



Larval morphology of the Western Balkans endemic caddisflies *Drusus krusniki* Malicky 1981, *D. vernonensis* Malicky 1989, and *D. vespertinus* Marinković-Gospodnetić 1976 (Trichoptera, Limnephilidae, Drusinae)

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Abstract

Drusinae (Trichoptera, Limnephilidae) are highland caddisflies inhabiting high-gradient, turbulent running water and spring habitats. They are disjunctly distributed over the Eurasian mountain ranges, and the majority of species are endemic to particular mountain areas. The most diverse of three main groups of the Drusinae, the grazer clade, consists of species in which larvae feed on epilithic biofilm and algae. In this paper we describe three previously unknown grazer-clade Drusinae larvae: *Drusus krusniki* Malicky 1981 (endemic to the Dinaric Western Balkans), *D. vernonensis* Malicky 1989 (endemic to the Hellenic Western Balkans), and *D. vespertinus* Marinković-Gospodnetić 1976 (endemic to the Dinaric Western Balkans). The larvae of these species have toothless mandibles typical of the Drusinae grazer clade. Larvae and adults were unambiguously associated using molecular genetic data, i.e., the mitochondrial cytochrome oxidase I gene fragment (mtCOI3-P). Morphological characteristics of the larvae are described and the diagnostic features enabling species-level identification are illustrated. We further discuss the ecology and distribution of the three Western Balkan endemic species.

Key words: 5th instar larva, description, identification, distribution, endemism

Introduction

Drusinae are restricted to mountain springs and high-gradient, turbulent running waters in hard-substrate channels covering the Eurasian mountain ranges from the Iberian Peninsula to the Iranian Highlands. Three quarters of the known species are endemic to a single or very few mountain ranges, making the group an ideal model for studying evolutionary processes like speciation, diversification and cryptic species evolution (Schmid 1956; Kumanski 1973; Marinković-Gospodnetić 1971a, b, 1976; Sipahiler 2002; Malicky 2005a; Pauls *et al.* 2006; Previšić *et al.* 2014a). Mountain areas of the Western Balkans harbor particularly high numbers of Drusinae endemics (Vitecek *et al.* 2015a). The fragmented montane sky-island populations of the Drusinae are also very sensitive to global change and their species are among the most threatened by climate warming (Previšić *et al.* 2009; Bálint *et al.* 2011). A molecular phylogeny of the subfamily yielded three well-supported clades that reflect the morphology and feeding ecology of larvae (Pauls *et al.* 2008; Graf *et al.* 2009; Vitecek *et al.* 2015b; Waringer *et al.* 2015): omnivorous

shredders, epilithic grazers, and carnivorous filter feeders. Drusinae comprises eight genera, with the genus *Drusus* Stephens 1837 containing the greatest number of species (92; e.g., Malicky 2004, 2005a; Morse 2015; Oláh *et al.* 2015; Vitecek *et al.* 2015a).

The genus *Drusus* was further divided into particular species groups of presumably closely related species (Schmid 1956). The *Drusus bosnicus* Group was first defined by Schmid (1956) based on the morphology of genitalia, comprising species from the Balkans and Central Europe. Many new species were described from the Western Balkans in the meantime, most of them small-scale endemics with genital morphology highly similar to the *Drusus bosnicus* Group (e.g., Marinković-Gospodnetić 1971a, b, 1976; Malicky 1989; Oláh 2010, 2011; Vitecek *et al.* 2015a).

Drusinae larvae exhibit high niche specificity, mostly inhabiting xenosaprobic to oligosaprobic headwater sections of streams or springs (Graf *et al.* 2008), and are comparatively easily identified. The group is therefore well-suited for water quality assessment (Barbour *et al.* 1999; Barbour & Yoder 2000; AQEM Consortium 2002). However, larval stages of many Drusinae species including some of the *Drusus bosnicus* Group are still unknown, and larvae of only seven species have been described previously [*D. bosnicus* Klapálek 1899, *D. crenophylax* Graf & Vitecek 2015 (in Vitecek *et al.* 2015a), *D. klapaleki* Marinković-Gospodnetić 1971b, *D. medianus* Marinković-Gospodnetić 1976, *D. radovanovici* Marinković-Gospodnetić 1971b, *D. ramae* Marinković-Gospodnetić 1971b, and *D. septentrionis* (Marinković-Gospodnetić 1976); Kučinić *et al.* 2008, 2010, 2011a, 2011b, 2015; Vitecek *et al.* 2015a].

In this contribution we extend the knowledge of larval taxonomy of the Drusinae by presenting descriptions of the hitherto unknown larvae of *Drusus krusniki* Malicky 1981, *D. vernonensis* Malicky 1989, and *D. vespertinus* Marinković-Gospodnetić 1976. The putative larvae of these three species were associated with co-occurring adults using molecular genetic sequence data [mitochondrial cytochrome oxidase *c* subunit I (mtCOI3-P)]. We also highlight the most important diagnostic features enabling separation of these larvae from those of other European Drusinae species. Additionally, we discuss the ecology and distribution of the three Western Balkan endemic species and the *Drusus bosnicus* Group in general.

Material and methods

Sampling, collected material and taxonomy. Adults and larvae were collected by A.P., W.G. and M.K. in Bosnia and Herzegovina, Macedonia, and Montenegro using a hand net and kick sampling at the following locations: *Drusus krusniki*: Montenegro: Alipaša's spring, 42°33'N, 19°49'E, 942 m a.s.l., 31 May 2009, sixteen 5th instar larvae; Ibra spring, 42°48'N, 20°05'E, 1271 m a.s.l., 31 May 2009, nine 5th instar larvae. *D. vernonensis*: Macedonia: Pelister Mt. Tributary of Caparska reka, 40°58'N, 21°12'E, 2323 m a.s.l., 8 July 2010, two 5th instar larvae. *D. vespertinus*: Bosnia and Herzegovina: Gornji Ribnik, 44°25'N, 16°49'E, 316 m a.s.l., 3 June 2008, eight 5th instar larvae. Additionally, temperature loggers (Hobo Onset) logging ambient water temperature at 0200 h and 1400 h were exposed at the same sites and recovered the following year. Material intended for sequencing was stored in 100% ethanol, the material for morphological analyses in 70% ethanol in order to keep the specimens more flexible. Information on examined voucher specimens and other *Drusus* species larvae used as comparative material is given in Table 1 (all in collection of the first author). The larvae were studied and photographed using a Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software for combining 8 to 42 frames in one focused image. In addition, SEM microscopy was used for some figures where specimens were air dried, gold coated using a BAL-TEC SCD 005 sputter coater, and examined using a JEOL JSM-6390lv scanning electron microscope. Larval morphological nomenclature follows Wiggins (1998).

Species affiliation was based on two criteria: Larval and adult specimens were collected at the same sites, close to the type localities of the respective species where other Drusinae species are absent or already known due to existing descriptions.

Molecular sequence data from the mitochondrial cytochrome oxidase *c* subunit I (mtCOI3-P) supports conspecificity of the larvae and adults.

DNA extraction and PCR amplification. For the association of larvae and adults both published and new sequences of a 541-bp-long fragment of the mitochondrial cytochrome oxidase *c* subunit I (mtCOI3-P) were used. Whole genomic DNA was extracted from part of the thorax or abdomen of adult or larval specimens, respectively,

TABLE 1. Information on larval *Drusus* specimens (fifth instar larvae) from the Western Balkans used in this paper.

Species	Locality (country, name)	Latitude (°)	Longitude (°)	No of larvae examined	Collector
<i>Drusus bosniacus</i>	(BIH) Paljanska Miljacka spring	N 43.9234	E 18.5973	1	Kučinić & Previšić
<i>Drusus crenophylax</i>	(BIH) Cvrcka river	N 44.5489	E 17.3927	2	Dmitrović & Šukalo
<i>Drusus klapaleki</i>	(BIH) Toplica spring	N 43.5943	E 18.4949	2	Graf & Previšić
<i>Drusus krusniki</i>	(MNE) Alipaša's springs, Gusinje	N 42.5501	E 19.8259	16	Previšić
<i>Drusus krusniki</i>	(MNE) Ibar spring	N 42.7975	E 20.0904	9	Previšić
<i>Drusus medianus</i>	(BIH) Plava voda spring	N 44.2303	E 17.6717	1	Kučinić & Previšić
<i>Drusus radovanovici</i>	(BIH) Sutjeska NP, stream close to Čemerno	N 43.2650	E 18.5928	1	Graf & Previšić
<i>Drusus septentrionis</i>	(BIH) Bistrica spring, Livno	N 43.8325	E 17.0084	2	Kučinić
<i>Drusus serbicus</i>	(SRB) Golija Mt, spring Ilinac	N 43.3333	E 20.2819	3	Bjelanović, Kučinić & Živić
<i>Drusus vermonensis</i>	(MKD) Pelister, tributary of Caparska reka	N 41.0148	E 21.1741	2	Previšić
<i>Drusus vespertinus</i>	(BIH) Ribnik, spring reach	N 44.4655	E 16.8380	8	Kučinić & Previšić

TABLE 2. Minimal and maximal values of nucleotide differences and uncorrected *p*-distance of the mtCOI3-P gene segment within populations of *Drusus krusniki* (data from Previšić *et al.* 2014b).

<i>Drusus krusniki</i> populations	Number of adult males	Number of larvae	Number of nucleotide difference (min-max)	Uncorrected <i>p</i> -distance (min-max)	GenBank accession nos
Alipaša springs	11	2	0-1	0-0.002	KC881401 - KC881413
Biogradska rijeka	8	5	0-6	0-0.011	KC881414 - KC881426
Bukovica spring	2	14	0-2	0-0.004	KC881427 - KC881442
Ibar spring	1	14	0-4	0-0.004	KC881443 - KC881457
Murinska rijeka	6	8	0	0	KC881458 - KC881471

using the DNEasy Blood and Tissue Kit or QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's protocol. PCR amplifications were accomplished using primers S20 and Jerry (Simon *et al.* 1994; Pauls *et al.* 2006). DNA extraction and amplification were performed as outlined by Pauls *et al.* (2008) and Previšić *et al.* (2009).

For *D. krusniki*, 71 published mtCOI3-P sequences from five populations (Previšić *et al.* 2014b; Table 2) were aligned. For *D. vernonensis* mtCOI3-P sequences of 3 adults (Previšić *et al.* 2014b; Vitecek *et al.* 2015a; GenBank accession nos KC881524, KP793086 and KP793087) and 3 larvae (GenBank accession nos KT598011-KT598013) from one population were aligned. For *D. vespertinus* mtCOI3-P sequences of 5 adults and 3 larvae from two populations were aligned (Previšić *et al.* 2009 and new sequences, Table 4). New sequences were edited manually using Geneious R7 (Kearse *et al.* 2012) and all were aligned using Muscle algorithm in Mega 6.0.1 (Tamura *et al.* 2013). Intraspecific uncorrected *p*-distances were calculated in Mega 6.0.1 for each species.

Results

Association of adults and larvae of *Drusus krusniki*

Comparison of haplotypes of the mtCOI3-P segment between a total of 28 adult males and 44 larvae belonging to 5 different populations of *Drusus krusniki* supported the association of adults and larvae. Within each particular population, haplotypes differed by maximally 6 nucleotides between adults and larvae (Table 2; data from Previšić *et al.* 2014b).

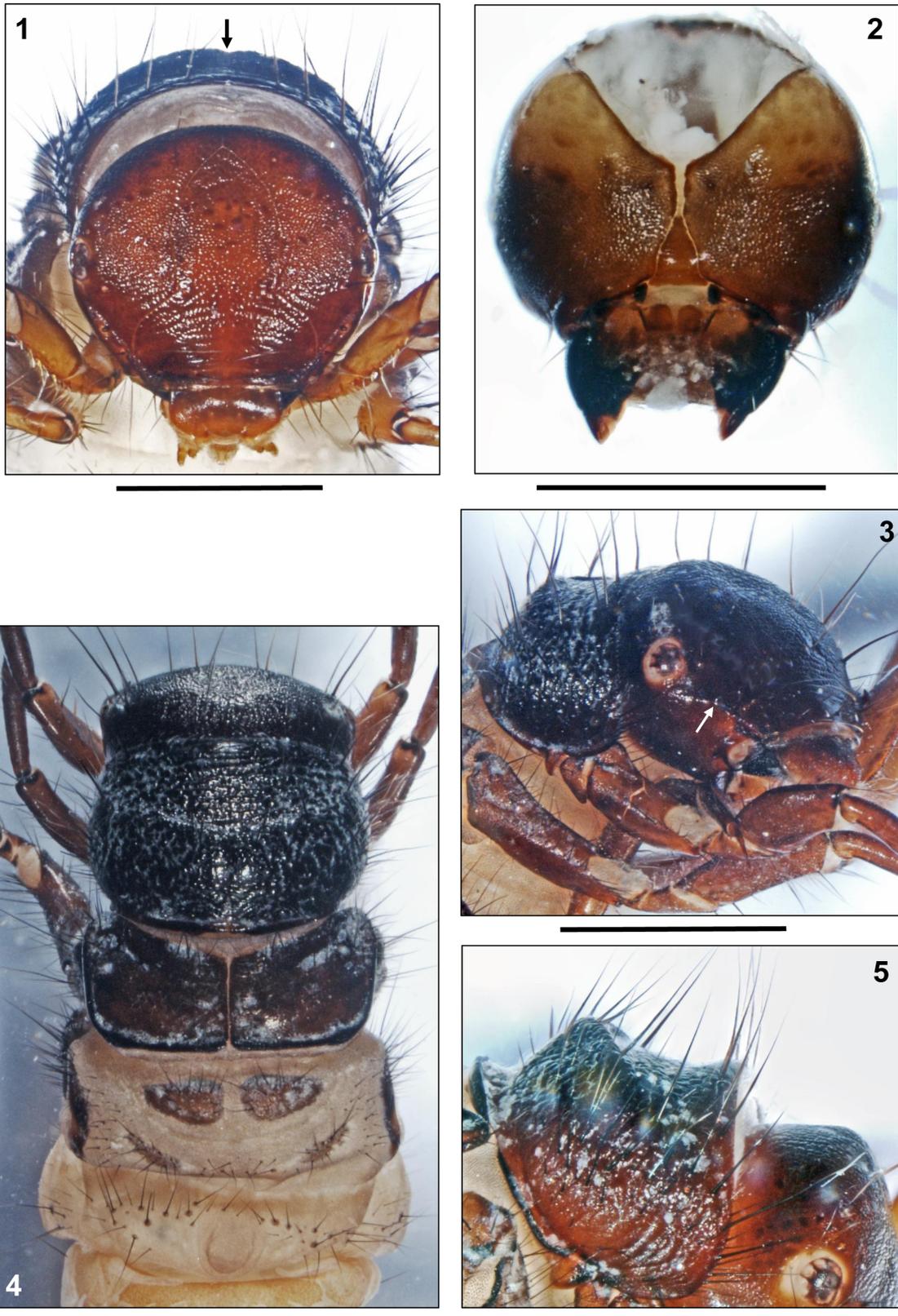
Description of the fifth instar larva of *Drusus krusniki*

Biometry. Body length of final instar larva 7.1–12.3 mm, head width 1.60–1.83 mm (*n* = 25).

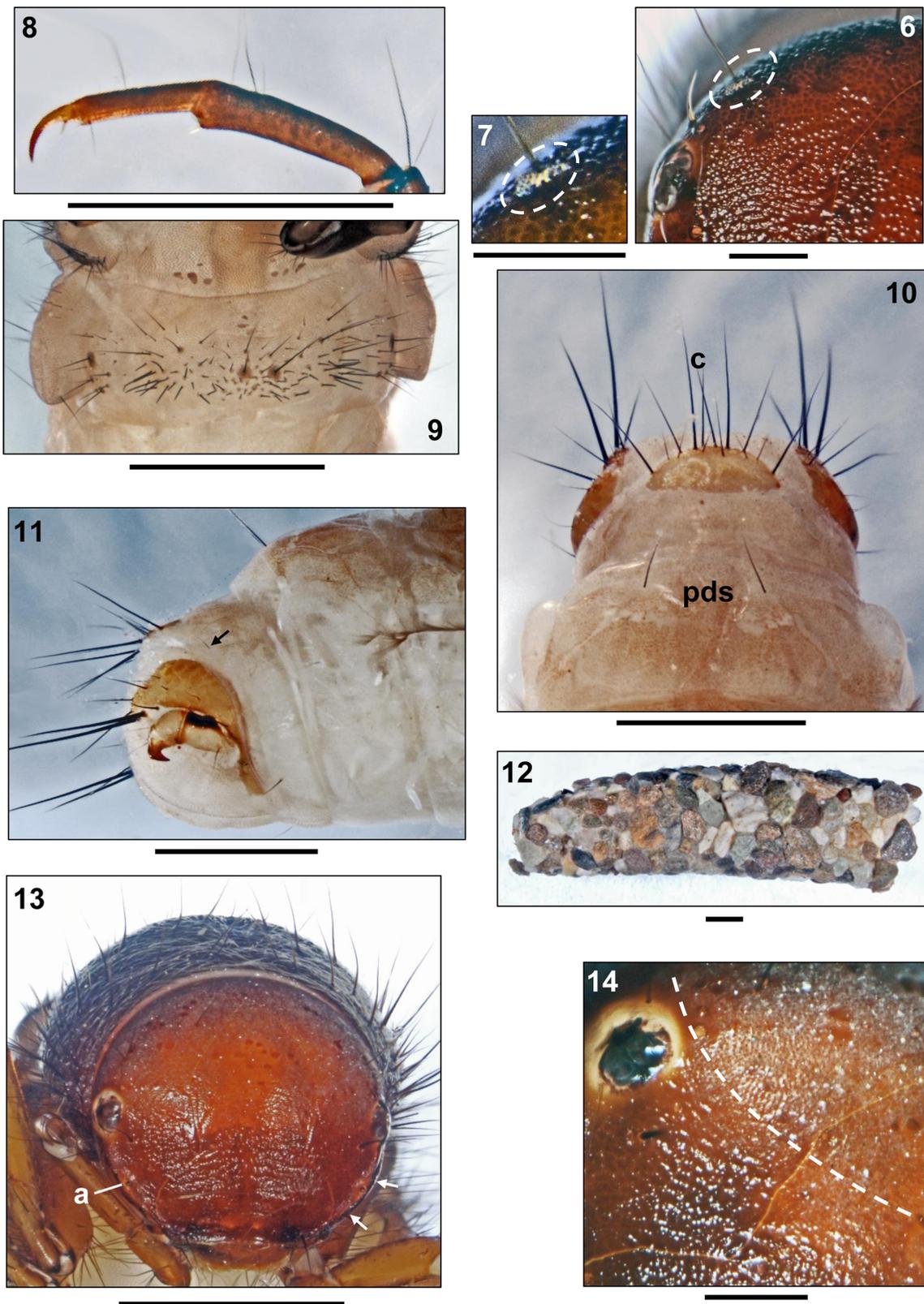
Head. Head capsule coarsely granulated, almost circular in shape (Figs. 1–3), dorsally with dark brown to very dark brown coloration and black muscle attachment spots (Fig. 1). Vertex rounded (Fig. 1). Ventral parietalia sections, submentum, maxillolabial sclerites and premandibular areas yellowish brown (Figs. 2, 3). White ring around each eye (Fig. 3). In lateral view, head capsule with straight carina (approximately 0.09 mm wide) extending from anterior eye margin to frontomedian corner of parietale (Fig. 3). Head capsule with complete set of 18 pairs of primary setae (nomenclature by Wiggins 1998) and lacking any additional spines or bristles known to occur in other Drusinae larvae (e.g., some *Ecclisopteryx* spp.). However, posterior to each eye, with oval areas (diameter approximately 0.05 x 0.09 mm) of spinules (= small spines approximately 0.03 mm long) surrounding bases of setae #15 and #16 (Figs. 6, 7; white ovals); such spinule areas occurring in most members of the *Drusus bosnicus* Group *sensu* Marinković-Gospodnetić (1971a). Frontoclypeus similar to other species of *Drusus bosnicus* Group (Fig. 1). Antennae located at small conical dorsal outcrops of lateral carina (Figs. 1, 3 arrow, 17), each consisting of 1 short cylindrical base and 1 short flagellum. At each parietal, 10 dorsal and 2 ventral primary setae present, with primary setae #2, 3, 7, 9, 15, and 16 long and conspicuous (setation numbers following the morphological standard nomenclature for caddis larvae by Wiggins 1998; Figs. 1, 3). Each side of frontoclypeus with 6 pairs of primary setae, 3 of them along anterior border. Labrum brown, with setal brush and primary setae #1–3 at anterolateral margins; on dorsal area, setation consisting of primary setae #4–6 (Fig. 1). Ventral apotome elongate-triangular, bell-shaped, brown, postgenal suture approximately 45% of apotome length (Fig. 2). Mandibles black with dark brown tips, lacking terminal teeth along edges as well as lacking ridges in central concavity (Figs. 2, 17).

Thorax. Pronotum very dark brown, very coarsely granulated (Figs. 3–5); at lateral and posterior pronotal surface, adjacent series of granuli creating ribbed structures (Fig. 5). Posterior margin thickened and darkly striped (Fig. 5). In frontal view, small dorsocentral pronotal notch present (Fig. 1, arrow). Pronotal transverse groove at end of anterior 3rd lacking. Pronotum type D (Table 3) in lateral view: median hump prominent, high, with anterior-looking crest gradually fading laterally; posterior slope straight, anterior slope concave (Fig. 5). Two setal rows along anterior border of pronotum: (1) Dense fringe of short, curved, fine, yellow setae; (2) widely-spaced, continuous row of long, straight, dark setae meeting at anterior pronotal midline (Figs. 1, 3, 5); in total, 50–60 dark

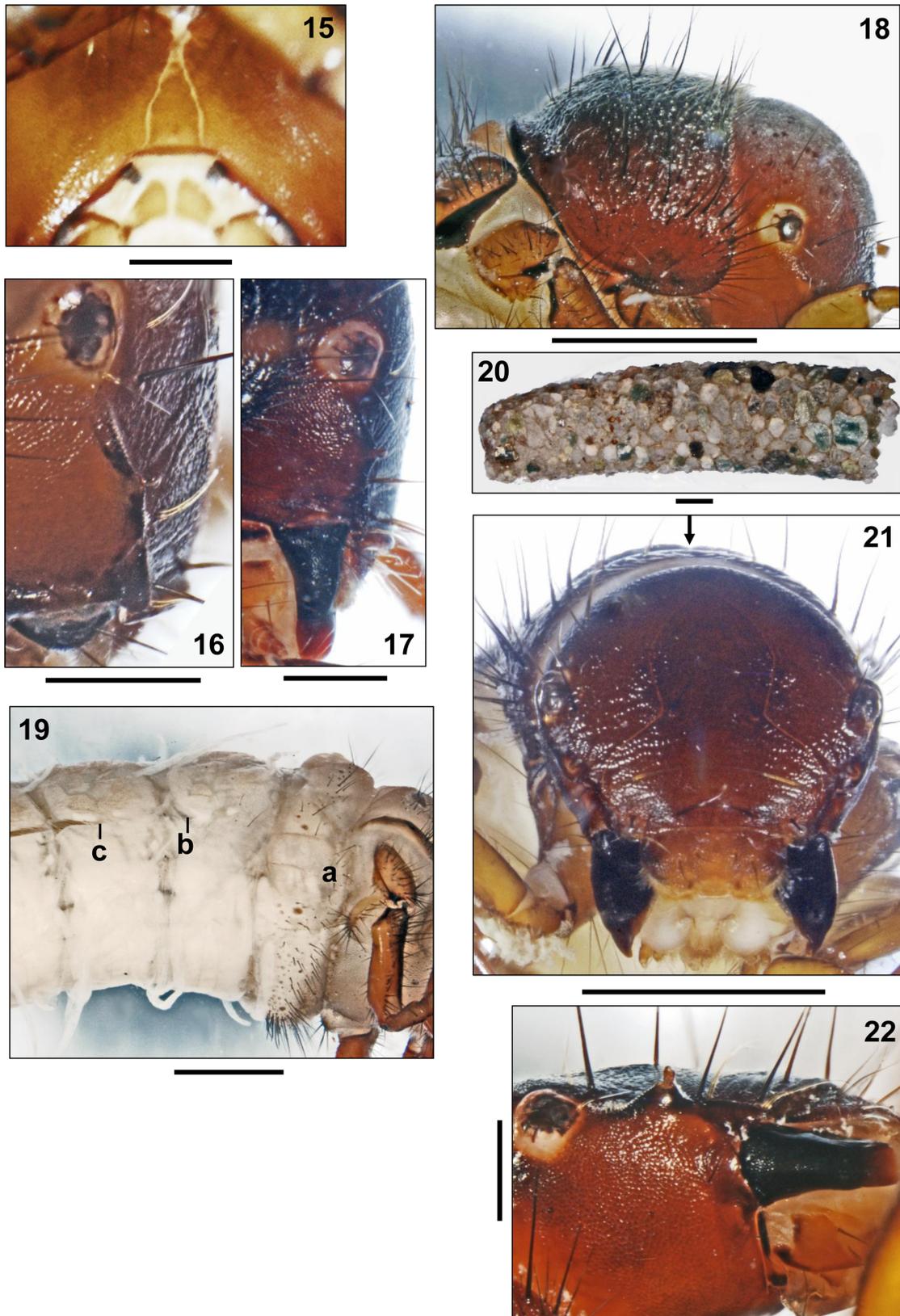
setae of varying lengths distributed over each pronotal half. In addition, pronotal surface covered by high number of tiny, pale, recumbent setae (Figs. 3, 4); spines present in other Drusinae (e.g., *D. trifidus*) lacking. Prosternite pentangular, pale yellow, darker (light brown) along posterior border; prosternal horn present.



FIGURES 1–5. *Drusus krusniki* Malicky 1981, 5th instar larva. 1, head, dorsal (arrow: pronotal notch); 2, head, ventral; 3, head and prothorax, right anterolateral (arrow: antenna on lateral carina); 4, head, thorax, and abdominal segment I, dorsal; 5, pronotum, right lateral. Scale bars: 1 mm.



FIGURES 6–14. *Drusus* spp., 5th instar larvae. **6–12**, *Drusus krusniki* Malicky 1981: **6**, head, frontal (dotted oval: spinule field); **7**, head, detail of spinule field (dotted oval); **8**, right hind leg, anterior; **9**, abdominal sternum I, ventral; **10**, abdominal terga VIII and IX, dorsal (pds: posterodorsal setae; c: position of c setae); **11**, tip of abdomen, right lateral (arrow: posterolateral seta); **12**, larval case, right lateral. **13–14**, *Drusus vernonensis* Malicky 1989: **13**, head, dorsal (arrows: left carina; a: antenna); **14**, head, detail of spinule field (dotted oval). Scale bars: 1 mm (except Figs. 6, 7, 14: 0.25mm).



FIGURES 15–22. *Drusus* spp. 5th instar larvae. **15–16**, *Drusus vernonensis* Malicky 1989: **15**, head (submentum), ventral, with anterior at bottom of image; **16**, head, left frontolateral, carina with antenna. **17**, *Drusus krusniki* Malicky 1981, head, right lateral, carina with antenna. **18–20**, *Drusus vernonensis* Malicky 1989: **18**, head and pronotum, right lateral; **19**, metathorax and anterior half of abdomen, right lateral (a: row of setae anterior of lateral protuberance; b: small portion of lateral fringe on segment II, c: start of lateral fringe on segment III); **20**, larval case, right lateral. **21–22**, *Drusus vespertinus* Marinković-Gospodnetić 1976: **21**, head, dorsal (arrow: pronotal notch); **22**, head, right lateral, carina with antenna. Scale bars: 1 mm (except Figs. 15, 16, 17, 22: 0.25mm).

Mesonotum completely covered by 2 dark brown sclerites with darker brown muscle attachment spots and slightly paler posterior and lateral sections; their anterolateral corners, lateral and posterior margins darkly sclerotized (Fig. 4). Counts for mesonotal setae on each sclerite are as follows: anteromesal setal group *sa1*: 12–15, posteromesal group *sa2*: 20–25, lateral group *sa3*: 25–30.

Metanotum partially covered by 3 pairs of yellowish brown sclerites. Anteromesal metanotal sclerites (*sa1*) very large, broadly triangular, their intermediate separation distinctly smaller than length of each of them; each with black anterior margin; approximately 15 setae per sclerite (Fig. 4). Row of setae present between small posteromesal sclerites (*sa2*); 15–20 setae per sclerite. Small setal group present between each lateral (*sa3*) and posteromesal sclerite (*sa2*); *sa3* sclerites crescent-shaped, dark brown at center and along dorsal border, with approximately 25–30 setae per sclerite, concentrated anteriorly (Fig. 4).

Legs yellowish brown; several proximodorsal setae on all femora. Setae present only at distal sections of trochanters. Additional setae present on both anterior and posterior faces of all femora; ventral trochanteral brush present on distal sections of fore- and midtrochanters. Forefemora each with 4 yellow ventral-edge setae, mid- and hind femora each with 3–4 dark ventral-edge setae. Dorsal setae only at distal third of mid- and hind tibiae (Fig. 8).

Abdomen. Dorsal setal areas *sa1*, *sa2* and *sa3* of abdominal segment I fused, thereby creating continuous transverse row of setae anterior to dorsal protuberance until dorsal section of each lateral protuberance, sharply delimited basal sclerites present for about 50% of these setae. Without setal group posterior to dorsal protuberance (Fig. 4). Posterior sclerites at lateral protuberances absent. In front of each lateral protuberance, continuous band of anterolateral setae (as Fig. 19a) linking with each dorsal and ventral *sa3* setal group (Figs. 4, 9). On abdominal sternum I, ventral setal areas *sa1*, *sa2* and *sa3* fused, creating continuous field of setae; setal bases at central section of abdominal sternum I mostly small and inconspicuous except two larger bases near midline which occasionally fuse with 1–2 neighbouring smaller setal bases (Fig. 9). On abdominal dorsum VIII, 2 long and 2 very tiny posterodorsal setae (pds) present (Fig. 10, pds). Only 1 posterolateral seta on each half of abdominal dorsum IX (Fig. 11, arrow).

All gills single filaments. Dorsal gills present at most from segment II (presegmental position) to segment VI (postsegmental position). Ventral gills ranging from segment II (presegmental) to segment VII (postsegmental). Dorsolateral gills ranging from segment II (presegmental) to segment IV (presegmental), and ventrolateral gills ranging from segment II (postsegmental) to segment IV (postsegmental). Lateral fringe extending from last quarter of segment II to middle of abdominal segment VIII (Fig. 11). Yellowish brown sclerite on abdominal tergum IX semicircular (Fig. 10); 8 long and several shorter setae along its posterior border, 2 of these long setae having position of central intermediate *c* setae (Fig. 10c). Anal prolegs of limnephilid type, yellowish brown, with light brown muscle attachment spots. Ventral sole plate with black dorsal rim (Fig. 11). Tips of anal claws dark brown, each with 1 small accessory hook (Fig. 11).

Case. Larval case 10.3–13.3 mm long (n= 25), slightly curved, slightly conical (width at anterior opening 2.4–3.5 mm and at posterior opening 1.6–2.5 mm), consisting of mineral particles (sand grains of mixed size; Fig. 12).

Association of adults and larvae of *Drusus vernonensis*

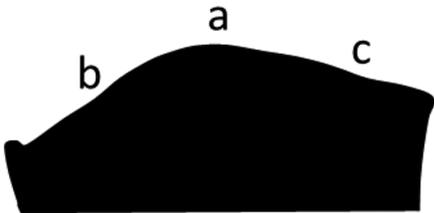
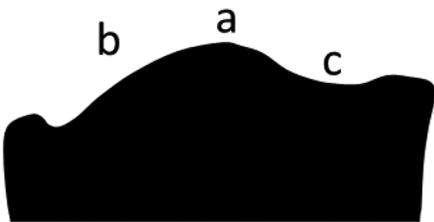
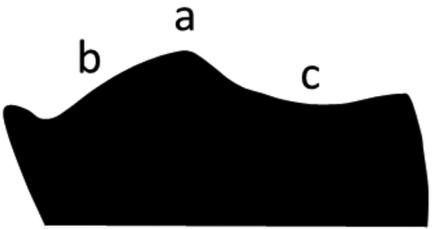
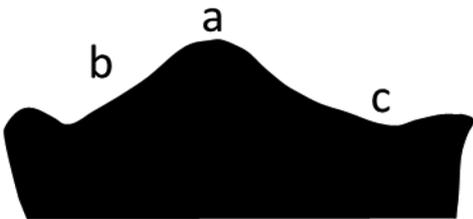
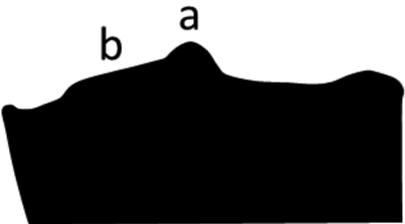
Haplotypes of the mtCOI3-P segment of 3 adult males and 3 larvae of *D. vernonensis* collected at the same site were completely identical. Moreover, the two different stages were sampled in different years (i.e., larvae in 2009 and adults in 2010). This confirms the conspecificity of the larvae and adults of *D. vernonensis* collected at Pelister Mt. in Macedonia.

Description of the fifth instar larva of *Drusus vernonensis*

Biometry. Body length of final instar larva 10.1–10.3 mm, head width 1.53–1.60 mm (n= 2). All morphological characters identical to those of *D. krusniki* except as noted below.

Head. Head capsule reddish to medium brown (Figs. 13, 14, 15, 16, 18), with dark brown muscle attachment spots. Large spinule field covering the dorsal sections of parietalia and frontoclypeus dorsal of eyes (Fig. 14). Labrum yellowish brown (Fig. 13). Ventral apotome elongated triangular, postgenal suture approximately 25–30% of apotome length (Fig. 15).

Table 3. Pronotum types observed in the *Drusus bosnicus* group.

Pronotum type	Lateral right profile of pronotum	Description
Type A		<p>Low median hump, evenly rounded (a); posterior (b) and anterior slopes (c) almost straight.</p>
Type B		<p>Median hump prominent, high, smoothly rounded (a); posterior slope convex (b); anterior slope concave (c).</p>
Type C		<p>Median hump prominent, high, with crest gradually fading laterally (a); posterior slope convex (b); anterior slope concave (c).</p>
Type D		<p>Median hump prominent, high, with crest gradually fading laterally (a); posterior slope straight (b); anterior slope concave (c).</p>
Type E		<p>Median hump annular, crest-like, highest at dorsal center, gradually fading laterally (a); with semicircular step (b) directly posterior of crest center and in front of posterior pronotal rim.</p>

Thorax. Pronotum dark brown, without pronotal notch (Figs. 13, 18). Pronotum type A (Table 3) in lateral view: low median hump, evenly rounded; steep posterior and flat anterior slope almost straight (Fig. 18). In addition to 50–60 dark setae of varying lengths distributed over each pronotal half, pronotal surface covered by (1) tiny, pale, recumbent setae, and (2) thin, long, yellowish setae concentrated at anterior center of pronotum (Fig. 18).

Mesonotum completely covered by 2 yellowish brown sclerites. Counts for mesonotal setae on each sclerite are as follows: anteromesal setal group *sa1*: 16–20, posteromesal group *sa2*: 22–26, lateral group *sa3*: 35–40.

Anteromesal metanotal sclerites large, broadly triangular, each with brown anterior margin; approximately 20 setae per sclerite. Setae between posteromesal sclerites scarce; 12–15 setae per posteromesal sclerite. Legs yellowish brown; setation as in *D. krusniki*.

Abdomen. Abdominal segment I with a small setal group sometimes posterior to dorsal protuberance. Two long, 2 intermediate, and up to 4 minute posterodorsal setae (pds) on abdominal dorsum VIII.

All gills single filaments. Dorsal gills present at most from segment II (presegmental position) to segment VI (presegmental position). Ventral gills ranging from segment II (presegmental) to segment VII (presegmental). Dorsolateral gills present on segment III only (presegmental), and ventrolateral gills ranging from segment II (postsegmental) to segment III (postsegmental). Lateral fringe starting at last quarter of segment II (Fig. 19b), followed by gap until mid-segment III (Fig. 19c); from this point extending to middle of abdominal segment VIII.

Case. Larval case 10.0–10.1 mm long (n = 2), slightly curved, slightly conical (width at anterior opening 2.7–2.9 mm and at posterior opening 1.9–2.0 mm), consisting of mix of mineral particles (Fig. 20).

Association of adults and larvae of *Drusus vespertinus*

Comparison of the mtCOI sequences from one adult male *D. vespertinus* and three larvae collected at the Ribnik spring reach supports the association of larvae to this species as they differed by maximally 2 nucleotide positions (Table 4). Additionally, uncorrected *p*-distances between haplotypes from two known populations of *D. vespertinus* (0.9–1.3%; Table 4) agree with variability recorded in other *Drusus* endemics in the Western Balkans (e.g., Previšić *et al.* 2009, 2014b).

Description of the fifth instar larva of *Drusus vespertinus*

Biometry. Body length of final instar larva 10.9–12.1 mm, head width 1.40–1.54 mm (n = 8). All morphological characters identical to those of *D. krusniki* except as noted below.

Head. Head capsule broadly oval (Fig. 21–24), dorsally with dark brown to very dark brown coloration and black muscle attachment spots. Vertex rounded (Figs. 21, 24). Oval area of spinules (= small spines approximately 0.03 mm long) posterior of each eye (Fig. 23; white oval). Ventral apotome scutiform, anterior half parallel-sided, posterior half triangular; postgenal suture approximately 33% of apotome length.

Thorax. Very dark brown pronotal surface very coarsely granulated, covered by white recumbent setae (Figs. 24–26). In frontal view, small dorsocentral pronotal notch present (Fig. 21, arrow). Pronotum type C (Table 3) in lateral view: median hump prominent, high, with edged crest gradually fading laterally; posterior slope convex, anterior slope concave (Figs. 24, 25). In total, 45–50 dark setae of varying lengths distributed over each pronotal half.

Counts for mesonotal setae on each sclerite are as follows: anteromesal setal group *sa1*: 15–20, posteromesal group *sa2*: 20–25, lateral group *sa3*: 20–25 (Fig. 26).

Anteromesal metanotal sclerites with 15–20 setae per sclerite (Fig. 26), posteromesal sclerites with 15–18 setae.

Forefemora each with 5–6 yellow ventral-edge setae, mid- and hind femora each with 5–6 ventral edge setae. Dorsal setae only at distal third of mid- and hind tibiae.

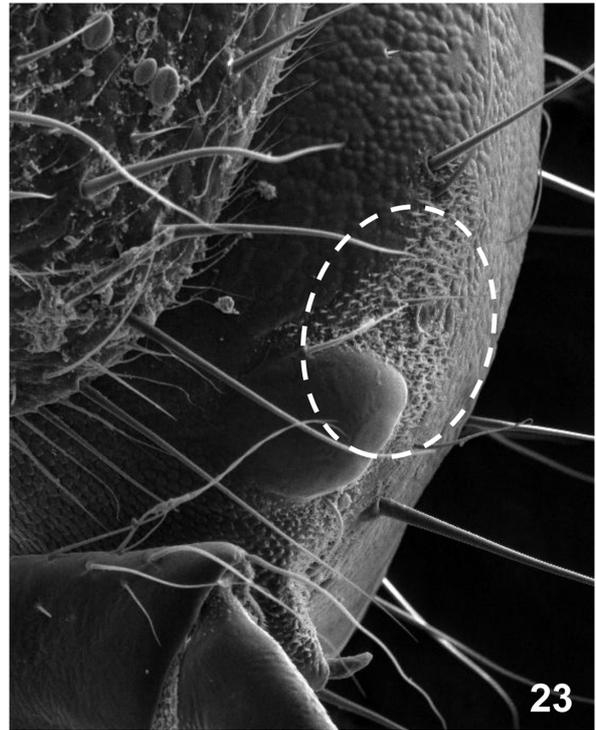
Abdomen. Abdominal segment I with small setal group sometimes posterior to dorsal protuberance. Two long posterodorsal setae (pds) on abdominal dorsum VIII. Only 1 posterolateral seta on each half of abdominal dorsum IX.

TABLE 4. Number of nucleotide differences (below diagonal) and uncorrected *p*-distance (above diagonal) of the mtCOI3-P gene segment within and between populations of *Drusus vespertinus*. For details on specimen sampling sites see Table 1.

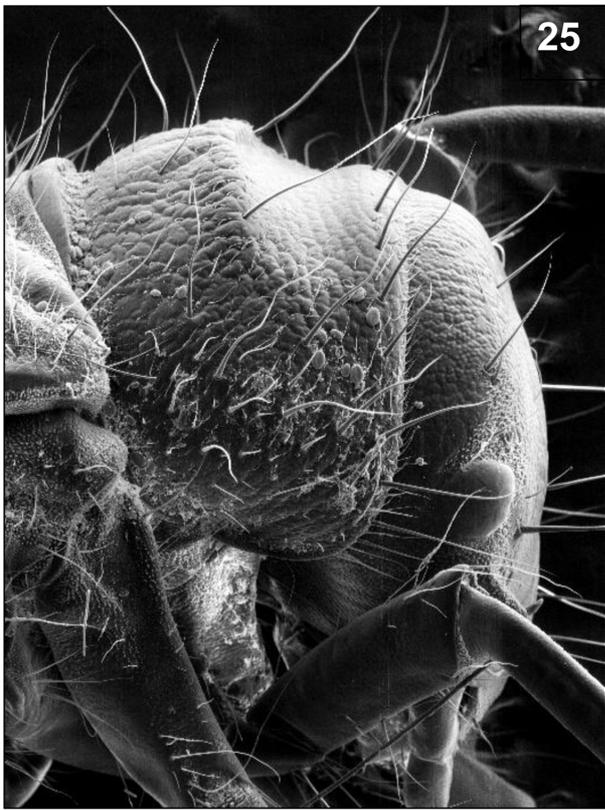
Population	DvRIM1	DvRL1	fDvs0103L	fDvs0104L	fDvs0202M	fDvs0203F	fDvs0205F	fDvs0206F	GenBank access. nos
Ribnik spring, BIH	DvRIM1	0.002	0.004	0.004	0.013	0.013	0.013	0.013	KT598004
Ribnik spring, BIH	DvRL1	1	0.002	0.002	0.011	0.011	0.011	0.011	FJ002685
Ribnik spring, BIH	fDvs0103L	2	1	0.004	0.013	0.013	0.013	0.013	KT598005
Ribnik spring, BIH	fDvs0104L	2	1	2	0.013	0.013	0.013	0.009	KT598006
Una spring, HRV	fDvs0202M	7	6	7	7	0	0.004	0.007	KT598007
Una spring, HRV	fDvs0203F	7	6	7	7	0	0.004	0.007	KT598008
Una spring, HRV	fDvs0205F	7	6	7	7	2	0.007	0.007	KT598009
Una spring, HRV	fDvs0206F	7	6	7	7	4	4	4	KT598010



24



23



25

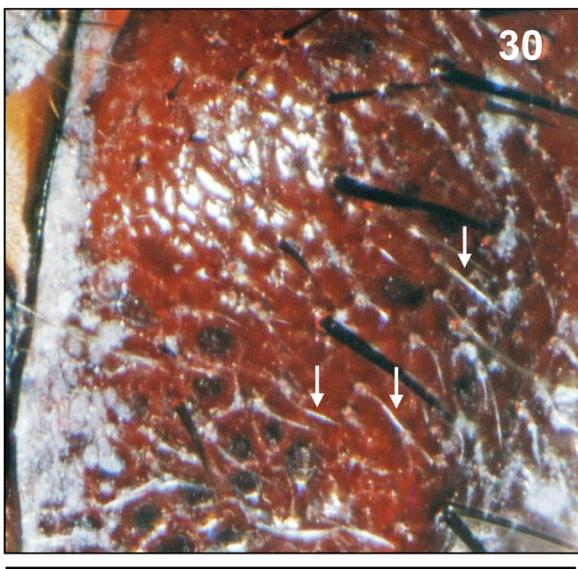
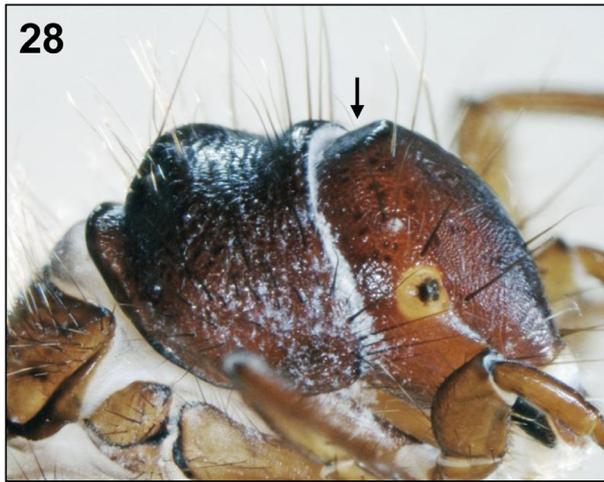


26



27

FIGURES 23–27. *Drusus vespertinus* Marinković 1976, 5th instar larva. **23**, head, right posterolateral, detail of spinule field (dotted oval); **24**, **25**, pronotum, right lateral; **26**, head, thorax, and abdominal segment I, dorsal; **27**, larval case, right lateral. Scale bars: 1 mm (except Fig. 23: 0.25 mm).



FIGURES 28–30. *Drusus* spp., 5th instar larvae. **28**, *Drusus bosnicus* Klapálek 1899, head and pronotum, right lateral (arrow: vertex flattened). **29**, *Drusus radovanovici* Marinković 1970, pronotum, right lateral (arrows: thin long yellowish setae). **30**, *Drusus medianus* Marinković 1976, detail of pronotum, right lateral (arrows: white recumbent setae). Scale bars: 1 mm (except Fig. 30: 0.5 mm).

All gills single filaments. Dorsal gills present at most from segment II (presegmental position) to segment VII (presegmental position). Ventral gills ranging from segment II (presegmental) to segment VII (postsegmental). Dorsolateral gills ranging from segment II (presegmental) to segment IV (presegmental), and ventrolateral gills ranging from segment II (postsegmental) to segment IV (postsegmental). Lateral fringe extending from last quarter of segment II to first quarter of abdominal segment VIII.

Case. Larval case 9.9–10.8 mm long ($n = 8$), distinctly curved, slightly conical (width at anterior opening 2.5–3.9 mm and at posterior opening 1.7–2.3 mm), consisting of mineral particles (sand grains of mixed size; Fig. 27).

Morphological separation of fifth instar larvae of *Drusus krusniki*, *D. vernonensis* and *D. vespertinus* from other European Trichoptera

Within the framework of the limnephilid key by Waringer & Graf (2011) and Waringer *et al.* (2010), *Drusus krusniki*, *D. vernonensis* and *D. vespertinus* are separable from other limnephilid species by the following features:

- gills consisting of single filaments only; dorsal gills present (Fig. 19);
- metanotum covered by 3 pairs of small sclerites (Fig. 4);
- mandibles spoon-shaped (terminal teeth and central cavity ridges lacking; Figs. 17, 21);
- head capsule with fields of spinules (= small spines approximately 0.03 mm long; Figs. 6, 7, 14, 23);
- anterior-row setae present near dorsal midline of pronotum (Figs. 1, 13, 18);
- dorsal-edge setae restricted to distal 3rd of mid- and hind tibiae (Fig. 8);
- center of first abdominal sternum without large sclerotized patches or concentrations of fused basal sclerites of setae (Fig. 9).

Based on this character list, *Drusus krusniki*, *D. vernonensis* and *D. vespertinus* key together with the other members of the *Drusus bosnicus* Group which have spinule fields on the head (so far, only *Drusus ramae* Marinković-Gospodnetić 1971b, also a member of the *Drusus bosnicus* Group, was found to lack such spinule fields). These species are easily separated by differences in the vertex structure of the head (Figs. 21, 28), dorsal profile (Table 3; Figs. 5, 18, 24), sculpturing (Fig. 5) and setation of the pronotum (Figs. 18, 29, 30) and presence/absence of a dorsocentral pronotal notch (Figs. 1, 13). A synopsis of differentiation characters in the *Drusus bosnicus* Group with spinule fields on the head is given in Table 5.

Discussion

Larval morphology of the Western Balkans *Drusus* endemics. The three larvae described in the present paper bear morphological characters common to members of the *Drusus bosnicus* Group, such as the fields of spinules of various sizes posterior to each eye. Marinković-Gospodnetić (1971a) assigned *D. bosnicus*, *D. klapaleki*, *D. plicatus* Radovanović 1942, *D. radovanovici* and *D. ramae* to this group of species, based on similarities of the main structures of the male genitalia. Later, *D. krusniki*, *D. medianus*, *D. septentrionis*, and *D. vespertinus* were added (see discussion by Kučinić *et al.* 2011a). Moreover, the morphology of some *Drusus* species described in recent years from Albania indicates close relatedness with the *Drusus bosnicus* Group (Oláh 2010, 2011) in adult morphology; larvae of these species are still unknown. Furthermore, the newly described endemic *D. crenophylax* and also *D. serbicus* Marinković-Gospodnetić 1971a share some of the common larval morphological features of this group (Vitecek *et al.* 2015a, Waringer *et al.* 2015). In all these species, including the three described herein, the spinule fields on the larval head are absent in only *D. ramae*, but are present in *D. serbicus* (Waringer *et al.* 2015) and the hitherto unknown larvae of *Drusus krusniki*, *D. vernonensis*, and *D. vespertinus*. Thus, the spinules most probably represent a synapomorphic character in this Group, and may also be present in the unknown larvae of other species.

Drusus vernonensis is unique in that the spinule field covers large areas of the parietalia and the frontoclypeus dorsal of eye level; in all other known larvae, the spinule fields are small oval areas posterior of the eyes (Kučinić *et al.* 2008, 2010, 2011a, 2011b, 2015; Vitecek *et al.* 2015a). Additionally, larval *D. vernonensis* have other unique characters (Table 3) that are inconsistent with characters usually observed in *Drusus bosnicus* Group taxa. Interestingly, these differences are consistent with differences in male adult genitalia morphology which suggest a close relationship of *D. vernonensis* with species of the *Drusus discophorus* Group (Malicky 1989). However, the larval stages of the majority of species of the latter group are still unknown. Thus, a more comprehensive discussion on the significance of the presented larval morphological characters in “fine scale” Drusinae systematics is not possible at this point. Nevertheless, our findings are consistent with previous studies implying a close link of larval morphology, feeding ecology, and phylogenetic relationships within Drusinae in general (Pauls *et al.* 2008; Graf *et al.* 2009), and more specifically within the carnivorous clade (Vitecek *et al.* 2015b). Hence, the improvement of the knowledge of the larval taxonomy presented in the current paper adds valuable information to an overall puzzle within the largest clade of Drusinae species, the epilithic grazers.

Distribution and ecology of *Drusus krusniki*, *D. vernonensis*, and *D. vespertinus*. Species included in the *Drusus bosnicus* Group are all endemic to the Dinaric Balkans, i.e. to the ecoregions ER5 (Dinaric Western Balkan) and ER6 (Hellenic Western Balkan) (*sensu* Illies 1978; Table 5). *Drusus krusniki* is endemic to the Western Balkans, inhabiting the southeastern parts of the Dinaric Alps in Montenegro and Kosovo (Graf *et al.* 2008; Previšić *et al.* 2014b; Gashi *et al.* 2015) and the Prokletje Mts. in Albania (Oláh 2010). *Drusus vespertinus* is a (micro-) endemic of the Dinaric Alps (ER 5), known from the western part of Bosnia and Herzegovina

TABLE 5. Synopsis of characters separating the currently known *Drusinae* larvae (5th instars) which share the following group morphomatrix: spoon-shaped mandibles; with spinule areas at the head capsule; anterior-row setae present near dorsal pronotal midline; dorsal gills present; dorsal edge setae restricted to distal third of mid- and hind tibiae; basal sclerites of setae at first abdominal sternum not fusing into sclerotized plates or multilobed patterns. Pronotum types are defined in Table 3. Distribution in ecoregions according to Illies 1978; ER5—Dinaric Western Balkans, ER6—Hellenic Western Balkans.

Species/character	Head with flat vertex (Fig. 28)?	Pronotum with long, yellowish setae (Figs. 18, 29)?	Pronotum with thin, numerous white, recumbent setae (Fig. 30)?	Pronotum type (Table 3)	Dorsocentral pronotum notch present (Fig. 1)?	Distribution (ecoregions <i>sensu</i> Illies 1978)	References
<i>Drusus bosnicus</i>	yes	no	yes	C	yes	ER5	Kučinić <i>et al.</i> 2015
<i>Drusus crenophylax</i> ¹	no	no	yes	B	yes	ER5	Vitecek <i>et al.</i> 2015a
<i>Drusus klapaleki</i> ¹	no	no	yes	B	yes	ER5	Kučinić <i>et al.</i> 2011b
<i>Drusus krusniki</i>	no	no	yes	D	yes	ER5, ER6	this paper
<i>Drusus medianus</i>	no	no	yes	B	no	ER5	Kučinić <i>et al.</i> 2010
<i>Drusus radovanovici</i>	no	yes	no	B	no	ER5	Kučinić <i>et al.</i> 2011a
<i>Drusus septentrionis</i>	no	no	no	C	yes	ER5	Kučinić <i>et al.</i> 2008
<i>Drusus serbicus</i>	no	no	yes	E	yes	ER5	Waringer <i>et al.</i> 2015
<i>Drusus vernonensis</i>	no	yes	yes	A	no	ER6	this paper
<i>Drusus vespertinus</i>	no	no	yes	C	yes	ER5	this paper

¹ In *Drusus klapaleki*, white recumbent setae are distributed over the whole pronotal surface (Kučinić *et al.* 2011b) whereas in *D. crenophylax* those setae are lacking in a semicircular area anterior of the pronotal dorsal hump (Vitecek *et al.* 2015a).

(Marinković-Gospodnetić 1976) and Una River spring in Croatia (Kučinić *et al.* 2014). *Drusus vernonensis* was considered endemic to the Vernon Mountains in Greece (Malicky 2005b), but the current data show that its distribution extends northwards to Pelister Mt. in Macedonia. However, it is an endemic species of ecoregion 6 (Graf *et al.* 2008).

All these species prefer spring areas and the headwaters of cold, oxygen-rich streams with high to moderate currents at high altitudes in the mountains. *Drusus vespertinus* occurs in large karstic springs, like the spring of the river Ribnik, a tributary of the river Sana (Marinković-Gospodnetić 1976), as well as the Una River spring (Kučinić *et al.* 2014). Mean annual water temperatures obtained by permanently exposed data loggers for sites inhabited by *D. vernonensis* (e.g., Pelister, 1805 m a.s.l., Macedonia) were 3.78°C (annual range 2.07–6.84°C) and for *D. krusniki* (e.g., Montenegro: Ibar spring, 1256 m a.s.l.; Alipaša's springs, 932 m a.s.l.) 6.22°C (annual range 5.88–8.20°C) and 6.13°C (annual range 3.60–14.35°C), respectively.

Mandible morphology of *Drusus krusniki*, *D. vernonensis*, and *D. vespertinus* larvae suggests a grazing lifestyle, with larvae feeding on biofilms and epilithic algae. The adults and larvae of *Drusus krusniki*, *D. vernonensis*, and *D. vespertinus* were collected in late May, June, and early July in 2008–2010. The latter species may be on the wing as early as late March since the holotype, paratypes, and allotypes were collected on 26 March 1968 (Marinković-Gospodnetić 1976). The holotype of *D. krusniki* was collected at the end of May (Malicky 1981). Only a few samples of *D. vernonensis* exist which were obtained in June and July (Malicky 1989, 2005b); this is in accordance with the reported short summer flight period of *D. vernonensis* by Graf *et al.* (2008).

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