NORTH-WESTERN JOURNAL OF ZOOLOGY 14 (1): 17-24
Article No.: e171201
©NWJZ, Oradea, Romania, 2018
http://biozoojournals.ro/nwjz/index.html

Revealing unknown larvae of the *maxima* species group of the genus *Acutipula* Alexander, 1924 (*Tipula*, Tipuloidae, Diptera) using an integrative approach

Lujza KERESZTES[1,*], Levente-Péter KOLCSÁR[1], Avar-Lehel DÉNES[1,2] and Edina TÖRÖK[1,2,3]

1. Hungarian Department of Biology and Ecology, Centre of Systems Biology, Biodiversity and Bioresources, University of Babeş-Bolyai Cluj-Napoca, Clinicilor 5-7, Romania; e-mail: keresztes2012@gmail.com, kolcsar.peter@gmail.com, edinatorok7@gmail.com
2. Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeş–Bolyai University, Treboniu Laurian 42, 400271, Cluj-Napoca, Romania; e-mail: avar.lehel@gmail.com
3. Romanian Academy Institute of Biology, Splaiul Independenţei 296, 060031 Bucureşti, Romania; e-mail: edinatorok7@gmail.com
* Corresponding author, E. Török, E-mail: edinatorok7@gmail.com

Received: 02. December 2016 / Accepted: 15. January 2017 / Available online: 15. March 2017 / Printed: June 2018

Abstract. Integrative tools should accelerate identification of "difficult" stages using genetic similarities between larvae and already known adult forms. *Acutipula* Alexander, 1924 larvae, which belong to the genus *Tipula* Linnaeus, 1758 (Diptera, Tipulidae), are common in aquatic, semi-aquatic or helobiont habitats in leaf litter or mud in the margins of small rivers. The subgenus is represented by 31 species in the Western Palaearctic area, but only seven larvae have been described so far, and the majority, of 77%, remains unknown. A B/MCMC and ML phylogenetic approach was applied based on mitochondrial DNA barcode sequences to detect unknown larvae of the South-Eastern European *T. balcanica* Vermoolen, 1983 and *T. latifiurca* Vermoolen, 1938 and then compared with the mostly Central and Western European *T. maxima* Poda, 1761 for morphological discrimination. Information on the morphology of the larvae are given and compared with other five European *Acutipula* larvae, presented in a synoptic key and illustrated. *Tipula bosnica* Strobl, 1898 and *Tipula luna* Westhoff, 1879 are recorded for the first time from Bulgaria.

Key words: larvae taxonomy, molecular tools, COI, morphology, phylogeny.

Introduction

The taxonomy of insect larvae offers new perspectives, since molecular methods are presently largely available and easy to apply (Jackson et al. 2014). *Acutipula* Alexander, 1924 is a subgenus of *Tipula* Linnaeus, 1758 characterized mainly by adult morphological traits: spur formula 1-2-2, the presence of the male tarsal claws toothed, squama with setae (excepting *bosnica* species group), wings with or without dark markings, veins R_{1+2} and R_3 tends to be crowded toward the costa, cell r_{1+2} being very small with its proximal end pointed, discoidal cell with veins more or less arcuate, male hypopygium with nine tergite and sternite fused into a continuous ring, having in the middle of the dorsum a conspicuous single or bifid projection bearing apically a dense surface with black thorns (Alexander 1935, Savchenko, 1961). Some characters are also important in *Acutipula* female's delimitations. For instance, it has an ovipositor with a slender, nonserrated, straight cerci and a subequal hypovalvae, in combination with a peculiar 10th tergite and cerci (Vermoolen 1983). Geographically the subgenus *Acutipula* has predominantly Palaearctic, Afrotropical and Oriental distribution with 195 recognized species, of which only 31 species occurs in the Western Palaearctic (Young et al. 2013, Oosterbroek 2016). In Europe only 7 species have larger distribution north of the "Mediterranean sanctuaries". The majority of the species are micro-endemics or have patchy distributions in some well known southern core areas, like Iberia, the Apennines, the southern Balkans, Anatolia, the Caucasus, northern Africa, and the Mediterranean islands.

The phylogeny of West Palaearctic *Acutipula* is still problematic because of the lack of molecular data and because morphological characteristics are less available in the case of a high number of larvae and pupae. However, an important contribution based on adult morphological features in combination with a few larvae or pupae characteristics pointed out important evolutionary trends within this subgenus (Vermoolen 1983, Oosterbroek & Theowald 1991, De Jong 1994, Koc et al. 1998). Combinations of some morphological features are useful to identify larvae belonging to this subgenus. For example the presence of four conspicuous and well-developed anal papillae around the anal opening, one pair of papillae above the anal opening and one pair below. The lower pairs of papillae were in the middle two pairs of short, unequal membranous processes in some species. The dorsal lobes of the spiracular disc are not fully sclerotized, and between the dorsal and lateral lobes there are tergite without dark markings, body segments with a conspicuous chaetotaxy, and no hair-bushes (Theowald 1967, Savchenko 1983).

Among *Acutipula* the *bosnica* species group was identified (Savchenko, 1967; Koc et al. 1998) on the basis of both adult (eg. tibial spurs formula, squama without macrotrichia and no transparent lunule in front of pterostigma) and larval characters (number of anal papillae) (*T. bosnica, T. aktashi* Koc, Hasbenli and De Jong, 1998, *T. aureola* Mannheims, 1952 from Central Europe to the Balkans, western Anatolia and southern Spain, respectively). In a morphologically-based phylogenetic analysis, the *bosnica* species group has a basal position among the Western Palaearctic *Acutipula*, with a completely sclerotized margin of the foramen at the base of the inner and outer gonostylus Koc et al. (1998). However, this character was also shared with the widely distributed *T. luna* Westhoff, 1879, *T. tenuicornis* Schummel, 1833 and *T. vittata* Meigen, 1804 (Mannheims 1952, Vermoolen 1983). Later, De Jong (1994) distinguished a highly divergent *maxima* species group based on a unique 9th tergite with two lateral processes, a specific male hypopygal structure (the presence of posterior directed projections on the lateral corners of the ninth tergite), female ovipositor details, a very characteristic zigzag wing pattern, and 2 pairs of additional short anal projections in the middle of the lower anal papillae in case of the larvae (this character is also present, however in *bosnica* and *fulvipennis* species groups). At the moment, the *maxima* species group includes the widely distributed *T. maxima, T. balcanica* and the other 16 mostly Mediterranean species: *T.*

anormalipennis Pierre, 1924, *T. citae* Oosterbroek and Vermoolen, 1990, *T. corsica* Pierre, 1921, *T. cretensis* Vermoolen, 1983, *T. cypriensis* Vermoolen, 1983, *T. doriae* Pierre, 1926, *T. isparta* Vermoolen, 1983, *T. latifurca* Vermoolen, 1983, *T. libanica* Vermoolen, 1983, *T. luctuosa* Mannheims, 1964, *T. macra* Savchenko, 1961, *T. repanda* Loew, 1864, *T. repentina* Mannheims, 1952, *T. rifensis* Theowald and Oosterbroek, 1980, *T. transcaucasica* Savchenko, 1961 and *T. triangulifera* Loew, 1864. The sister group of the *maxima* is the *fulvipennis* group, with four species with Western Palaearctic distribution: *T. fulvipennis*, *T. nevada* Dufour, 1990, *T. niethammeri* Mannheims, 1969 and *T. nigroantennata* Savchenko, 1961. The sister groups of the combined *fulvipennis* and *maxima* groups are the species *T. irrequieta* Alexander, 1936 (from the Caucasus and the western Himalayas), *T. ismene* Mannheims, 1969 and *T. schmidti* Mannheims, 1952 (both from the Western Balkan area).

Due to their rather uniform external appearance, *Acutipula* larvae have been highly neglected, compared to its adult forms (Chiswell 1956, Brindle 1957, Lantsov 2012, Podeniene 2001, Theowald 1967, Savchenko 1983). So far, data on 7 species from the West Palaearctic area have been published (namely *T. bosnica*, *T. fulvipennis*, *T. luna*, *T. maxima*, *T. nigroantennata*, *T. teniucornis* and *T. vittata*). These studies highlight the importance of the chaetotaxy of the thoracic and first seven abdominal segments and the shape of sclera on the lobes of the larval spiracular disc. Anal papillae, however, have important evolutionary implications; their number and length are important adaptations to the water content of the larvae's environment (Brindle 1957). In this respect, *Acutipula* larvae should be classified in two distinct ecological groups, *T. bosnica, T. fulvipennis T. maxima* and *T. nigroantennata* larvae, which have four pairs of papillae, two pairs of which are very long, are able to have a free aquatic life, and are frequently found under stones or detritus close to the river margins. *T. luna, T. tenuicornis* and *T. vittata* larvae have only two pairs of papillae and are mostly helobionts, frequenting marshy soils near riverbanks (Podeniene 2001, Lantsov 2012, Brindle 1957). Head capsules with sensory organs and mouth parts are less variable. The shredder-type mouth parts, also involved in the retention of fine particulate organic matter (FPOM), use a complicated filtering apparatus (Neugart et al. 2009). Despite a highly complex appearance, this kind of less specialized shredder-filtering apparatus has not been involved in adaptive radiation within *Acutipula* and is hence less useful for species level identifications.

Unlike the morphologies of adults, these characters were never subjected to a comprehensive phylogenetic analysis, with the exception of a general evaluation of tipuloid immature stages (Oosterbroek & Theowald 1991), and all available keys for larvae fail because of a high number of unknown larvae.

In the present study we added to knowledge of the larvae taxonomy of *Acutipula* by presenting descriptions of the hitherto unknown larvae of *T. balcanica* and *T. latifurca*. The putative larvae of these species were associated with co-occurring adults using molecular data from the most widespread mitochondrial DNA barcode region in a phylogenetic framework.

Material and methods

Specimen collections

Adults and larvae of 8 *Acutipula* species were collected by hand, mostly from different regions of Europe 1999-2016 (Supplement 1). Larvae and adults (mostly males) involved in genetic analyses were preserved in 96% ethanol. Fourth and third instar larvae were collected. Some larvae were reared (in the case of *T. bosnica, T. balcanica* and *T. vittata*) in glass dishes of 500 mL containing moist mould with abundant vegetable material picked from their original habitats. The majority of the larvae were immersed in hot water (50-60 °C) to expand the anal papillae and spread the lobes of the spiracular disc. The postabdomen of the male individuals and larvae head capsule was macerated in KOH 10% and then put on glycerol prior to morphological studies. Material for morphological analyses (both adults and larvae) was stored in 70% alcohol in order to keep the specimen more flexible and deposited in the Zoological Museum of the Babeş-Bolyai University, Cluj Napoca, Romania.

Morphological study

Morphological structures were examined using an Olympus SZ61 stereomicroscope equipped with a Canon 650D camera and an LM Digital SLR Adapter (Micro Tech Lab, Austria). Layer photos were combined using the software Combine ZP and drawings were executed with the use of Adobe Photoshop CS4. Morphological terminology and chaetotaxy of the larvae follows Theowald (1967) and Savchenko (1983). The larvae were described in terms of a set of morphological characters for Tipulidae, chaetotaxy, anal papillae and the spiracular area as defined by Chiswell (1956), Theowald (1967), Savchenko (1983) and Gelhaus (1986). Head capsules were compared based on Neugart et al. (2009) and Den Hollander (1975).

Molecular study

We used phylogenetic analyses to associate adult males with their putative larvae. We inferred phylogenetic trees based on molecular sequence data from DNA barcoding regions of 658 bp of the mitochondrial cytochrome c oxidase subunit I (COI) for a number of 28 adults and 11 larvae belonging to 8 European widespread *Acutipula* species (Will & Rubinoff 2004). We extracted DNA from larvae and adult males using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. PCRs were performed in a volume of 50 µl solution, visualized on a 1% agarose gel and purified with the Wizard SV Gel and PCR Clean–Up System (Promega). PCR products were sequenced by Macrogen Inc. (Korea) (10 sequences) and Barcode of Life Data System (BOLD; http://www.boldsystems.org) (29 sequences). Sequences were verified at the NCBI website using a Basic Local Alignment Search Tool (BLAST) of Johnson et al. (2008) and deposited in GenBank. Sequences in the BOLD system can be found under the EUTIP (34 sequences), SATIP (3 sequences) and FINTI (2 sequences) cods. The sequences were downloaded and aligned using Clustal W in MEGA6 (Tamura et al. 2013). We applied the Bayesian Markov-Chain Monte-Carlo (B/MCMC) method (1000000 generations) in MrBayes v. 3.2.6 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003), using a General Time Reversible (GTR) evolution model with a gamma-distributed variation rate across sites (G), and a proportion of invariable sites (I) was selected from a pool of 88 models with jModelTest v. 2.1.4 (Darriba et al. 2012). The B/MCMC tree was visualized with FigTree v 1.4. A Maximum Likelihood (ML) tree was also estimated using the same model in MEGA6 (Tamura et al. 2013). Genetic distance between *Acutipula* species was calculated in MEGA6 using p-distance algorithm (Tamura et al. 2013). Adults and larvae associations were accepted when posterior probabilities were high and bootstrap value raised to 95-100% or clustered together in a monophyletic unit. The identified larvae were then compared morphologically to detect taxonomically informative characters.

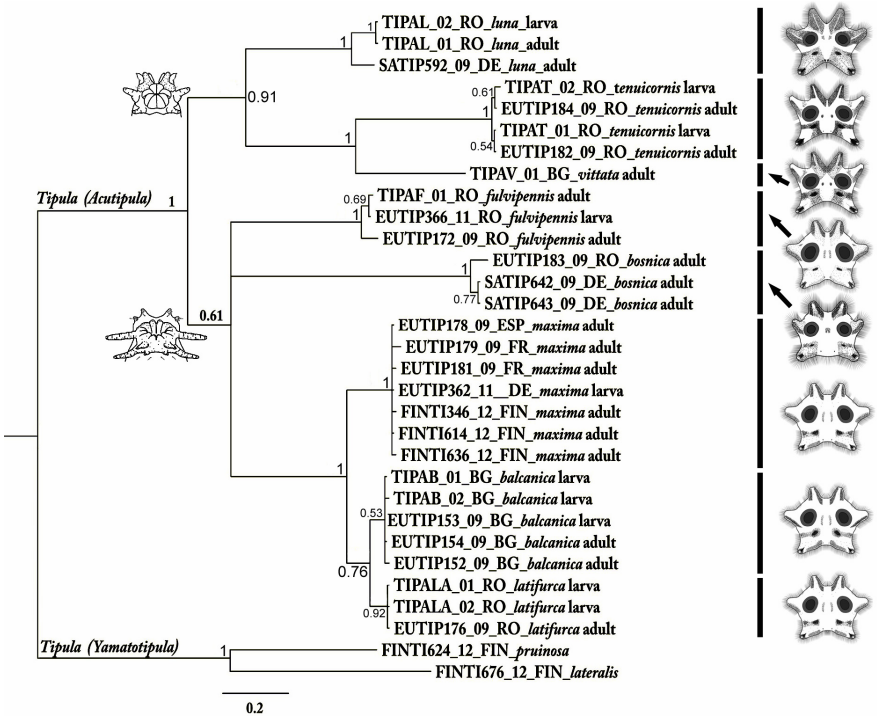

Figure 1. Molecular B/MCMC and ML combined tree of *Tipula (Acutipula)* species group, with *Tipula (Yamato-tipula) pruinosa* and *Tipula (Yamato-tipula) lateralis* as outgroups. Posterior probabilities (PP) and bootstrap values (BP, %) for the nodes are shown under the branches.

Results

Identification of the larvae

The two phylogenetic trees show the same lineages and topology, except that on the ML tree, the subgenus *Yamatotipula* Matsumura, 1916 species acts like a basal clade only for the group formed by the *maxima* species, the *T. fulvipennis* and *T. bosnica* (Fig. 1). Due to the unclear relationships and low support shown by the ML tree, we discuss it only for the larvae association and consider that it has no phylogenetic value. The putative conspecific larvae have 95-100% genetic similarity with adults and always clustered together in a monospecific clades (exceptions are *T. balcanica* and *T. latifurca* having monospecific clades, but low statistical support) (Fig. 1). Monophyly with identical haplotypes and strong support was detected in the case of *T. bosnica*, *T. fulvipennis*, *T. maxima*, *T. luna* and *T. tenuicornis.* There are however, some weaknesses in the resolution of the larvae association clusters, as the clades including *T. balcanica* and *T. latifurca* were not significantly supported (PP=0.53 and BP=73%, respective PP=0.92 and BP=95%). MtCOI sequences of *T. latifurca* and *T. balcanica* are clustered together, as was also suggested by adult morphologies, and are basal for a highly supported (PP=1 and BP=98%) clade of *T. maxima.* Further multigene analyses and the introduction of more sequences from both closely related *balcanica/latifurca* and the mostly Mediterranean species belonging to *maxima* group are required to achieve better resolution and clarify the phylogenetic position of this important south-eastern European clade. However, this weaker statistical support didn't considerably influence the association of adults with larvae, supported by identical haplotypes in all of the species that were analysed.

In three cases, *T. nigroantennata, T. bosnica* and *T. vittata* putative larvae were analyzed and identified based only on data in the literature, or we reared them (the latter two), as molecular sequences for B/MCMC analyses were not available in the framework of our inquiry.

Larva of *Tipula* (*Acutipula*) *balcanica* Vermoolen, 1983

Tipula balcanica belongs to the *maxima* species group and was first described as a subspecies of *T. maxima* (Vermoolen, 1983) and later raised to species rank (De Jong 1994).

Material examined: 38 fourth instars larvae were analyzed. Additionally, 12 males were examined and included in our molecular analyses (Supplement 1).

Description of the fourth instar larva: General coloration is brown, length of the larva 35-62 mm (average: 47 mm). Habitus and abdominal setal arrangements (chaetotaxy) illustrated on Fig. 2A-C, 2J. Head capsule have a length 3.4-3.5 mm and a width 1.3-1.4 mm (Fig. 3). Coloration in larvae preserved in alcohol is generally brownish-grey due to the dense and short pubescence of the body. The head is ovular in dorsal view and compressed dorsoventrally with a general dark brown or blackish coloration, with some lateral, less strongly pigmented oval areas. The prognathous and hemicephalic head capsule is deeply retracted into the prothorax and fixed by the cervical membrane at the articulatory fold close to its anterior margin (Fig. 3). The larvae antennae are conspicuous and composed from a single cylindrical and slightly curved antennomere (Fig. 3B, D). The apex of the antennomere is rather complicated, having a moderately large globular sensillum and a group of smaller sensilla. The tripartite labrum has a complex structure and is separated from the anterior margin of the clypeal region by a deep fold (Fig. 3A, B). Dorsally, it is composed of two sclerotized lobes, having brushes of long setae. A conspicuous stout process is also visible in the apical region of each lobe. The convex membranous median part of the labrum is densely covered with anteriorly directed short microthrichia and two small sclerotized rings. The mandibles are one-segmented, strongly sclerotized, and slightly curved inwards (Fig. 4E-H). Three distinct, rounded teeth are present on the apical edge. A fourth small, rounded tooth is present

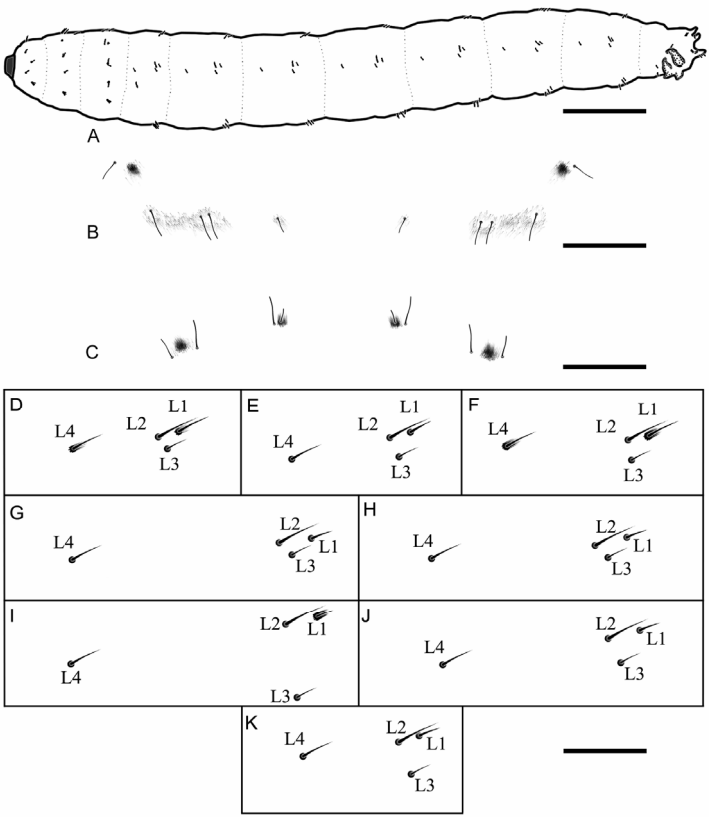

Figure 2. A. Habitus of *Tipula (Acutipula) balcanica* larvae in lateral view. B. Chaetotaxy of abdominal tergite in *T. balcanica*. C. Chaetotaxy of abdominal sternit in *T. balcanica*. Chaetotaxy of abdominal pleural parts: D. *T. tenuicornis*. E. *T. vittata*. F. *T. luna*. G. *T. maxima*. H. *T. latifurca*. I. *T. fulvipennis*. J. *T. balcanica*. K. *T. bosnica*. Scale bars: A = 4 mm; B-K = 0.1 mm.

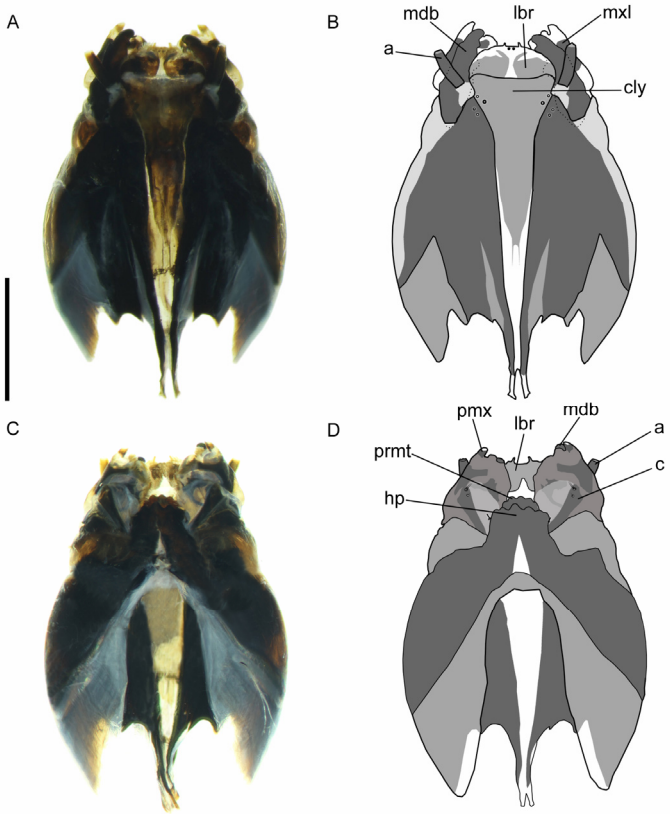

Figure 3. Head capsule morphology of the fourth instar larvae of *Tipula (Acutipula) balcanica*. A-B. Head capsule in dorsal view. C-D. Head capsule in ventral view. Abbreviation: a - antenna, mdb - mandible, lbr - labrum, mxl - maxilla, cly - clypeus, hp - hypostomium, prmt - prementum, pmx - maxillary palp, c - cardo. Scale bar: 1 mm.

near the lacinia mobile. However, the shape of the teeth on the mandible proved to be less useful in larvae taxonomy, as the teeth vary considerably depending on the age of the larvae (meaning attrition by usage). A well-developed lacinia mobile is present on the dorsal side of the middle of the mandible (Fig. 4E-H, see lm). It is sclerotized, distinctly widening distally, and glabrous in its apical margin. The hypostomal plate is strongly sclerotized, having seven strongly developed triangular teeth on its anterior margin (Fig. 4I-J). The prementum is almost entirely covered by hypostomium

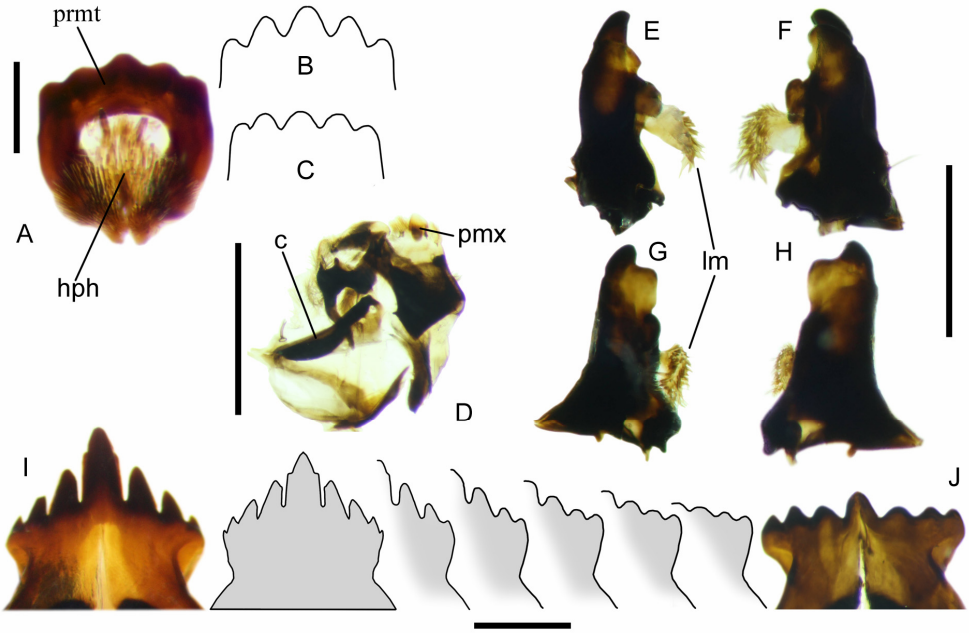

Figure 4. A-B, D-J. Details in head capsule of *Tipula (Acutipula) balcanica*. A. Prelabio-hypopharyngeal complex. B. Shape of prementum of *T. balcanica*. C. Shape of prementum of *T. fulvipennis*. D. Maxilla, ventral view. E. Mandible, ventral view. F. Mandible, dorsal view. G. Mandible, inner lateral view. H. Mandible outer lateral view. J-I. Shape of hypostomium plate in different wear rates. Abbreviation: prmt - prementum, hph - hypopharynx, c - cardo, pmx - maxillary palp, lm - lacinia mobilis. Scale bars: A-C: 0.2 mm. D-H: 0.5 mm, I-J: 0.25 mm.

from below. It bears five slightly rounded teeth at its anterior margin (Fig. 4A-B). Maxillae with maxillar palps were less important in our study, as they are covered by dense fringes of long microtrichiae or brushes of long hairs with no interspecific variability (Fig. 4D). The chaetotaxy of the dorsal and ventral side of each abdominal segment IV-VII are presented in Fig. 2B, C and Fig. 2J as are the pleurae**.** The spiracle area of the anal segment has 6 lobes and edges with a fringe of short setae. Spiracles are slightly ovular, with dark brown interior and outer circles. Further details on the spiracle area are presented in Fig 5K. Anal papillae surrounding the anal opening are long slender processes. There are two pairs of long lateral papillae and two pairs of shorter processes in the middle (Fig. 5B, D).

Molecular distance: molecular distance between *T. balcanica* and the closely related *T. latifurca* is only 0.7%, which shows only a minimal difference in mtCOI sequences, with no signs of introgression, but this difference is much lower than the conventional barcoding gap of 2% generally accepted for insects (Brower, 2006). Molecular similarity between the two species needs a multigene approach, involving a wide range of analyses of geographically distant populations.

Habitat: The larvae were found in mud or in leaf litter and plant residues in water.

Tipula (*A.*) *balcanica* is a monophyletic unit in our phylogenetic analyses, and it is closely related to *T. latifurca* with low statistic support (PP=0.76) (Fig.1). Morphologically, the presence of two pairs of long papillae around the anal opening and the additional two pairs of rather short middle papillae right under the anal openings suggest that the larvae belong to the aquatic *T. fulvipennis* + *T. bosnica* + *T. maxima* groups and show clear differences from the mostly helobiont *T. vittata* + *T. tenuicornis* + *T. luna* groups (Fig. 1). However, the larvae of *T. balcanica* (Fig. 2J) are distinct from *T. fulvipennis* (Fig. 2I) by the chaetaotaxy of the pleurae. *T. balcanica* is also differentiated from *T. bosnica* by a distinct chaetotaxy of the pleurae (Fig. 2K) and a rather long distance between the L4 and L2 setae. However, these characters were also shared with *T. maxima* and *T. latifurca*, from which it should be separated by a combination of characters, such as the shape of the interior chitinous band of the ventral lobes of the spiracular area and the blackish, mostly triangular spot on *T. balcanica* and *T. latifurca* in contrast with the mostly rounded black spot on *T. maxima.*

Larva of *Tipula (Acutipula) latifurca* Vermoolen, 1983

T. latifurca was first described as a subspecies of *T. (A.) transcaucasica* (Savchenko, 1961) and later raised to species rank (De Jong, 1994).

Material examined: 33 fourth instar larvae and 19 adult males were collected and compared (Supplement 1).

Description of the fourth larvae: The fourth instars larvae are very similar to the closely related *T. balcanica* and *T. maxima*. Body length is 50-60 mm, head capsule has a length of 3.6-3.8 mm, head capsule width is 1.7-1.8 mm. Coloration in larvae that were preserved in alcohol is generally brownish-grey due to the dense and short pubescence of the body. It is also a member of the mostly aquatic group among *Acutipula*, with the presence of four well-developed pairs of anal papillae. The head capsule is very similar to that of *T. balcanica* (Fig. 2-3) and *T. maxima*, with extremely variable chitinous parts, ex. hypostomium (Fig. 4I-J), and the teeth of mandible (Fig. 4E-H) are more or less rounded in relation to the age of the larvae (usage by attrition). The chaetotaxy of the pleurae is presented in Fig. 2H. The spiracle area of the anal segment is showing in Fig. 5L.

Molecular distance: for molecular divergences see Table

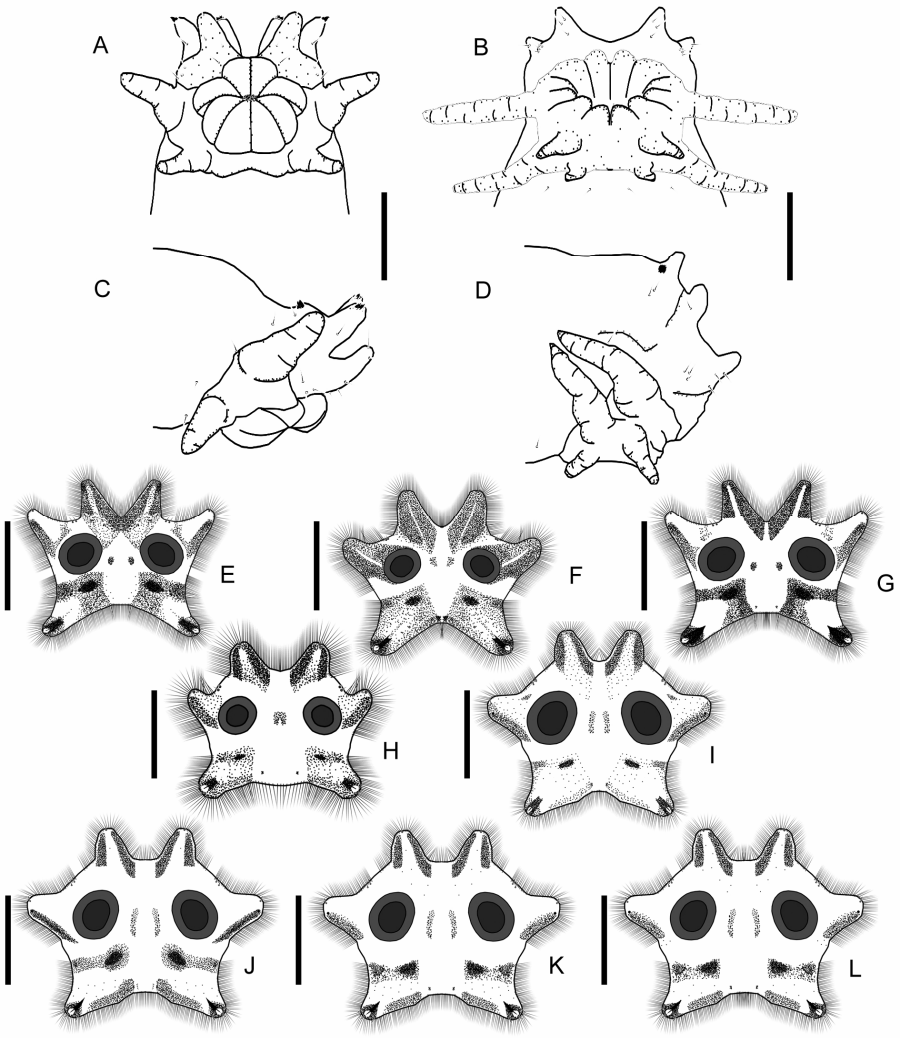

Figure 5. Tracheal gills (anal papillae) around anal openings in *Tipula (Acutipula) tenuicornis*: A. ventral view, C. lateral view; in *T. balcanica*: B. ventral view, D. lateral view. E-L. Spiracular area of different *Acutipula* species: E. *T. tenuicornis*. F. *T. luna*. G. *T. vittata*. H. *T. bosnica*. I. *T. fulvipennis*. J. *T. balcanica*. K. *T. maxima*. L. *T. latifurca*. Scale bar: A,C, E-G: 2.5 mm. B,D, J-L: 4 mm. H: 2 mm. I: 3 mm.

1 and comments in the description of *T. balcanica* larvae.

Habitat: The collecting larvae were found submerged under stones at the edge of the small marshy brook in Fagilor valley (Luncavita, Macin Mts., Romania), which is rich in leaf litter and plant residues in water.

T. (A.) latifurca should be differentiated from all other widely distributed European *Acutipula* larvae by the presence of the following combination of characters: four pairs of anal papillae; lack of a bush of short hair around pleural seta L1 (Fig. 2H), the presence of a dark patch at the tip of the ventral lobes of the spiracular disc which does not bifurcate at the base (Fig. 5L);

Key for the currently known Western Palaearctic *Acutipula* larvae (fourth instars):

1. Anal field with four pairs of anal lobes (papillae) (Fig. 5B, D) **2**
– Anal field with two pairs of anal lobes (papillae) (Fig. 5A, C) **6**
2. Lateral seta 1 (L1) at the base with a small tuft; the distance between L2 and L3 at least twice as large as the distance between L1 and L2, usually more than 2.5-3 times (Fig. 2I), spiracular disc as in Fig. 5I ..... ***T. fulvipennis* De Geer**
– L1 without tuft and the distance between L2 and L3 almost the same as the distance between L1 and L2 (or unknown in the case of *T. nigroantennata*) (Fig. 2G, H, J, K) **3**
3. Ventral lobe at the base with small elongated ovular or sometimes line-like sclerite; the sclerite at the end of the ventral lobe elongated with obtuse edge or with a notch on the base (Fig. 5H) ..... ***T. bosnica* Strobl**
– Ventral lobe at the base with a large ovular (Fig. 5J) or triangle-like (Fig. 5K, L) sclerite; the sclerite at end base of the ventral lobe with a spike (if less sclerotized) or drop of water like **4**
4. Ventral lobe at the base with ovular dark sclerite **5**
– Ventral lobe at the base with triangle-like dark sclerite ..... ***T. balcanica* Vermoolen and *T. latifurca* Vermoolen**
note: the only small difference between *T. balcanica* and *T. latifurca* larvae is the presence of the interior small peak of the patch at the end of the ventral lobe, but this character is less visible in many cases

5. Length of the last instar larvae between 26-31 mm; the patch in the inner margin of ventral lobe uniform does not broaden at the base (see Lantsov 2012, Fig. 2, 3) ..... ***T. nigroantennata*** **Savchenko**

– Length of the last instar larvae always larger than 32 mm; the patch in the inner margin of ventral lobe broadens at the base (Fig. 5J) ..... ***T. maxima*** **Poda**

6. The base of L1 and L2 setae with no small tuft (Fig. 2E); the sclerite at the end of the ventral lobe with a spike (if less sclerotized) or drop of water like (Fig. 5G) ..... ***T. vittata*** **Meigen**

– The base of L1 and L2 seta with a small tuft (Fig. 2D, F); the sclerite at the end of the ventral lobe elongated with a notch on the tip (Fig. 5E, F) ***7***

7. Both margins of the dorsal and lateral lobe with very wide sclerites almost the same length (Fig. 5F) ..... ***T. luna*** **Westhoff**

– Only the lower margin of lateral lobe sclerotized, forming an "L" like patch. The inner margin of the dorsal lobe with a larger sclerite than the outer margin (Fig. 5E) ..... ***T. tenuicornis*** **Schummel**

Table 1. Genetic distance with p-distance between *Tipula (Acutipula)* species group and outgroup *Tipula (Yamatotipula).*

Tipula species	*T. latifurca*	*T. balcanica*	*T. maxima*	*T. bosnica*	*T. luna*	*T. tenuicornis*	*T. fulvipennis*	*T. vittata*	*T. (Yamatotipula) pruinosa*
T.(Acutipula)latifurca									
T. (Acutipula)balcanica	0.70								
T. (Acutipula)maxima	3.74	3.64							
T.(Acutipula)bosnica	12.43	12.53	13.49						
T.(Acutipula)luna	10.89	10.90	10.85	12.74					
T. (Acutipula)tenuicornis	14.79	15.02	14.73	14.74	14.30				
T. (Acutipula)fulvipennis	9.00	10.07	10.61	11.89	10.90	14.08			
T.(Acutipula)vittata	14.85	14.68	14.61	13.86	11.00	10.28	12.87		
T.(Yamatotipula)pruinosa	10.56	10.33	10.11	13.36	11.60	13.31	10.72	13.36	
T.(Yamatotipula)lateralis	12.21	11.91	11.64	12.65	11.82	13.47	12.65	13.20	10.72

New faunistic records

After processing 215 *Acutipula* specimens (belonging to 8 species) from different regions of Europe, we identified *Tipula bosnica* and *T. luna* that are new to the Bulgarian fauna (see Supplement 1).

Discussions

This is the first contribution to the *Acutipula* larvae taxonomy using a phylogenetic analysis based on the widely used mtCOI DNA barcode region. Our analyses support the hypothesis that *Acutipula* is a well-established monophyletic unit, with two highly divergent lineages, a hypothesis that is also supported by larvae morphologies (see also Brindle 1957). A group of larvae with two pairs of long anal papillae was identified in the mostly helobiont *T. luna, T. tenuicornis* and *T. vittata*, and the identification of these larvae as a distinct group was also supported by our molecular data. The other group is composed of larvae with two pairs of long anal papillae and two additional pairs of conic processes at the base of the lower pair of anal papillae, in relation to the aquatic environment of the immature stages of *T. bosnica, T. fulvipennis, T. maxima, T. balcanica* and *T. latifurca.* The aquatic larvae constitute a highly supported monophyletic unit in our B/MCMC tree. Similarly, the reduced number of anal lobes to 4 represents another well differentiated evolutionary unit in relation to the ecology of larvae. Hence, both the free-aquatic life forms and the mostly terrestrial, helobiont forms are closely related to some divergent evolutionary ecological processes of soil moisture or water content Young et al. (2013).

On the phylogenetic tree a monophyletic clade can be observed that includes larvae of the very similar *maxima* complex (Fig. 1), also supported by a highly divergent male hypopygium (De Jong, 1994). Two larvae that belong to *T. balcanica* and *T. latifurca* were first detected with the use of molecular tools and clustered together with the species-rich *maxima* species group (having 8 anal lobes, a spike like spot on the ventral lobe of the spiracular area, and no small hairs at the base of L1 and L4). However, *T. maxima* differ from both *T. latifurca* and *T. balcanica* by the ovular shape of sclerite on the ventral lobe at the base, and the patch in the inner margin of ventral lobe broadens at the base. On the other hand, *T. latifurca* and *T. balcanica* share identical characters, the triangle-like sclerite on ventral lobe at the base, and the patch in the inner margin of ventral lobe is uniform and does not broaden at the base. We found only a small in and many cases less visible difference between the two species, which is the presence of the interior small peak of the patch at the end of the lateral lobe (Fig. 5K, L).

Although the phylogenetic relationship between *T. balcanica* and *T. latifurca* and their position on the tree was supported by a low posterior probability value (0.76) and minimal genetic distance (0.7), the strong support of the individual groups suggests good isolation, shown also by unique haplotypes of these monospecific clades (Fig. 1). Thus, molecular tools help discriminate between morphologically highly cryptic *T. latifurca* and *T. balcanica*, along with a weakly established morphological divergence of the larvae. Further details on larvae body characters and more molecular data on *T. latifurca*, including data on Mediterranean

populations, need to be examined for better taxonomic resolution.

The "helobiont" group representing a clade with *T. luna* + *T. tenuicornis* and *T. vittata* is well supported genetically and also confirmed by larvae characters (the presence of only 4 anal lobes).

The less specialized but highly complicated shredder-like mouthparts of larvae, well equipped for passive filter feeding, were less useful for species level identification and do not follow deeply divergent structures detected in adult morphologies or molecular data. In general, feeding ecology in Tipulidae larvae is very similar between different taxonomic groups, supporting less severe evolutionary pressure, similar to Ephemeroptera, but different from Trichoptera. In the case of some caddis-fly groups, like Drusinae, larvae have important adaptive radiations, leading finally to some morphologically highly specialized and phylogenetically well supported evolutionary ecology units (from shredders and filter feeders to carnivores), in contrast with very similar adult morphologies Pauls et al. (2008), Vitecek et al. (2015), Waringer et al. (2015).

In conclusion, morphology based taxonomy of *Acutipula* larvae still remains a challenge, because of a high number of closely related cryptic larvae (ex. the *maxima* group) as well as a high number of unknown larvae (mostly Mediterranean species) and phylogenetic inconsistencies (Fig. 1). Even so, phylogenetic analyses based on mitochondrial markers provide an effective tool to detect larvae not only in fourth stages, but even earlier. These analyses add to our repositories of taxonomic information and further our understanding of evolutionary trends in these conspicuous dipteran larvae belongs to *Acutipula*.

Acknowledgements. This research was supported by a grant of the Ministry of National Education, CNCS-UEFISCDI, PN-II-ID-2012-4-0595. We would like to express our sincerest gratitude to the collectors and also to thank Jukka Salmela and Chen W. Young for for genetic data.

Reference

Alexander, C.P. (1935): New or little-known Tipulidae from eastern Asia (Diptera). XXV. Philippine Journal of Science 57: 81-148.

Brindle, A. (1957): The ecological significance of the anal papillae of *Tipula* larvae (Diptera, Tipulidae). Entomologists Monthly Magazine 93: 202-204.

Brower, A.V.Z. (2006): Problems with DNA barcodes for species delimitation: 'ten species' of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). Systematics and Biodiversity 4(2): 127-132.

Chiswell, J.R. (1956): A taxonomic account of the last instar larvae of some British Tipulinae (Diptera: Tipulidae). Transactions of the Royal Entomological Society of London 108: 409-484.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D. (2012): jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9(8): 772.

De Jong, H. (1994): The phylogeny of the *Tipula (Acutipula) maxima* species group, with notes on its distribution (Diptera: Tipulidae). Entomologica Scandinavica 24: 433-457.

Gelhaus, J.K. (1986): Larvae of the crane fly genus *Tipula* in North America (Diptera: Tipulidae). University of Kansas Science Bulletin 53: 121-182.

Hollander, J.D. (1975): The growth of larvae of *Tipula oleracea* Linnaeus. Tijdschrift voor Entomologie 118: 67-82.

Huelsenbeck, J.P., Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754-755.

Jackson, J.K., Battle, J.M., White, B.P., Pilgrim, E.M., Stein, E.D., Miller, P.E., Sweeney, B.W. (2014): Cryptic biodiversity in streams: a comparison of macroinvertebrate communities based on morphological and DNA barcode identifications. Freshwater Science 33(1): 312-324.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden T.L. (2008): NCBI BLAST: a better web interface. Nucleic Acids Research 36 (2): W5-W9.

Koc, H., Hasbenli, A., De Jong, H. (1998): The *Tipula (Acutipula) bosnica* species group, with the description of a new species from Turkey (Diptera: Tipulidae). Entomologica Scandinavica 29: 267-274.

Lantsov, V.I. (2012): Ecology of the Caucasus populations and preimaginal spages of the crane-fly *Tipula nigroantennata* Sav. (Diptera, Tipulidae). Russian Entomological Journal 21: 269-272.

Neugart, C., Schneeberg, K., Beutel, R.G. (2009): The morphology of the larval head of Tipulidae (Diptera, Insecta), the dipteran ground plan and evolutionary trends. Zoologischer Anzeiger 248: 213-235.

Mannheims, B. (1952): Tipulidae. In: Lindner, E. (ed.), Die Fliegen der palaearktischen. Region Lief 3(5)1: 65-112.

Oosterbroek, P., Theowald, B. (1991): Phylogeny of the Tipuloidea based on characters of larvae and pupae (Diptera, Nematocera) with an index to the literature except Tipulidae. Tijdschrift voor Entomologie 134: 211-267.

Oosterbroek, P. (2016): Catalogue of the Craneflies of the World. <http://ccw.naturalis.nl/>, accessed at: 2016.05.29.

Pauls, S.U., Graf, W., Haase, P., Lumbsch, H.T., Waringer, J. (2008): Grazers, shredders and filtering carnivores – The evolution of feeding ecology in Drusinae (Trichoptera: Limnephilidae): Insights from a molecular phylogeny. Molecular Phylogenetics and Evolution 46: 776–791.

Podeniene, V. (2001): Notes on the larvae of Lithuanian Tipulidae from the subgenera *Tipula (Acutipula)*, *T. (Beringotipula)* and *T. (Platytipula)*. Ekologija 2: 81-89.

Ronquist, F., Huelsenbeck, J.P. (2003): MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Savchenko, E.N. (1961): Crane-flies (Diptera, Tipulidae), Subfam. Tipulinae, Genus *Tipula* L., (part 1) Fauna USSR, N.S. 79. Nasekomye Dvukrylye Diptera 2 (3): 1-487.

Savchenko, E.N. (1967): The immature stages and the taxonomic position of the crane-fly *Tipula* (? Subgen.) *decipiens* Cz. (Diptera, Tipulidae). Vestnik Zoologii 1967(3): 22-28.

Savchenko, E.N. (1983): Crane-flies (Fam. Tipulidae), Introduction, Subfam. Dolichopezinae, subfam. Tipulinae (start). Fauna USSR, N.S. 127. Nasekomye Dvukrylye Diptera 2(1-2): 1-585.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Theowald, B. (1967): Familie Tipulidae (Diptera, Nematocera). Larven und Puppen. Bestimmungsbucher zur Bodenfauna Europas 7: 1-100.

Vermoolen, D. (1983): The *Tipula (Acutipula) maxima* group (Insecta, Diptera, Tipulidae). 1. Taxonomy and Distribution. Bijdragen tot de Dierkunde 53: 49-81.

Vitecek, S., Graf, W., Kučinić, M., Oláh, J., Bálint, M., Previšić, A., Keresztes, L., Pauls, S.U., Waringer, J. (2015): A hairy case: The evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera). Molecular Phylogenetics and Evolution 93: 249-260.

Young, C.W., Li, Y., Chu, W.C., Fang, H.S. (2013): Review of *Acutipula* crane flies of Taiwan with description of new species and immature instars (Diptera: Tipulidae: Tipulinae: *Tipula*). Annals of the Carnegie Museum 82: 115-148.

Waringer, J., Graf, W., Bálint, M., Kucinic, M., Pauls, S.U., Previsic, A., Keresztes, L., Ibrahimi, H., Zivic, I., Bjelanovic, K. (2015): Larval morphology and phylogenetic position of *Drusus balcanicus, D. botosaneanui, D. serbicus* and *D. tenellus* (Trichoptera: Limnephilidae: Drusinae). European Journal of Entomology 112(2): 344-361.

Will, K.W., Rubinoff, D. (2004): Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20(1): 47-55.

+ Supplementary material – available exclusive online (4 pages, 1 table)