2005; Ibrahimi *et al*., 2013; Oláh, 2010; Stanić-Koštroman *et al*. 2015;). In Croatian fauna 6 species have been found so far (e.g. Langhoffer, 1915; Radovanović, 1935; Marinković-Gospodnetić, 1979; Kučinić & Ilić, 1993; Kučinić, 2002; Cerjanec, 2012) (Tab. 3). The

Tab. 3. Biodiversity and distribution of the genus *Micropterna* in three geographical regions in Croatia (*species which have DNA barcoding analysis from specimens collected in Croatia), some DNA barcoding data are unpublished.

Species of genus *Micropterna*	Pannonian-peripannonian	Mountainous part part of Croatia	Mediterranean part of Croatia
M. fissa McLachlan	–	–	●
M. lateralis Stephens*	●	●	–
M. nycterobia McLachlan*	–	●	●
M. sequax McLachlan*	●	●	●
M. testacea Gmelin*	–	●	●
M. wageneri Malicky *	–	–	●
TOTAL	2	4	5

highest number represented by 5 species was recorded in the Mediterranean part of Croatia, while in the mountain area there are 4 recorded species and in the Pannonian-peripannonian part of Croatia only two species (Tab. 3). The species that appears in all three biogeographic regions of Croatia is the species *M. sequax* (Tab. 3). Two species recorded are distributed only in the Mediterranean area: *M. fissa* and *M. wageneri* (Tab. 3). Three species were recorded in two regions of Croatia: *M. lateralis* (Pannonian-peripanonian and mountainous part), *M. nycterobia* and *M. testacea* (mountainous and Mediterranean part) (Tab. 3).

Ecological data

According to Graf *et al* (2008) *Micropterna wageneri* belongs to submountain and mountain species. During our investigations of Mt. Biokovo, we found *M. wageneri* in middle and high altitudes (550 m and 1270 m above sea level) but in Konavle region we found this species at significantly lower altitudes (220 m and 250 m above sea level). These results were expected because this Mediterranean species probably has the ability to migrate into higher mountain regions, like some other limnephilid species (e.g. Novák & Sehnal, 1963). There are no natural aquatic habitats, such as springs or small streams, in higher mountainous areas of Mt. Biokovo. These types of aquatic habitats were established only at lower altitudes on Biokovo, where *M. wageneri* has not been established yet. In Konavle region *M. wageneri* probably inhabits springs and small streams, habitats in which we have collected adults. In small manmade canals in the Konavle valley we found interesting aquatic insect fauna (M. Kučinić unpublished data).

The larva of *M. wageneri* is not described yet (Waringer & Graf, 1997, 2011; Waringer & Malicky, 2016). Future investigations of caddisflies in the Konavle region will be focused on finding and possibly describing morphological features of larvae of this species. Very interesting ethological and ecological feature of the genus *Micropterna* is the ability of adults to live in pits and caves like troglophilic species (if such a habitat type exists in that area). The first finding of the genus *Micropterna* in Croatian subbteranean habitats is from the beginning of the XX. century, with the species: *M. lateralis*, *M. nycterobia* and *M. sequax* (e.g. Radovanović, 1935; Langhoffer, 1915; Gottstein *et al.*, 2002; Kučinić *et al.*, 2012). The fourth species from the genus *Micropterna* in subterranean habitats in Croatia was a species *M. testacea* (Kučinić & Ilić, 1993). This species was found on Mt. Biokovo for the first time for Croatian fauna (Kučinić & Ilić, 1993). Caddisflies are frequent faunistic element in caves and pits of the Dinaric karst, although in a relatively small number of species (Gottstein-Matočec, 2002; Kučinić *et al.*, 2012, M. Kučinić unpublished data). So far 12 species have been found in the fauna of Croatia (Gottstein-Matočec, 2002; Kučinić *et al.*, 2012). They belong to the group of troglophilic organisms that spend only one period of their life cycle in subterranean habitats. The most common species of caddisflies in speleological objects in Croatia are *M. sequax*, *M. nycetrobia* and *Stenophylax permistus* McLachlan (Gottstein *et al.*, 2002; Kučinić *et al.*, 2012). *Micropterna wageneri* was not found in these types of habitats in Croatia, but Moretti recorded this species as element of subterranean fauna in Italy (Moretti, 1988). The reason why caddisflies are entering speleological objects are not entirely clear. The occurrence of this two genera in caves (*Micropterna* and *Stenophylax* Kolenati) is not accidental and that has been proved by overwhelming number of the finds so far (e.g. Gottstein–Matočec *et al.*, 2002; Kučinić *et al.*, 2012). We can assume that they found shelter in subterranean habitats. The biological reasons behind the migration of species of the genera *Micropterna* and *Stenophylax* to speleological objects remain to be found.

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