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Larval description of *Drusus bosnicus* Klapálek 1899 (Trichoptera: Limnephilidae), with distributional, molecular and ecological features

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Abstract

In this study we present morphological, molecular and ecological features of the last instar larvae
of *Drusus bosnicus* with data about distribution of this species in Bosnia and Herzegovina. We also
included are the most important diagnostic features enabling separation of larvae of *D. bosnicus*
from larvae of the other European Drusinae and Trichoptera species.

Keywords

Drusinae; 5th instar larva; identification; morphology; Bosnia and Herzegovina

Introduction

The Balkan Peninsula is one of the most interesting centres of diversity for different animal
groups in Europe (e.g., Gottstein-Matoec *et al.* 2002; Džuki & Kalezi 2004; Kryštufek
2004; Bedek *et al.* 2006; Bilandžija *et al.* 2013) including Trichoptera (e.g., Kumanski &
Malicky 1999; Malicky 2005; Oláh 2010, 2011). In this region (Albania, Bosnia and
Herzegovina, Bulgaria, Croatia, Kosovo, Macedonia, Montenegro, Serbia, Greece) 40

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species from the Eurasian genus *Drusus* (family Limnephilidae) have been recorded (Schmid 1956; Radovanovi 1942; Krušnik 1987; Kumanski 1988; Malicky 2004, 2005; Oláh 2010, 2011; Oláh & Kovács 2013). Except *Drusus botosaneanui* Kumanski 1968, *Drusus discolor* (Rambur 1842), *Drusus biguttatus* (Pictet 1834) and *Drusus tenellus* (Klapálek 1898), all other *Drusus* species are endemic to this region. *Drusus tenellus* and *D. botosaneanui* also occur in Romania, *D. biguttatus* and especially *D. discolor* are widespread throughout Europe. *Drusus* larvae usually inhabit springs and the crenal section of mountain streams and rivers with low water temperature.

Within family Limnephilidae, one of the most interesting groups is the *Drusus bosnicus* Group (Schmid 1956) with numerous endemic species described from the Balkan Peninsula (e.g., Radovanovi 1942; Marinkovi -Gospodneti 1976). All species of the *Drusus bosnicus* Group share some morphological and behavioural features like the shape of male genitalia, the dark coloration of adults and daylight activity. The first species described from this group at the end of the 19th century was *Drusus bosnicus* Klapálek 1899 (Fig. 1), with *locus typicus* at the spring of the River Bosna situated in Sarajevo, central Bosnia and Herzegovina (Fig. 2). Recently, eight the newly described species from Albania and Greece (Oláh 2010, 2011) were found to belong to the *Drusus bosnicus* Group. The main distribution area of the *Drusus bosnicus* Group is the Balkan Peninsula with more than 20 described species (e.g., Radovanovi 1942; Marinkovi -Gospodneti 1976; Kumanski 1988). So far, larvae of 5 species of the *Drusus bosnicus* Group from the Balkan Peninsula have been described: *Drusus klapaleki* Marinkovi -Gospodneti 1971a, *Drusus medianus* Marinkovi -Gospodneti 1976, *Drusus radovanovici* Marinkovi -Gospodneti 1971a, *Drusus ramae* Marinkovi -Gospodneti 1971a, and *Drusus septentrionis* Marinkovi -Gospodneti 1976 (Ku ini et al. 2008, 2010, 2011a, 2011b).

In this study we present morphological features of the last instar larvae of *Drusus bosnicus*, combined with molecular and ecological notes and distribution data on this species. We also present the most important diagnostic features enabling separation of larvae of *D. bosnicus* from larvae of the other European Drusinae species.

Material and Methods

Fieldwork and sampling

The studied material of *D. bosnicus* comprises 10 larvae and one adult, collected on 17 May 2008, all of them from the spring of the Paljanska Miljacka River (Fig. 3), situated 15 kilometres southeast of Sarajevo, and two adults of *D. bosnicus* collected at the spring of the Bosna River situated in Sarajevo. Larvae were collected by handpicking and adults with an entomological net. Collected specimens were stored in containers with 80 and 96% EtOH, for morphological and molecular analysis, respectively. All collected specimens are deposited in the Faculty of Science in Zagreb, Croatian Natural History Museum in Zagreb and the Institute of Hydrobiology and Aquatic Ecology Management in Vienna (Austria). Larval morphological features were analysed according to Wiggins (1996) and Ku ini et al. (2010, 2011a, 2011b). Systematic presentation follows Morse (2015). We use *Drusus adustus* McLachan 1867 as a valid name (syn. *Drusus destitutus* Kolenati 1848). In this

paper, we include species *Leptodrusus budtzi* (Ulmer 1913), but it is possible that this species does not belong to Drusinae (J. Waringer, personal communication, 2015).

DNA extraction and PCR amplification

One adult male and two larvae of *D. bosnicus* from the spring of the Paljanska Miljacka River were genetically analysed in order to support the association of the larvae with the adults. Additionally we included two adult males from the spring of the Bosna River. Association was based on 541-bp-long fragment of the mitochondrial cytochrome oxidase I (mtCOI) gene and sequences were taken from Ku ini et al. (2010), Previši et al. (2009) and Previši et al. (2014b). Intraspecific *p*-distances were calculated in Mega 4.0.1 (Tamura et al. 2007).

Electronic microscopy, macrophotography and measuring

Morphological observations of samples were accomplished using a Tescan TS 5136 variable pressure scanning electron microscope (SEM). Samples were mounted with graphitic adhesive tape on the SEM stub and coated with carbon. The samples were examined by SEM operating in secondary electron (SE), or back-scattered (BSE) mode at an accelerating voltage of 20 kV, running current of 110 pA and variable pressure of 30 Pa to 5×10^{-1} Pa, sometimes pressure was increased to 10 Pa to eliminate sample charging.

Macrophotographing and measurements of larvae and larval cases were accomplished using a Leica Wild MZ8 stereomicroscope and Olympus SP-500 UZ digital camera, processed with computer programme Olympus Quick Photo Camera 2.2. In larvae of *D. bosnicus* the following features were measured: head width, total body length, length of the anterior-median metadorsal sclerites, their width in the widest median part and distance between them, and also the length of the posterior metadorsal sclerites (N=10). The following characters of cases were measured: total length, width of the anterior part, and width of the posterior part.

Description of fifth instar larva of *Drusus bosnicus*

Case and Larva

Case constructed completely of mineral particles (Fig. 4), slightly curving, total length 9.50–13.60 mm, width of anterior part 2.92–3.76 mm, width of posterior part 1.66–2.62 mm (N=10). Overall body shape eruciform (Fig. 5), total length without case 8.50–13.34 mm (N=10).

Head

Head capsule hypognathous, with width 1.57–1.77 mm (N=10) (Figs 5–8). In lateral view, posterior part of head capsule (vertex) flat with slight median concavity (Figs 5–8). Head brown, dorsally darker and laterally lighter, with granular surface sculpturing (Fig. 9). Genae of parietals reddish-brown to yellowish with lighter ring around each eye (Fig. 5). Dorsum of head capsule with dark muscle attachment spots (Fig. 9). Distinct area with small number of spinules (small spines) positioned laterally on each side of head capsule in areas between setae 13 and 16, but some specimens without visible spinules. Frontoclypeal apotome bell-

shaped with narrow central region (Fig. 9). Antennae short, brown to dark brown, each positioned on small, noticeable prominences. Labrum symmetrical, brown posteriorly to yellowish anteriorly (Fig. 9), with setal brush at anterolateral margins (Fig. 9) and thin primary setae on dorsal surface. Mandibles black, distally reddish (Fig. 9); typical for grazers, mesal margin of each mandible with setal brush (Fig. 10); two setae present laterobasally on each mandible (Fig. 10). Labium and maxillae light-brown (yellowish). Maxillary palps 5-segmented.

Thorax

Pronotum dark brown to black (Fig. 5) with granular sculptured surface. Posterior margin rounded, both posterior and lateral margins thick and darkly sclerotized. Anterior part (50–60%) of pronotum slightly concave and rising, posterior part with prominent median hump (Figs. 5, 7–8). Pronotum bearing dark setae, especially laterally and on anterior margin, some of them long and conspicuous (Figs. 5–8). Dorsal (posterior part) and lateral regions of pronotum with white recumbent setae (Fig. 8); in some specimens, pronotum with small number of white recumbent setae. Prosternal horn present.

Mesonotal sclerites brown, lighter than pronotum, with dark setae and uneven rugous surface (Figs. 5–6, 11). Posterior and lateral margins thick and darkly sclerotized (Fig. 11).

Metanotum with 3 pairs of dorsal sclerites (Fig. 11). Anteromedian (*sa1*) sclerites elongated, triangular with rounded apices, inter-sclerite distance less than width of either sclerite (Fig. 11), covered by setae, mainly anteriorly, color similar to mesonotum. Length of anteromedian sclerites 0.51–0.65 mm (mean 0.59 mm); width of anteromedian sclerites 0.26–0.34 mm (mean 0.30 mm); distance between anteromedian sclerites 0.07–0.14 mm (mean 0.10 mm). Posteromedian (*sa2*) sclerites smaller and lighter than *sa1* sclerites (Fig. 11), triangularly or irregularly ellipsoid and with many setae. Length of posteromedian sclerites 0.32–0.51 mm (mean 0.45 mm). Group of setae present on membranes between *sa2* sclerites and between *sa2* and *sa3* sclerites. Lateral (*sa3*) sclerites longitudinally prolonged, sickle-shaped, lighter brown with dark median region, and group of setae anteriorly.

Legs yellow-brown to dark brown or black, with dark ventral and dorsal margins (Figs. 5, 12–14). Coxae and femora of all legs with dark setae on both dorsal and ventral edges (Figs. 12–14). Trochanters of all legs without setae on dorsal margins (Figs. 12–14). Ventral margin on distal part of each foreleg trochanter with row of fine, yellowish setae (trochanteral brush). Midleg trochanters with few, fine, yellowish setae apicoventrally. Foreleg coxae and femora wide compared to those of mid- and hind legs (Figs. 12–14). Additional setae present on anterior and posterior faces of all femora. Mid- and hind legs similar in shape and size (Figs. 13–14), with slender coxae, trochanters, and femora. Setae on dorsal edges of tibiae present only distally in all legs (Figs. 13–14). Tarsi each with claw and basal seta, and tibial spurs light brown, almost yellowish (Figs. 12–14).

Abdomen

Abdominal segment I with well-developed dorsal (Fig. 11) and lateral humps (protuberances). Numerous setae present anterior and lateral to dorsal hump. Distinction

between dorsal setal areas *sa1* and *sa2* not possible (Fig. 11). Ventrally with numerous setae, some of them with small sclerites at bases.

Single filamentous gills present on segments II–VII (Figs. 15–16). Dorsal pre-segmental gills present on segments II–VII; dorsal post-segmental gills present on segments II–VI, ventral pre- and post-segmental gills present on segments II–VII (Fig. 15). Lateral pre- and post-segmental gills present on segments II–IV (Fig. 16). On abdominal segment VIII, four dorsal setae present. Lateral fringe extending from last third of segment II to segment VIII (Fig. 16); in some specimens, only few setae forming lateral fringe on segment II or setae not visible.

Segment IX bearing irregular, semicircular, light brown dorsal sclerite, generally with 8 long, dark setae on posterior margin and several shorter, lighter setae on posterior half of sclerite (Fig. 17). Anal prolegs typical of limnephilids (Fig. 18), each with longitudinally prolonged lateral sclerite, sickle-shaped, yellowish, with setae mainly in posterior part and 2 large, dark setae at posterior end (Fig. 18). Anal claws and accessory hook brown to dark brown (Fig. 18).

Discussion

Association of *Drusus bosnicus* larvae and adults

COI haplotypes of adult male and larvae from the spring of the Paljanska Miljacka River were either completely identical or differed in one nucleotide position (Table 1). Overall, intraspecific variability recorded in *D. bosnicus* (Table 1) is in line with the observed variability of the same COI fragment in populations of some other *Drusus* species (e.g., Pauls *et al.* 2009; Previši *et al.* 2009), including the *D. bosnicus* Group (e.g., Ku ini *et al.* 2008). However, the association of larvae and adults are not completely reliable based solely on comparisons of sequences of a single gene from one specimen each (e.g., Zhou *et al.* 2007). In addition to the molecular genetic data, association of adults and larvae of *D. bosnicus* are further supported by:

1. Species distribution data on the *Drusus bosnicus* Group in Bosnia and Herzegovina, in which all species have restricted ranges and generally show allopatric distribution (Marinkovi -Gospodneti 1979, Ku ini *et al.* 2008, 2011a, 2011b; Previši *et al.* 2009),
2. Presence of *D. bosnicus* as exclusive *Drusus* species in the Paljanska Miljacka River spring (Marinkovi -Gospodneti 1979).

Morphological separation of *Drusus bosnicus* larvae from other European Trichoptera larvae

In Europe, larvae of numerous different species of Trichoptera have been described (e.g., Urbani *et al.* 2003a, 2003b; Graf *et al.* 2011; Sáinz-Bariáin & Zamora-Muñoz 2012; Waringer *et al.* 2012a, 2012b) including larvae of species in the genus *Drusus* (e.g., Lepneva 1966; Decamps & Pujol 1975; Szczesny 1978; Waringer 1987; Wallace *et al.* 1990; Waringer & Graf 1997; Waringer *et al.* 2000, 2007a, 2007b, 2011; Graf *et al.* 2005, 2009;

Ku ini et al. 2008). The larvae of the subfamily Drusinae share common morphological features such as:

- a fully sclerotised pronotum and mesonotum;
- metanotum with six sclerites;
- additional setae present on anterior and posterior faces of mid- and hind leg femora;
- gills with one filament;
- slightly curved case, etc.

Distinctive features of *D. bosnicus* larvae are the specific shape of the head, pronotum, and anteromedian sclerites, enabling separation from other known larvae of European Trichoptera. Also, larvae of *D. bosnicus* differ from other larvae of the *Drusus bosnicus* Group in the shape of the head, except for *D. ramae* larvae. *Drusus bosnicus* and *D. ramae* exhibit similar head shapes in lateral view, but differ in pronotal shape (Figs. 19–21). Larvae of *D. ramae* have two very prominent and widely separated humps posteriorly on the pronotum (Figs. 20–21), whereas the pronotum in *D. bosnicus* larvae has a ridge in this position (Figs. 6–7, 19).

The following features clearly distinguish larvae of *D. bosnicus* from other described Drusinae larvae (Lepneva 1966; Hickin 1967; Decamps & Pujol 1975; Szczesny 1978; Sedlák 1980; Waringer 1987; Wallace et al. 1990; Pitsch 1993; Urbani et al. 2003c; Graf et al. 2005, 2009; Waringer & Graf 1997; Waringer et al. 2000, 2007a, 2007b, 2008a, 2008b, 2010, 2013a, 2013b, 2015; Vieira-Lanero et al. 2005; Ku ini et al. 2008, 2010, 2011a, 2011b; Previši et al. 2014a):

Anomalopterygella chauviniana (Stein 1874), *D. bolivari* (McLachlan 1880) and *D. brunneus* Klapálek 1898, have a different shape of the posterior part of the pronotum than the larva of *D. bosnicus*. These species have a very pronounced mid-dorsal concavity in the dorsal hump which is lacking in *D. bosnicus*.

- *Cryptothrix nebulicola* McLachlan 1867, *D. alpinus* (Meyer-Dür 1875), *D. chrysotus* (Rambur 1842), *D. discolor*, *D. franzi* Schmid 1956, *D. muelleri* McLachlan 1868, and *D. romanicus* Murgoci & Botosaneanu 1954 have teeth along the mesal margin of each mandible which are lacking in *D. bosnicus*.

- In *Drusus adustus*, *D. alpinus*, *Drusus annulatus* (Stephens 1837), *D. biguttatus*, *D. camerinus* Moretti 1981, *D. balcanicus* Kumanski 1973, *D. croaticus* Marinkovi - Gospodneti 1971b, *D. franzressli* Malicky 1974 (in Malicky & Kumanski 1974), *Drusus franzi*, *D. improvisus* McLachlan 1884, *D. klapaleki*, *D. medianus*, *D. mixtus* (Pictet 1834), *Drusus monticola* McLachlan 1876, *D. nigrescens* Meyer-Dür 1875, *D. rectus* McLachlan 1868, *D. septentrionis*, *D. trifidus* McLachlan 1868, *D. vinconi* Sipahiler 1992, and *Ecclisopteryx malickyi* Moretti 1991 the head vertex is evenly rounded. In *D. bosnicus* this part of the head is flat with a slight median concavity.

- *Drusus alpinus*, *D. aprutiensis* Moretti 1981, *D. camerinus* Moretti 1981, *D. croaticus*, *D. franzi* Schmid 1956, and *D. mixtus* lack prominent, long median setae dorsally on the anterior border of the pronotum; *D. bosnicus* larvae have long, median setae in this part of the pronotum.
- *Drusus biguttatus*, *D. croaticus*, *D. ingridae* Sipahiler 1993, *D. vinconi* Sipahiler 1992, and *Hadimina torosensis* Sipahiler 2002 have an evenly rounded pronotum in lateral view. The pronotum of *D. bosnicus*, in lateral view, has a prominent median hump.
- *Drusus botosaneanui*, *Drusus tenellus*, *Drusus schmidt* Botosaneanu 1960, *Ecclisopteryx dalecarlica* Kolenati 1848, *E. guttulata* (Pictet 1834), *E. ivkae* Previši , Vitecek & Graf 2014, *E. keroveci* Previši , Vitecek & Graf 2014, and *E. madida* (McLachlan 1867) have prominent spines on the head and/or pronotum; *D. bosnicus* lacks these prominent spines on the head and pronotum.
- *Drusus discolor* and *D. romanicus* have hair-like structures on the head and pronotum; these structures are absent in *D. bosnicus*.
- *Drusus carpathicus* Dziedzielewicz 1911 lacks dorsal gills on the abdomen; *D. bosnicus* has such gills.
- In *Drusus adustus* and *D. melanchaeltes* McLachlan 1876, setae along the whole length on the dorsal side of mid- and hind-leg tibiae are present; in *D. bosnicus* such setae are present only distally.
- *Drusus franzressli*, *D. spelaeus* (Ulmer 1920), *Metanoea rhaetica* Schmid 1956 and *M. flavipennis* (Pictet 1834) have a large median sclerotized patch on abdominal sternum I which is lacking in *D. bosnicus*.
- *Drusus radovanovici* has numerous thin, long, yellow (yellowish) setae on the dorsal and lateral parts of the pronotum. Such long setae are lacking in *D. bosnicus*.
- In *Drusus serbicus* Marinkovi -Gospodneti 1971b, the dorsal profile of the pronotum in lateral view has an annular crest that is highest at its dorsal centre and gradually declining laterally. This crest is lacking in *D. bosnicus*.
- *Drusus trifidus* has numerous light spines on the pronotum which are lacking in *D. bosnicus*.
- *Ecclisopteryx asterix* Malicky 1979 has ovoidal metanotal anteromedian sclerites (metanotal *sal* sclerites) which are more triangular in *D. bosnicus*.
- *Leptodrusus budtzi* has a rounded pronotum in lateral view with a clear ridge in its anterior part. The pronotum of *D. bosnicus* does not have this shape and lacks a ridge.

Distribution, protection status, and feeding ecology of *Drusus bosnicus*

According to literature data (Marinkovi -Gospodneti 1979) *Drusus bosnicus* was found in central Bosnia and Herzegovina at three localities: the Bosna River spring, the Paljanska

River spring, and the Mokra ka Miljacka River spring. The distance between springs of the Paljanska and Mokra ka Miljancka Rivers is 21 kilometres, and the distance between these springs and the spring of the Bosna River is about 34 kilometres. This species thus occupies the smallest area known in the *Drusus bosnicus* Group, except for *D. ramae* (Ku ini et al. 2010).

In our investigation of caddisflies in Bosnia and Herzegovina, we found *D. bosnicus* in all three localities listed in literature (Marinkovi -Gospodneti 1979), but not elsewhere (Stani -Koštroman 2009). Considering the locations of these springs, i.e. the spring of the River Bosna is situated in Sarajevo and two other springs are located near villages, it is very important to protect them. A spring of the Paljanska Miljacka River is used as a source for drinking water, but the habitat was not considerably disturbed, so aquatic fauna is preserved, including the population of *D. bosnicus* (Ku ini et al. 2010). The other two springs are natural, at the moment without visible negative anthropogenic influence.

According to morphological features of mandibles, *D. bosnicus* belongs to the group of grazers, like larvae of the other species that form the *Drusus bosnicus* Group from the Balkan Peninsula (Ku ini et al. 2008, 2010, 2011a, 2011b). Larvae of these species are feeding mainly on epilithic algae in the periphyton of mosses, cobbles, pebbles and gravel. This consistent feeding ecology of all species from the *Drusus bosnicus* Group in Bosnia and Herzegovina is possibly a result of the monophyletic origin of the species (Marinkovi -Gospodneti 1976; Ku ini et al. 2011a). The taxa of the *Drusus bosnicus* Group were separated in different periods during the Pleistocene, probably from one ancestral species (Marinkovi -Gospodneti 1976; Previši et al. 2009; Ku ini et al. 2011a, 2014). Recent molecular study showed that two different species from the *Drusus bosnicus* Group inhabit the spring of the Bosna River (Ku ini et al. 2014; Previši et al., 2014b). This is possibly an indication of a more complex distribution pattern and evolutionary history of the *Drusus bosnicus* Group than previously recognised (e.g., Ku ini et al. 2011a, 2014). So far, we have not found adults or last instar larvae of this possibly new species in this spring.

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FIGURES 1-3.

1, *Drusus bosnicus* Klapálek 1898, adults in copula; 2, Bosnia and Herzegovina with distribution of *D. bosnicus*; 3, spring of the Paljanska Miljacka River, site where larvae and adults of *D. klapaleki* Marinkovi -Gospodneti 1971a were collected.

FIGURES 4–10.

Drusus bosnicus Klapálek 1898. 4, case, right lateral; 5, larva, left lateral; 6 head, pronotum and mesonotum, right lateral; 7, head and pronotum, right lateral; 8 head, right lateral; 9, head, frontal; 10, left mandible, ventral.

FIGURES 11–18.

Drusus bosnicus Klapálek 1898. 11, thorax (pronotum, mesonotum and metanotum), dorsal; 12, left foreleg, posterior; 13, left midleg, posterior; 14, left hind leg, posterior; 15, abdominal gill diagram: position of filaments on left side of abdominal segments I-VIII, with ● = gill present; ; 16, abdomen with gills, right lateral; 17, abdominal segment IX, dorsal; 18, left anal proleg, left lateral.

FIGURES 19–21.

Pronotum. 19, *Drusus bosnicus* Klapálek 1898, right lateral; 20–21, *Drusus ramae* Marinkovi -Gospodneti 1971a: 20, right lateral; 21, right dorsolateral.

Table 1

Intraspecific uncorrected pairwise distances (p) shown as percents and number of nucleotide differences (in brackets) of the mitochondrial cytochrome oxidase I (mtCOI) gene recorded in *D. bosnicus*. Abbreviations are used to denote life stages; IM(M) = adult male, L = larva.

Sampling site	Specimen code	Stage	DbsIM1	DbsIBIM2	DbsVMIM1	DbsVML1	DbsVML2	GenBank accession nos
Bosna spring	DbsIM1	IM(M)						FJ002689
Bosna spring	DbsIBIM2	IM(M)	0.2% (1)					KC881518
Paljanska Miljacka spring	DbsVMIM1	IM(M)	0.0% (0)	0.2% (1)				GQ470609
Paljanska Miljacka spring	DbsVML1	L	0.0% (0)	0.2% (1)	0.0% (0)			GQ470610
Paljanska Miljacka spring	DbsVML2	L	0.2% (1)	0.4% (2)	0.2% (1)	0.2% (1)		GQ470611