NAT. CROAT. | VOL. 28 | No 2 | 305-323 | ZAGREB | December 31, 2019

original scientific paper/ izvorni znanstveni rad
DOI 10.20302/NC.2019.28.22

DNA BARCODING OF THE FAMILY PHRYGANEIDAE (INSECTA, TRICHOPTERA) IN CROATIA WITH PARTICULAR REFERENCE TO PHYLOGENY, DISTRIBUTION AND CONSERVATION BIOLOGY

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Kučinić, M., Ćukušić, A., Cerjanec, D., Podnar, M., Plantak, M., Žalac, S., Ćuk, R., Vučković, I., Ibrahimi, H. & Delić, A.: DNA barcoding of the family Phryganeidae (Insecta, Trichoptera) in Croatia with particular reference to phylogeny, distribution and conservation biology. Nat. Croat., Vol. 28, No. 2., 305-323, 2019, Zagreb.

In Europe the Phryganeidae family comprises 7 genera and 20 species. In Croatia, 6 species have been recorded from this family, and four of them recorded and collected in the last five years have been DNA barcoded. In terms of faunistic research, most interesting is the record of *Trichostegia minor* Curtis, 1834 from the region of the Gacka River in Lika, in upland Croatia. Up to now four species have been included in the BOLD system with 6 DNA-barcoded specimens. From the Gacka River area, 11 specimens of 8 species, including *T. minor,* have been barcoded and included into the BOLD system. Phylogenetic research based on DNA barcode data has shown some interesting results. Specimen of *T. minor* collected in the area of the Gacka River is clustered with a specimen of this species from Austria into a separate subgroup, which shows minor molecular specifics. Phylogenetic analysis has confirmed the justification of the status of a subspecies from Mongolia, *Phryganea grandis rotundata* Ulmer, 1905.

Future Trichoptera fauna research in Croatia will continue investigations of the family Phryganeidae in various parts of the country for the purpose of ascertaining the detailed distribution of all the species recorded, to enable finding some other previously missing species, as well as collecting and DNA barcoding of *Hagenella clathrata* (Kolenati, 1848) and *Oligostomis reticulata* (Linnaeus, 1761). With respect to these species, the data will also be useful for the area of conservation biology, necessary for their protection and for the protection of the sites in which they occur, for it is clear that these are very rare species in Croatia, *T. minor* also belonging to this group: 50% of the species from this family recorded in Croatia are rare and very rare species.

Key words: COI, *Phryganea grandis rotundata*, *Trichostegia minor*, south-east Europe, dinaric karst, the River Gacka

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Kučinić, M., Ćukušić, A., Cerjanec, D., Podnar, M., Plantak, M., Žalac, S., Ćuk, R., Vučković, I., Ibrahimi, H. & Delić, A.: DNA barkodiranje porodice Phryganeidae (Insecta, Trichoptera) u Hrvatskoj, s posebnim osvrtom na filogeniju, rasprostranjenost i konzervacijsku biologiju. Nat. Croat., Vol. 28, No. 2., 305-323, 2019, Zagreb.

Porodica Phryganeidae broji u Europi 7 rodova i 20 vrsta. Iz ove porodice na području Hrvatske utvrđeno je 6 vrsta, od kojih su DNA barkodirane četiri vrste, zabilježene i prikupljene u posljednjih pet godina. Faunistički najzanimljiviji je nalaz vrste *Trichostegia minor* Curtis, 1834 s područja rijeke Gacke (regija Lika), gorska Hrvatska. U BOLD bazu upisane su četiri vrste sa šest DNA barkodiranih primjeraka. S područja rijeke Gacke je DNA barkodirano i upisano u BOLD bazu 11 primjeraka s osam vrsta, uključujući i vrstu *T. minor*. Filogenetska istraživanja temeljena na analizi DNA barkod podataka pokazala su zanimljive rezultate. Primjerak vrste *T. minor* prikupljen na području rijeke Gacke grupira se s primjerkom te vrste iz Austrije u zasebnu podgrupu, što ukazuje na manje molekularne specifičnosti. Filogenetska analiza potvrdila je i opravdanost statusa podvrste iz Mongolije, *Phryganea grandis rotundata* Ulmer, 1905.

U budućim istraživanjima faune Trichoptera Hrvatske nastavit će se istraživanja porodice Phryganeidae u različitim dijelovima Hrvatske u svrhu utvrđivanja detaljnijeg rasprostranjenja svih zabilježenih vrsta, potencijalnog nalaza neke još nezabilježene vrste, ali i nalaza te DNA barkodiranja vrsta *Hagenella clathrata* (Kolenati, 1848) i *Oligostomis reticulata* (Linnaeus, 1761). Za te vrste bit će potrebno utvrditi i određene značajke u području konzervacijske biologije potrebne za njihovu zaštitu i zaštitu lokaliteta na kojima žive, jer je očito da su to vrlo rijetke vrste u fauni Hrvatske, u koje pripada i vrsta *T. minor*; naime, 50% zabilježenih vrsta porodice Phryganeidae u Hrvatskoj su rijetke ili vrlo rijetke vrste.

Ključne riječi: COI, *Phryganea grandis rotundata*, *Trichostegia minor*, jugoistočna Europa, dinarski krš, rijeka Gacka

INTRODUCTION

The family Phryganeidae belongs to the order Trichoptera, superfamily Phryganeoidea suborder Integripalpia. The superfamily Phryganeoidea includes 7 families: Baissoferidae, Dysoneuridae, Kalophryganeidae, Kokiriidae, Lepidostomatidae, Oeconesidae and Phryganeidae, the first three of which are extinct (Morse, 2019). The family Phryganeidae is a not very species-rich family of Trichoptera, with about 80 described species in 15 genera (Holzenthal *et al*., 2007). In Europe there are seven genera and 20 described species (Malicky, 2004). Lot of species have adults with large or very large forewings, 20 mm or more, for example *Phryganea grandis* Linnaeus, 1758 or *Agrypnia varia* (Fabricius, 1793) (Malicky, 2004). The genus *Eubasilissa* from this family includes the largest species of caddisflies (Holzenthal *et al*., 2007). Larvae from family Phryganeidae produce long, spiral or ring cases from parts of plants (stems), sometimes with small pebbles. The family is distributed in the Palearctic, Nearctic and Holacrtic regions (Holzenthal *et al*., 2007).

This paper consists of (1) a depiction of the distribution and biodiversity of family Phryganeidae in Croatia, with a reference to the rare species *Trichostegia minor* (Curtis, 1834); (2) DNA barcode data and a phylogenetic depiction of the family Phryganeidae as compared with other genera and species included in the list of barcoded Trichoptera from Croatia; (3) faunistic features of the Trichoptera fauna of the Gacka River where some interesting species of the family Phryganeidae were collected, including *T. minor* together with a presentation of the DNA barcoded specimens and species collected from this area included in the BOLD database.

The study of the Earth's biodiversity is a very long process that started with the development of humankind and attained scientific dimensions with the establishment of binomial nomenclature, the taxonomic, and the basic evolutionary model for the depiction of this diversity (Linnaeus, 1758). Since this period (the 18th century), and according to this model and the rules of taxonomy (for example, the ICZN 2000), a large number of organisms on Earth have been described, with more than a million known species, which is considered as just a part of total existing biodiversity. Each year thousands of new species within various groups of organisms are described (for example Brehm *et al*., 2019; da Silva *et al*., 2014; Fang & Xing, 2019; Malicky, 2017; Suwannarat *et al*., 2019; Vitecek *et al*., 2015c; Yánez-Muñoz *et al*., 2018) which leads to further knowledge of biodiversity on Earth. Such a model of describing new species using primarily a morphological approach and an analysis of morphological features of organisms was significantly changed when the DNA barcoding method was discovered and began to be applied (Hebert *et al*., 2003a, 2003b). DNA barcoding and other molecular analyses made it possible to study biodiversity, taxonomy and phyologeny not only based on the morphological, but also on the genetic level (DAYRAT, 2005). The employment of morphological and genetic methods made possible the development of what is called integrative taxonomy (for example, Bilandžija *et al*., 2013., Bogdanović *et al*., 2014, 2019; Dayrat, 2005; Ibrahimi *et al*., 2016; Vitecek *et al*., 2017; Will *et al*., 2005), which is much more comprehensive, for it involves various morphological and genetic analyses and enables the taxonomic, phylogenetic, phylogeographic and biogeographic features of any given taxon or group of organisms to be established much more accurately. In the last 15 years, DNA barcoding has become one of the essential methods in taxonomy, phylogeny, and phylogeography (for example, Geraci *et al*., 2011; Hjalmarsson *et al*., 2019; Kučinić *et al*., 2015; Pauls *et al*., 2010; Pfeiler, 2018; Vitecek *et al*., 2015a, 2015b; Will *et al*. 2005). As well as DNA barcoding, which analyses a certain segment of mitochondrial DNA, there are other kinds of genetic analysis that involve other genes which makes possible a further and better quality presentation of the evolutionary process and the taxonomic and phylogenetic position of certain taxa (for examples Pauls *et al*., 2006, 2009; Previšić *et al*., 2009, 2014; Vitecek *et al*., 2017).

The DNA barcoding method is used in Trichoptera taxonomy and phylogeny in various regions of the world and in the last few years has been used in caddisfly research in Croatia as well (Ćukušić *et al*., 2017; Kučinić *et al.* 2013; Szivák *et al*., 2017). Systematic implementation of DNA barcoding of Trichoptera in Croatia started with the studies of e.g. Kučinić *et al*. (2013) and has lasted with larger or smaller lacunae to this day (Ćukušić *et al*., 2017; Kučinić, 2019). Currently the ongoing scientific project „DNA barcoding of faunal biodiversity in Croatia" has placed the Trichoptera as one of the target groups. The present paper is a part of this scientific project.

MATERIAL AND METHODS

Field work

The sampling of Trichoptera for DNA barcoding was conducted during the last few years in various parts of Croatia (Pannonian and Peri-Pannonian part, central-mountain part and Mediterranean part) (Fig. 1), including the area of the Gacka River (2012, 2015, 2016) (Fig. 2) and the Kostelka River (in 2019). These rivers are situated in the central-mountain part of Croatia, in a Dinaric karst region called Lika (Bertić *et al*., 2001). The sampling at the Gacka River included four sites: Tonkovića vrilo (spring), Majerovo vrilo (spring), Sinac vrilo (spring) and the middle course of the Gacka River near Otočac and at the spring of the Kostelka River. The Gacka River has three main springs: Majerovo vrilo, Sinac vrilo and Tonkovića vrilo.

Trichoptera were collected with an entomological net for a period of 30 minutes (i.e., the time that a single work effort lasted) and at night with 12 V UV lamps and a portable battery for a period of 60 minutes.

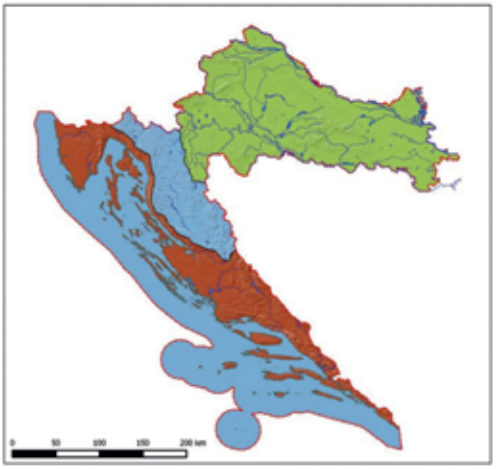

Fig. 1. Map of Croatia with three geographical parts: Pannonian and Peri-Pannonian part (green), Central-mountain part (i.e. upland part; blue) and Mediterranean part (red); according to Bertić *et al*. (2001).

Fig. 2. Middle part of the River Gacka, near the town of Otočac (photo M. Vuković).

Laboratory work

All the collected Trichoptera material was preserved in pure ethanol. Determination of the material was conducted after Malicky (2004) and Kumanski (1988), and a systematic presentation after Morse (2019) is provided. The material collected is a component part of the NIP Trichoptera Collection (Croatian Natural History Museum, Zagreb) and the Cerjanec Trichoptera Collection. DNA barcoded specimens collected from the area of the Gacka River are kept as vouchers in the Croatian Natural History Museum in Zagreb. Literature data (Marinković-Gospodnetić, 1979) are also used in the presentation of the Trichoptera fauna of the Gacka River.

Macrophotographing of Trichoptera adults was carried out using a Leica Wild MZ8 stereomicroscope and Olympus SP-500 UZ digital camera, processed with the computer program Olympus Quick Photo Camera 2.2 at the Laboratory for patology of trees (Department of Forest Protection and Wildlife Management) at the Faculty of Forestry, University of Zagreb.

DNA extraction, PCR amplification and phylogenetic analysis

Genomic DNA was extracted from legs of four specimens, one *T. minor* and the other caddisfly species listed in Appendix 1 with specimen IDs marked with bold letters. All specimens are kept as vouchers 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 50 µl of elution buffer. For the amplification of the COI-5P, barcode region primers LCO1490 and HCO2198 (C 1994) 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).

For phylogenetic analysis, all available barcode sequences from family Phryganeidae were retrived from the BOLD System database (BOLD IDs are given in Appendix 1). DNA sequences obtained in this study were submitted to Barcode of Life Data Systems (BOLD, Ratnasingham & Hebert 2007, Appendix 1, Tabs. 2 and 4). As outgroup taxon *Lepidostoma hirtum* (Fabricius, 1775) was selected (Appendix 1) which belongs to the same superfamily Phryganeoidea WE Leach, 1815. Indistinguishable sequences were collapsed into unique haplotypes using FaBox v.1.41 (Villesen, 2007). All haplotypes are listed in Appendix 1, Tabs. 2 and 4. 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 specimen identification. Details of phylogenetic analyses were performed as outlined by Ćukušić *et al*. (2017).

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 a 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 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

Biodiversity, distribution and some aspects of the conservation of Phryganeidae in Croatia

Six species from five genera of Phryganeidae are recorded in Croatia (Tab. 1). Three species were reported for the first time in the Plitvice Lakes National Park (Kučinić, 2002): *Agrypnia varia, Phryganea bipunctata* Retzius, 1783 and *P. grandis*. *Hagenella clathrata* (Kolenati, 1848) was first found around Zagreb (Radovanović, 1935) and subsequently in central Croatia (Previšić *et al*., 2013). *Oligostomis reticulata* (Linnaeus, 1761) was discovered at the spring of the Kostelka River (Marinković-Gospodnetić, 1979) and *Trichostegia minor* was established in northern Croatia only 89 years ago (leg. F. Košćec) (Malicky, 2009).

The number of recorded species indicates the relatively good state of research of this family in Croatia; one or two more species can be expected in Croatia based on the situation in well-researched neighbouring areas. In Slovenia, Italy and Hungary, for example, eight species of this family have been recorded (Cianficconi, 2002; Krušnik & Urbanič, 2002; Nógardi & Uherkovich, 2002).

In terms of species, 83% (5 species) of the Phryganeidae family have been found in the Pannonian and Peri-Pannonian as well as in the central mountainous area; only 2, or 33%, have been recorded in the Mediterranean area (Tab. 1). This kind of distribution is a consequence of the biological characteristics of the species within the family, the hydrological features of Croatia in the separate regions (Pannonian and Peri-Pannonian, upland and Mediterranean), and of certain geological processes that occurred in the past and had a considerable influence on the distribution and speciation of Trichoptera in south-eastern Europe. Only two species, *Agrypnia varia* and *Phryganea grandis*, are distributed in all the three geographical regions of Croatia (Tab. 1) whereas *Hagenella clathrata* and *Oligostomis reticulata* have been recorded in just one area: the first one in the Pannonian and Peri-Pannonian, and the second in the upland (central mountainous) region of Croatia (Marinković-Gospodnetić, 1979; Previšić *et al.*, 2013) (Tab. 1). Trichoptera research carried out in Croatia in the last years (Graf *et al*., 2008; Kučinić *et al.*, 2017b; Previšić *et al*., 2013; Waringer *et al*., 2009) resulted in 5 species from the family Phryganeidae (Tab. 1) but *O. reticulata* recorded in the area of the Gacka River (Marinković-Gospodnetić, 1979) is unknown elsewhere.

It is specific for the family of Phryganeidae in Croatia that 50% of the species are rare or very rare, and therefore endangered. Conservation measures will be necessary for those species (*O. reticulata, H. clathrata* and *T. minor*).

Tab. 1. Biodiversity and distribution of family Phryganeidae in Croatia.

Species	Pannonian and Peri-Pannonian part	Central-mountain part	Mediterranean part
Agrypnia varia	●	●	●
Haganella clathrata	●	-	-
Phryganea bipunctata	●	●	-
Phryganea grandis	●	●	●
Oligostomis reticulata	-	●	-
Trichostegia minor	●	●	-
TOTAL	5	5	2

For the sake of finding and DNA barcoding *O. reticulata* we collected additional Trichoptera in the Gacka River area in 2016 and 2017, and at the spring of the Kostelka River in 2019. For the same reasons, we carried out similar field sampling in the Banovina and in part of the Kordun region for detecting *H. clathrata* in the 2016-2018 period (Kučinić *et al*., 2020). During this research we found neither one of these two species (Kučinić *et al*., 2020) but without success; the research will be carried on during 2020 in the hope of finding and DNA barcoding these two species.

Tab. 2. GenBank BOLD COI data for DNA barcoding specimens from family Phryganeidae collected in Croatia.

Species	Specimen ID	Locality	BOLD Sequence ID	Species identification (%)
Agrypnia varia	TAVAR_1	River Mura (lower part) Goričan	CROAA013-18	*A. varia* 100 %
Agrypnia varia	TAVAR_2	spring Rude	CROTR078-19	*A. varia* 99.84 %
Agrypnia varia	TAVAR_3	Kozjak Lake, NP Plitvice Lakes	CROTR240-19	*A. varia* 99.81 %
Phryganea bipunctata	TPBIN_1	River Drava, Gornji Hrašćan	CROAA026-18	*P. bipunctata* 100 %
Phryganea grandis	TPGRA_1	River Danube, Zlatna Greda	CROAA134-18	*P. grandis* 99.05 %
Trichostegia minor	TTMIN_1	spring of the Gacka River, Majerovo vrilo	CROAA133-18	*T. minor* 98.93 %

DNA barcoding and phylogenetic features of Trichostegia minor *and other species from the Phryganeidae family*

With respect to molecular genetics, four Croatian Phryganeidae species were DNA barcoded until now (Tab. 2). In total, 6 specimens were analysed and the data obtained were entered in the BOLD system. The most interesting species that we recorded was *Trichostegia minor*, two specimens of which were collected in the area of the Gacka River (Fig. 3A-B). This species was found at only one site in northern Croatia in the first half of the 20th century (Malicky, 2009) and at one site at the beginning of the 21st century in the Cetina River (Graf *et al*., 2008). In the last 20 years (for example Kučinić *et al*., 2008, 2017b; Cerjanec, 2012; Previšić *et al*., 2009, 2013, 2014) the species was found only at one site, on the Cetina River (Graf *et al*., 2008) (Fig. 4).

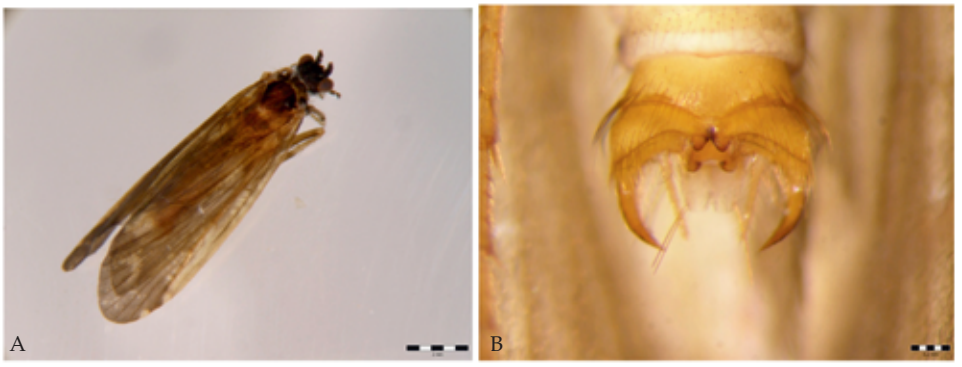

Fig. 3. *Trichostegia minor* Curtis, 1834, collected at the Majerovo vrilo (spring) of the Gacka River; A – adult, B – male genitalia.

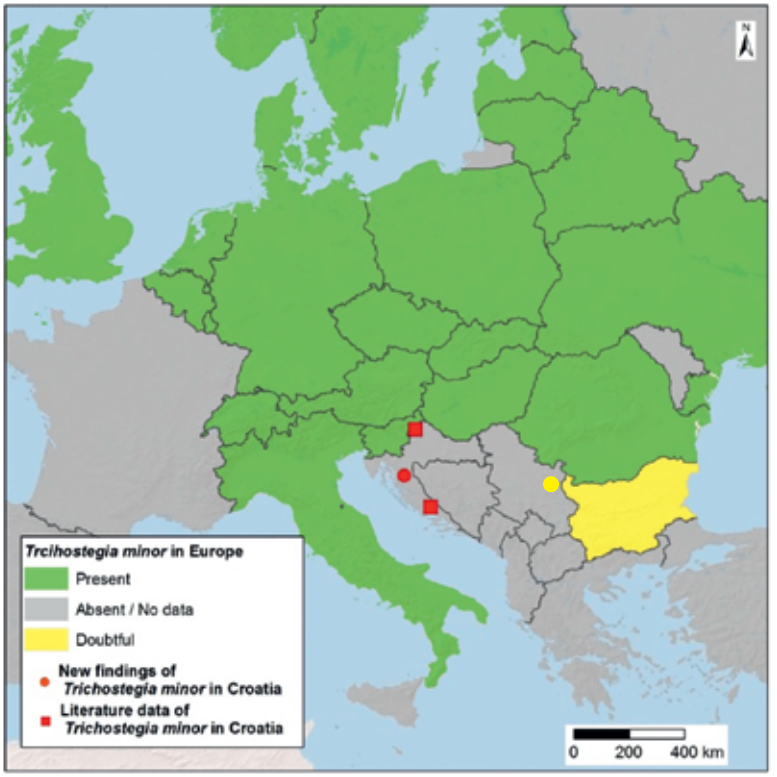

Fig. 4. Distributon of *Trichostegia minor* Curtis, 1834 according to the Fauna Europaea, with literature data from Graf *et al.*, 2008 and Malicky, 2009 (squares) and new data for the area of Croatia (red circle); yellow circle denotes finding from Serbia (Savić *et al.*, 2013).

The phylogenetic analysis of the Phryganeidae family conducted in the present study encompassed the species and genera recorded in Croatia, which were compared with the same species and genera from some other parts of Europe and from Asia (Fig. 5). The phylogenetic tree shows that *T. minor* from Croatia (TTMIN_1) is grouped with other *T. minor* specimens (Fig. 5). Specimen TTMIN_1 is highly similar to *T. minor* from Austria, collected from region Schutt, near Arnoldstein, river Gail, Tümpel bei Wehranlage, BOLD ID: KJTRI226-13. It is interesting how two specimens, TTMIN_1 from Croatia and KJTRI226-13 from Austria are separated from other *T. minor* specimens. We observed that two or more separate lineages occur in other species used in this study (*Phryganea bipunctata, Oligostomis reticulata, Hagenella clathrata, Agrypnia varia)* (Fig. 5).

The minimum interspecific difference between *T. minor* and other Phryganeidae is 0.1395 (14%), and with the outgroup species (*Lepidostoma hirtum*) is 0.1572 (16%) (Tab. 3), which is distinctly higher than the 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). As already noticed, the most similar specimen to *T. minor* (TTMIN_1) is KJTRI226-13 with only 0.0059 (0.6%) difference in the COI region of mtDNA, when compared to other *T. minor* specimens that on

average differ in 0.0227 (2%). The low value of the intraspecific differences (among all Phryganeidae) is 0.002 (0.2%) what is in line with the observed variability within *Drusus* species (Kučinić *et al*., 2015), *Anisogamus* species (Graf *et al*., 2015) and *Micropterna* species (Kučinić *et al*., 2017a). Intraspecific differences are up to 0.016 (1.6%) between *P. grandis* and *P. bipunctata*, 0.0275 (2.8%) between *T. minor* and *O. reticulata*, 0.0354 (3.5%) within *A. varia* and 0.045 (4.5%) within *H. clathrata.* In Tab 3. we can notice that high intraspecific differences between species *P. grandis* specimens from Europe and subspecies *P. grandis rotundata* from Mongolia is 0.016 (1.6%). If we compare to maximum intraspecific differences observed in the mtCOI barcode region reported in other studies (Hydropsychidae maximum intraspecific distance = 0.9% in Gereci *et al*., 2011, 3.19% between *Xanthochorema* species in Johanson *et al*., 2007, 3.9% between *Ceratopsyche* in Zhou *et al*., 2007, 5.3% between *Cheumatopsyche campyla* Ross, 1938 in Zhou, 2009) those observed distances are relatively high. More confusing is that the minimum interspecific difference is close to the maximum intraspecific distance (1.9% in Gereci *et al*., 2011, 3.5% in Zhou, 2009, 8% in Johanson *et al*., 2007, 9.6% in Zhou *et al*., 2007).

The ABGD analysis revealed 11 genetic groups (Fig. 5). Interspecific distances of *T. minor* with other specimens did not overlap with intraspecific divergences, as shown by the ABGD analysis (Fig. 6). We observed that ABGD groups do not completely reflect the pattern of branching in the phylogenetic tree. Although *P. grandis rotundata* Ulmer, 1905 specimens form well supported subclade within *P. grandis* clade, separated from *P. grandis grandis*, specimens belonging to both subspecies are encompassed within single ABGD group (Group 1). The same is true for two distinct lineages within *H. clathrata* (Group 6). On the other hand, the specimens belonging to the same species are separated in distinct ABGD groups in the case of (*P. bipunctata* in Group 2 and Group 3, *O. reticulata* in Group 4 and Group 5, *A. varia* in Group 9 and Group 10) (Fig. 5). *T. minor* from Croatia (TTMIN_1) and Austria (KJTRI226-13) formed separate group from the rest of *T. minor* specimens (Group 8) (Fig. 5).

Tab. 3. Uncorrected pairwise divergences (p distances) among mtCOI haplotypes (DNA barcode region) of the analysed Phryganeidae.

	T. minor (TTMIN_1 & KJTRI226-13	*T. minor*	*Agrypnia varia*	*Hagenella clathrata*	*Oligostomis reticulata*	*Phryganea bipunctata*	*Phryganea grandis*
T. minor (TTMIN_1 & KJTRI226-13	0.5						
T. minor	2-2.8	0-0.1					
Agrypnia varia	16.11-17	15.7-17.0	0.20- 3.5				
Hagenella clathrata	15.7-16.5	15.3-16.5	14.7-16.5	0.4-4.5			
Oligostomis reticulata	14.3-15.1	13.9-15.3	14.1-15.7	13.4-14.5	0.2-2.8		
Phryganea bipunctata	16.3-17.2	16.3-17.4	16.3-17.1	14.3-15.3	14.3-15.3	0.1-1.8	
Phryganea grandis	14.5-15.2	14.3-15.9	15.5-16.7	13.9-15.7	12.6-13.8	8.8-10.2	0.2-1.6
Lepidostoma hirtum	15.9-16.1	15.7-16.1	15.7-17.1	16.7-18.1	17.1-17.5	18.3-19.1	15.7-16.1

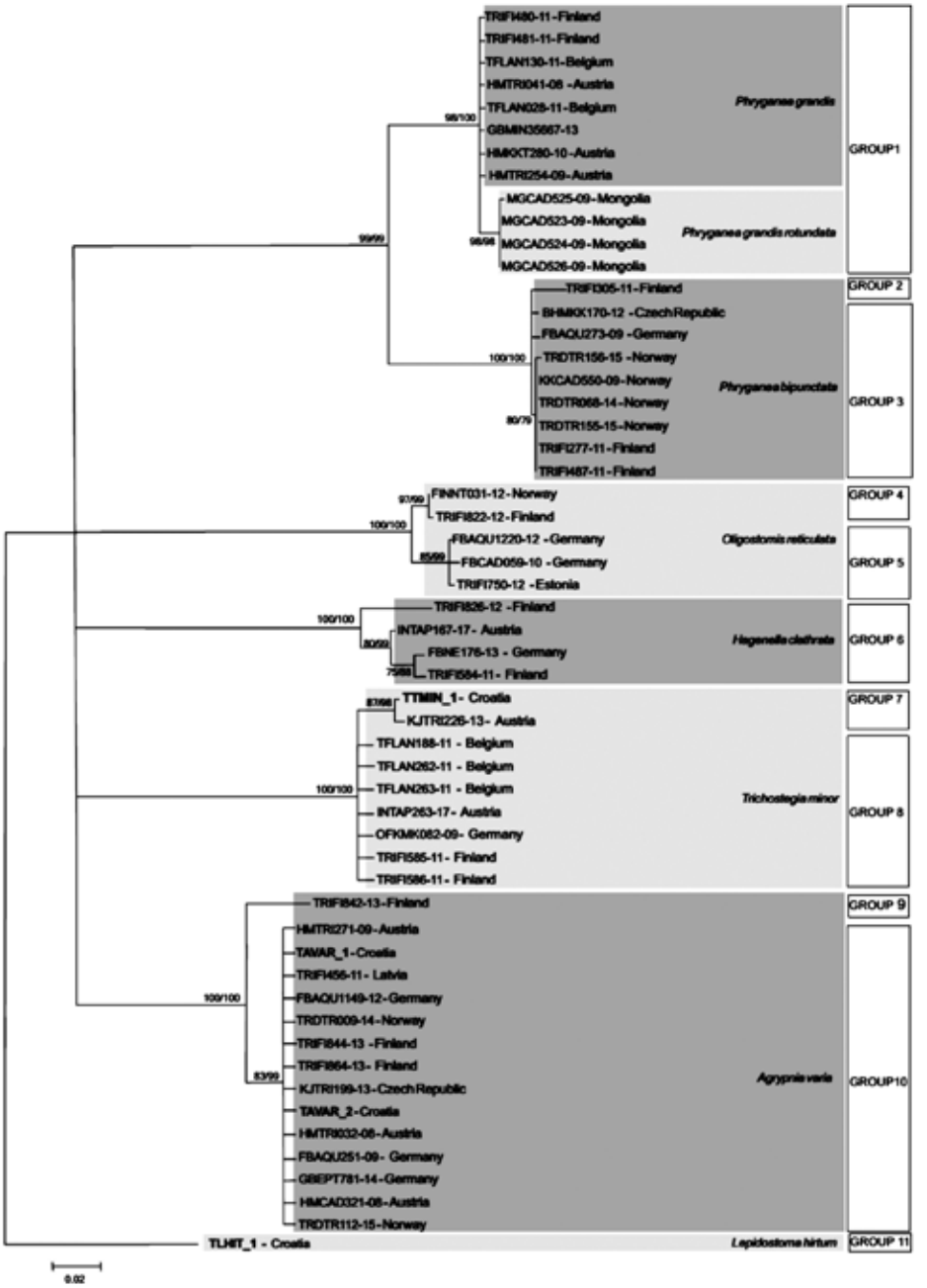

Fig. 5. Maximum likelihood (ML) phylogram based on a 658 bp long fragment of the DNA barcode region showing the relationships in Phryganeidae with species *Lepidostoma hirtum* as outgroup. Numbers above the branches represent bootstrap support (BS) for Neighbor-Joining (NJ) and ML analysis (NJ/ML). BS values less than 70 are not shown. The groups delineated by the Automatic Barcode Gap Discovery (ABGD) approach are shown on the right side of the tree.

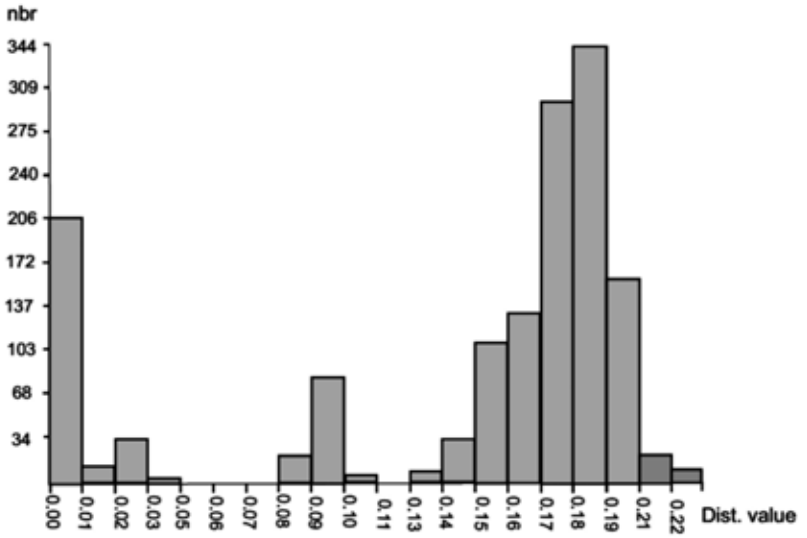

Fig. 6. Histogram depicting the frequency distribution of K2P distances for species of the Phryganeidae family used in this study, calculated by ABGD. The horizontal axis shows the pairwise K2P-distance, and the vertical axis shows the number of pairwise sequence comparisons.

DNA barcoding and faunistics of caddisflies at the River Gacka

In this recent study from the area of the Gacka River nine Trichoptera species were DNA barcoded (Tab. 4). In total 11 specimens were analysed and the data obtained were entered in the BOLD system (Tab. 4).

The studies of adult caddisflies in the area of the River Gacka was conducted by Mara Marinković-Gospodnetić, a professor from Sarajevo, who recorded 4 species at the Sinac and Majerov vrilo springs (Marinković-Gospodnetić, 1979), including the species *Drusus croaticus* Marinković-Gospodnetić, 1971. It was originally described by Professor Marinković-Gospodnetić from the area of the Plitvice lakes (Marinković-Gospodnetić, 1971); afer that the species was found in other areas of upland Croatia, including the springs of the Gacka River (Marinković-Gospodnetić, 1971).

Today, 17 caddisfly species from 15 genera and 11 families are known from the area of the Gacka River: family Rhyacophilidae - *Rhyacophila fasciata* Hagen, 1859 (the River Gacka, Otočac); family Glossostomatidae - *Agapetus ochripes* Curtis, 1834 (the River Gacka, Otočac); family Hydroptilidae - *Hydroptila martini* Marshall, 1977 (the River Gacka, Otočac), *Hydroptila tineoides* Dalman, 1819 (the River Gacka, Otočac); family Polycentropodidae - *Polycentropus flavomaculatus* (Pictet, 1834) (Tonkovića vrilo, spring); family Psychomyiidae - *Psychomia klapaleki* Malicky, 1995 (the River Gacka, Otočac); family Phryganeidae - *Oligostomis reticulata, Trichostegia minor*; family Hydropsychidae - *Hydropsyche bulbifera* McLachlan, 1878 (the River Gacka, Otočac); family Lepidostomatidae - *Lepidostoma hirtum* (the River Gacka, Otočac); family Limnephilidae - *Drusus croaticus* (Tonkovića

vrilo, spring), *Limnephilus flavicornis* (Fabricius, 1787) (Majerovo vrilo, spring), *Limnephilus rhombicus* (Linnaeus, 1758) (the River Gacka, Oročac), *Micropterna testacea* (Gmelin, 1789) (Majerovo vrilo, spring), *Potamophylax cf. latipennis* (Curtis, 1834) (Tonkovića vrilo, spring; the River Gacka, Otočac); family Odontoceridae - *Odontocerum albicorne* (Scopoli, 1763) (the River Gacka, Otočac); family Leptoceridae - *Oecetis notata* Rambur, 1842 (Majerovo vrilo, spring). Apart from *Trichostegia minor* the following species from the area of the Gacka River area are also interesting from a faunistic viewpoint: *Hydroptila martini*, *H. tineoides*, *Hydropsyche bulbifera* and *Oecetis notata*. *H. martini* and *H. bulbifera* were recorded for the first time in the upland part of Croatia, and the other two have been found very seldom in Croatia, at two to three sites only (Cerjanec, 2012).

On the River Kostelka 8 species were recorded (M-G 1979) of which 5 species were not found in the River Gacka: *Plectrconemia conspersa* (Curtis, 1834) (family Polycentropodidae), *Oligostomis reticulata* (family Phryganeidae), *Chaetopteryx bosniaca* Marinković-Gospodnetić, 1959 (family Limnephilidae), *Stenophylax vibex* (Curtis, 1834) (family Limnephilidae) and *Beraea pullata* (Curtis, 1834).

Tab. 4. GenBank and BOLD COI barcode data for DNA barcoding specimes collected from the area of the Gacka River.

Species	Specimen ID	BOLD Sequence ID	Location
Trichostegia minor	TTMIN_1	CROAA133-18	river Gacka, spring Majerovo vrilo
Hydroptila martini	THMAR_1	CROAA094-18	river Gacka, Otočac
Psychomyia klapaleki	TPKLA_2	CROAA112-18	river Gacka, Otočac
Drusus croaticus	TDCRO_3	CROTR019-19	river Gacka, spring Majerovo vrilo
Drusus croaticus	TDCRO_4	CROTR043-19	river Gacka, spring Majerovo vrilo
Limnephilus flavomaculatus	TLFLA_1	CROTR073-19	river Gacka, spring Majerovo vrilo
Limnephilus rhombicus	TLROM_1	CROAA097-18	river Gacka, Otočac
Limnephilus rhombicus	TLRHO_5	CROTR188-19	river Gacka, spring Majerovo vrilo
Micropterna testacea	TMTES_3	CROTR028-19	river Gacka, spring Majerovo vrilo
Oecetis notata	TONOT_2	CROTR072-19	river Gacka, spring Majerovo vrilo
Hydropsyche bulbifera	THBUL_3	CROTR039-19	river Gacka, Otočac

CONCLUSION

Six species from the family Phryganeidae have been recorded in the Croatian fauna (Tab. 1). In this recent research, four of them were collected and DNA barcoded (Tab. 2). In total, 6 specimens were analysed and the data obtained were entered in the BOLD system.

Phylogenetic analysis of the DNA barcoded specimens from Croatia showed that our species group fits well with European specimens of the same species put into the BOLD system, such as *Agrypnia varia* (Fig. 3).

T. minor collected in the Gacka River area and DNA barcoded (Tabs. 2, 5) forms, together with a specimen of the same species from Austria, distinct subclade within *T. minor* clade which indicates small molecular specificity. Our

phylogenetic analysis has confirmed the justifiability of the subspecies status of *Phryganea grandis rotundata* from Mongolia (Fig. 3).

In future Trichoptera research in Croatia, the Phryganeidae family will be included for the purpose of new records, a more detailed distribution of already recorded species and the re-discovery and barcoding of *Hagenella clathrata* and *Oligostomis reticulata*. It will be necessary establish conservation measurements necessary for their protection and the protection of their habitats as they seem to be very rare in the Croatian fauna, which refers to *T. minor* as well.

ACKNOWLEDGEMENT

We are very grateful to two anonymous referees for their useful suggestions and also to Graham McMaster for his assistance with English; we thank the Faculty of Forestry for the macrophotography.. This study was supported by the "EU Natura 2000 Integration Project (NIP)" funded by the Croatian Ministry of Environmental and Nature Protection (2012-2016), and the scientific project „DNA barcoding of Croatian faunal biodiversity" (IP-06-2016-9988)" (2017-2021) funded by the Croatian Science Fundation.

Received November 28, 2019

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Appendix 1. List of specimens used in this study, showing life stage, origin, BOLD Sequence ID number, specimen ID, number of unique haplotypes. Specimens which genomic DNA was extracted in this study are written in bold letters. Abbreviation used: ID = Identification number, BOLD = Barcode of Life data system, A = adult, I = imago, L = larvae, M = male, F = female, No. = number

Sex	Life Stage	Country	Location	BOLD Sequence ID	Specimen ID	No. haplotyp
Trichostegia minor Curtis, 1834						
M	A	Croatia	River Gacka, Spring Majerovo vrilo	CROAA133-18	**TTMIN_1**	1
M	I	Austria	Franstanz, Giessen	INTAP263-17	PE310	2
M	I	Austria	Franstanz, Giessen	INTAP264-17	PE311	
M	A	Austria	Tumpel bei Wehranlage	KJTRI226-13	HMCAD13-36	3
	A	Germany	Karinchensee nr. Ferch	OFKMK082-09	09OFKMK-0082	4
F	A	Belgium	Hobokense Polder, Hoboken	TFLAN188-11	UA-SG-TRICH-D80	5
	A	Belgium	Hobokense Polder, Hoboken	TFLAN262-11	UA-SG-TRICH-D94	6
	A	Belgium	Bospolder, Ekeren	TFLAN263-11	UA-SG-TRICH-D95	7
F	A	Finland	Kalkkimaeki	TRIFI585-11	JSlk-2011F045	8
M	A	Norway	Tjome, Mostranda	UMNEC454-08	RBCAD-2323	
F	A	Finland	Kalkkimaeki	TRIFI586-11	JSlk-2011F046	9
Agrypnia varia Fabricius, 1793						
		Croatia	River Mura, near Goričan		**TAVAR_1**	10
M		Germany	Donauaue 350 m W Giesenau, Donau Fkm 2436,125 [rec	FBAQU1149-12	BCZSMAQU00864	11
M		Germany	Gutendorf/Weimar, Klosterholz	GBEPT776-14	GBOL04390	
F		Austria	A-Salzburg,St. Georgen, Waidmoos	HMCAD320-08	07HMCAD-0320	
M		Austria	Stutz	INTAP120-17	PE143	
M		Belgium	Genk	TFLAN172-11	UA-SG-TRICH-D75	
F		Belgium	Dilsen-Stokkem	TFLAN173-11	UA-SG-TRICH-D76	
	L	Belgium	Gammelelva naturreservat	TFLAN252-11	UA-SG-TRICH-D74	
M		Germany	Tegernsee, Ufer unterhalb Kaltenbrunn (Gde. Gmund)	FBAQU251-09	BC ZSM AQU 00251	12
M		Germany	Zehdennick, Eichlerstich, Waldrand	GBEPT781-14	GBOL04395	13
F		Austria	A-Salzburg,St. Georgen, Waidmoos	HMCAD321-08	07HMCAD-0321	14
M		Austria	Mayrgraben, Lunz am See	HMTRI032-08	08HMCAD-032	15
M		Austria	Zell/Ybbs	HMTRI271-09	08HMCAD-271	16
M		Czech Republic	Blatenska slat` pod	KJTRI199-13	HMCAD13-9	17
M		Norway	Gammelelva naturreservat	TRDTR009-14	TRD-TRI88	18
M		Norway	Gammelelva naturreservat	TRDTR010-14	TRD-TRI89	
M		Norway	Gammelelva naturreservat	TRDTR011-14	TRD-TRI90	
M		Norway	Gammelelva naturreservat	TRDTR012-14	TRD-TRI91	
M		Norway	Gammelelva	TRDTR041-14	TRD-TRI87	
F		Norway	Genk	TRDTR113-15	TRD-TRI190	
F		Norway	Gammelelva	TRDTR112-15	TRD-TRI189	19

Sex	Life Stage	Country	Location	BOLD Sequence ID	Specimen ID	No. haplotyp
M		Latvia	river Gauja	TRIFI456-11	JSlk-20110108	20
	L	Finland	Loeyttynsuo lampi	TRIFI842-13	ARin-2012F312	21
	L	Finland	Virkkala	TRIFI844-13	ARin-2012F314	22
M		Finland	Loeyttynsuo lampi	TRIFI864-13	ARin-2012F334	23
Hagenella clathrate Kolenati, 1848						
	A	Germany	Bodenwoehr, Breitenbrucker Weiher	FBNE176-13	BC ZSM NEU 176	24
		Germany	Ueberacker	GBEPT332-14	GBOL03362	
		Germany	Ueberacker	GBEPT334-14	GBOL03364	
M		Austria	Bizauer Moos/Bach daneben	INTAP167-17	PE194	25
M		Finland	Ahamsuonlampi	TRIFI584-11	JSlk-2011F044	26
	L	Finland	Tappunen	TRIFI826-12	ARin-2012F291	27
M		Germany	Erlenbruch im Leutstettener Moos, 400 m N Heimaths	FBAQU1220-12	BCZSMAQU00935	28
M		Germany	Grabenartiger Bach zum Randlagg/Bruchwald S Knuepp	FBCAD059-10	BC ZSM AQU 00629	29
M		Norway	Tormajavri	FINNT031-12	FinnCAD-041	30
F		Estonia	Nomme	TRIFI750-12	JSlk-2012F215	31
	L	Finland	Jeesioenjoki	TRIFI822-12	ARin-2012F287	32
Phryganea bipunctata Retzius, 1783						
F		Germany	Tegernsee, Ufer unterhalb Kaltenbrunn (Gde. Gmund)	FBAQU273-09	BC ZSM AQU 00273	33
M		Czech Republic	Plesne jezero	BHMKK170-12	HMCAD1211-170	34
M		Austria	A-Salzburg, St. Georgen, Waidmoos	HMCAD322-08	07HMCAD-0322	
M		Austria	A-Salzburg, St. Georgen, Waidmoos	HMCAD323-08	07HMCAD-0323	
	A	Austria	Kogelsbach	HMCAD331-08	07HMCAD-0331	
		Norway	Morstadstolen	KKCAD550-09	GGCAD909-06	35
M		Norway	Storaastjoenna	TRDTR068-14	TRD-TRI145	36
M		Norway	Dam ved Engelsaastroea	TRDTR069-14	TRD-TRI146	
M		Norway	Store Skeistjern	TRDTR158-15	TRD-TRI235	
M		Norway	Store Skeistjern	TRDTR155-15	TRD-TRI232	37
M		Norway	Store Skeistjern	TRDTR157-15	TRD-TRI234	
F		Norway	Aasen	TRDTR156-15	TRD-TRI233	38
	L	Finland	Jeesioejoki	TRIFI277-11	JSlk-20100117	39
	L	Finland		TRIFI305-11	ARin-20100151	40
M		Finland	Ahmasuonlampi	TRIFI487-11	JSlk-20110139	41

Sex	Life Stage	Country	Location	BOLD Sequence ID	Specimen ID	No. haplotyp
Phryganea grandis Linnaeus, 1758						
M	adult	Croatia	River Dunav near Zlatna greda	CROAA134-18	**TPGRA_1**	42
	A	Finland	Bagaskaer	COLFH083-14	MM24011	
F		Germany	Donauaue 350 m W Giesenau, Donau Fkm 2436,125 [rec	FBAQU1224-12	BCZSMAQU00939	
M		Germany	Grosser Ostersee, Suedufer bei Mdg. Verbindungsbac	FBAQU274-09	BC ZSM AQU 00274	
F		Germany	Tegernsee, Ufer unterhalb Kaltenbrunn (Gde. Gmund)	FBAQU275-09	BC ZSM AQU 00275	
	L	Belgium	Geel	TFLAN036-11	UA-SG-TRICH-NA53	
	L	Belgium	Geel	TFLAN037-11	UA-SG-TRICH-NA54	
	L	Belgium	Mol	TFLAN116-11	UA-SG-TRICH-NA52	
	L	Belgium	Knokke-Heist	TFLAN124-11	UA-SG-TRICH-D77	
	L	Belgium	Kasterlee	TFLAN140-11	UA-SG-TRICH-D79	
	L	Belgium	Ekeren	TFLAN279-11	UA-SG-TRICH-X32	
	A	Norway	Rambjora	ZMBN036-15	Kurs2015-SR2	
				GBMIN35667-13	FN600940	43
	A	Austria	St. Konrad - Hausern	HMKKT280-10	10HMCAD-280	44
M		Austria	Mayrgraben, Lunz am See	HMTRI041-08	08HMCAD-041	45
F		Austria	Salzburg, Astenschmeide	HMTRI254-09	08HMCAD-254	46
	L	Belgium	Ranst	TFLAN028-11	UA-SG-TRICH-B42	47
	L	Belgium	Kortrijk	TFLAN130-11	UA-SG-TRICH-D78	48
M		Finland	Siikalahti	TRIFI480-11	JSlk-20110132	49
M		Finland	Siikalahti	TRIFI481-11	JSlk-20110133	50
Phryganea grandis rotundata G Ulmer, 1905						
M		Mongolia	Arhangay, Ogiy nuur	MGCAD523-09	ID-10340	51
M		Mongolia	Arhangay, Ogiy nuur	MGCAD524-09	ID-10341	52
M		Mongolia	Arhangay, Ogiy nuur	MGCAD525-09	ID-10342	53
M		Mongolia	Arhangay, Ogiy nuur	MGCAD526-09	ID-10343	54
M		Mongolia	Arhangay, Ogiy nuur	MGCAD527-09	ID-10344	
Lepidostoma hirtum (Fabricius, 1775)						
M		Croatia	River Kupa, near Pribanjci	CROAA126-18	**TLHIT_1**	55