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DNA based association and description of the larval stage of *Drusus melanchaetes* McLachlan, 1876 (Trichoptera: Limnephilidae: Drusinae) with notes on ecology and zoogeography

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Abstract

At three alpine locations in Switzerland adults of *Drusus melanchaetes* and unknown Drusinae larvae which could not be identified with existing keys were sampled. Based on DNA association with adults, we identified the unknown larvae as *D. melanchaetes*. To further support the association of specimens a phylogeny was estimated with the putative closest relatives of *D. melanchaetes* – *D. monticola* and *D. nigrescens* – and five other species of *Drusus* (*D. chrysotus, destitutus, discolor, muelleri* and *romanicus*). A highly supported monophyletic clade groups unknown larvae and *D. melanchaetes* specimens from the central Alps and Austria (Vorarlberg), confirming the association.

Based on morphology, larvae of *Drusus melanchaetes* key out together with *D. destitutus* in existing keys. *D. melanchaetes* is separated from the latter species by the shape of the lateral head profile which is almost straight and shows a small step at the height of the antenna, whereas in *D. destitutus* the lateral head profile is evenly rounded. In addition, in frontal view, the shape of the lateral head outline is straight in *D. melanchaetes* and rounded in *D. destititus*. There are also differences in the shape of the pronotum and in the number of the posterodorsal setae at the eighth abdominal dorsum.

Keywords

Trichoptera; *Drusus melanchaetes*; fifth instar larva; description; identification; distribution; ecology; mitochondrial DNA

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Introduction

Within the international context of the European Water Framework Directive (WFD; European Union 2000) as well as in the United States of America, Trichoptera represent an essential species group in various methododical approaches of assessing the ecological quality of water bodies (e.g. AQEM consortium 2002 (EPT-metrics); Barbour, Gerritsen, Snyder & Stribling 1999; Moog 2002; Moog & Chovanec 2000; Waringer 2003). This also fully applies to the subfamily Drusinae, where all members are restricted to water quality classes I or I-II and are used as bioindicators (sensitive species) (Moog, Graf, Janecek & Ofenböck 2002; Graf, Grasser & Waringer 2002).

In order to use the full bioindication potential of this groups, identification of larvae to species level is crucial (Goethals 2002; Margreiter-Kownacka, Pechlaner, Ritter & Saxl 1984; Pitsch 1993; Schmidt-Kloiber & Nijboer 2004). However, no comprehensive and integrated effort has been made to complete the available keys to larval Drusinae from which 24 species are reported from Austria, Germany and Switzerland (Lubini & Vicentini 2005; Malicky 1999; Robert 2001, 2004). Six of them (*Drusus alpinus* (Meyer-Dür), *D. chapmani* McL., *D. franzi* Schmid, *D. improvisus* McL., *D. melanchaetes* McL. and *D. noricus* Malicky) are still unknown in the larval stage.

In 2004-2006, however, we managed to collect larvae of *D. melanchaetes* at Swiss high alpine locations whose identity was confirmed by genetic association with adults (Pauls 2004). This material enabled us to work out reliable diagnostic characters permitting integration of *D. melanchaetes* in the identification key by Waringer & Graf (1997, 2004).

Material and methods

Larvae which obviously were not yet included in existing Drusinae keys were collected at three high alpine locations in Switzerland where the dominant adult was *D. melanchaetes.* To support conspecific association between larval and adult specimens we sequenced and analysed a 498bp long fragment of the mitochondrial cytochrome oxidase I gene (mtCOI) of three larvae and three adults from four localities (Table 1) following the methods outlined in Pauls (2004) and Pauls, Lumbsch, & Haase (2006). We generated uncorrected pairwise distances between individuals using the DNADist function as implemented in BioEdit 7.0.5.3 (Hall 1999). To further support the association of specimens we estimated a phylogeny using new and previously published sequence data of the putative closest relatives of *D. melanchaetes – D. monticola* and *D. nigrescens* (Pauls 2004) – and five other species of *Drusus* (*D. chrysotus*, *D. destitutus*, *D. discolor*, *D. muelleri* and *D. romanicus*). The Phylogeny was estimated using a Bayesian approach. Bayesian Markov chain Monte Carlo (B/MCMC) analysis was performed using the program MrBayes 3.1.2 (Huelsenbeck, Ronquist, Nielsen & Bollback 2001) assuming the GTR + I + G model. Two parallel MCMC samplings were performed with 4 simultaneous chains each for 5 million generations. Trees were sampled every 1000th generation for a total of 5000 trees from each sampling run. Log likelihood scores of samples were plotted against generation time using Tracer 1.3 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) to determine stationarity (Huelsenbeck, Ronquist, Nielsen & Bollback 2001). The initial 2500 trees of each run were discarded as ‘burn-in’. The phylogenetic tree was drawn in TreeView 1.6.6 (Page 1996).

To describe and characterise the larval morphology of *D. melanchaetes* and identify differentiating characters we examined three fifth instar larvae from Valetta di S. Gottardo (2270 m a.s.l.) collected by H.Vicentini on 8 September 2004; one fifth instar larva from Munt da San Franzesc, Poschiavo (2150 m a.s.l.) collected by V. Lubini on 27 June 2001; four fifth instar larvae from the Furkapaß (2386 m a.s.l.) collected by W.Graf on 13 October 2007.

Results

Genetic association

Within the central Alps, haplotypes between larvae and adults of *D. melanchaetes* differed by one or two base changes ($p = 0.002$-0.004). Maximum p within *D. melanchaetes* was found between a larva from the Sustenpass and an adult male from the Klostertal ($p = 0.041$). Minimum p between *D. melanchaetes* and other species was $p = 0.064$. The B/MCMC phylogeny of eight Drusinae species (Fig. 13) clearly shows that the unknown larvae fall within a highly supported monophyletic clade with *D. melanchaetes* adults from the central Alpine region (pp=1.0) and Vorarlberg (pp=0.97). *D. monticola* and *D. nigrescens* form a sister clade to *D. melanchaetes* (pp=1.0). The sister relationship is highly supported (pp=0.97), however the relationship between is *D. monticola* and *nigrescens* is resolved but not supported (pp=0.88). Basal to the *melanchaetes-nigrescens-monticola* clade is *D. destitutus* (pp=1.0). *D. chrysotus* and *D. muelleri* also build highly supported monospecific clades (pp=1.0). *D. discolor* and *D. romanicus* form a highly supported clade, however the relationship between these two species remains unresolved.

Description of the fifth instar larva of *Drusus melanchaetes*

The body length of final instar larvae ranges from 9.0 to 12.1 mm, the head width from 1.36 to 1.53 mm. The larval case is 8.7 – 11.8 mm in length, distinctly curved, tapering posteriorly (the width at anterior opening is 2.5 – 3.2 mm and at the posterior opening 1.6 – 1.9 mm), and consists completely of mineral particles with grain sizes increasing distinctly in anterior direction.

The head capsule and all body sclerites are dark brown to black brown. The head capsule (Figs. 1, 3) lacks the additional setae or spines known from other Drusinae larvae (e.g. *Ecclisopteryx* spp., *Drusus trifidus*). The mandibles lack terminal teeth along edges as well as ridges in the central concavity.

In profile, the dorsal line of the pronotum is evenly rounded, thereby creating a small dorsal hump in its posterior third (Fig. 7). In lateral and even more so in frontal view a ventrolateral bulge is clearly visible (Figs. 7, 9). The black brown pronotal surface is covered by prostrate, tiny white setae which are distinctly shorter and scarcer than in *D. destitutus*; in addition, larger black setae along the lateral and anterior borders are present. The prosternite is inconspicuous and a prosternal horn is present. The mesonotum is completely covered by two chestnut-brown sclerites. The metanotum is partially covered by three pairs of sclerites with the anterior metanotal sclerites being large and ovoidal; their median separation is distinctly smaller than their maximum extension along the body axis (Fig. 6). The setal bases at the central section of the first abdominal sternum are mostly small and inconspicuous except two larger bases near the midline which occasionally fuse with neighbouring smaller setal bases. However, a large sclerotized central plate as it is common in genus *Metanoea* (Waringer & Graf 1997, 2004; Waringer, Graf & Maier 2000) or in *Drusus nigrescens* (Waringer, Graf, Pauls & Lubini 2007) is lacking. At the eighth abdominal dorsum, the number of posterodorsal setae (pds) is 2-4, consisting of 2 long and 0-2 short setae (Fig. 12).

Dorsal gills are present from the second (presegmental position) to the sixth (presegmental position). Ventral gills range from second (presegmental) to seventh segment (presegmental). Lateral gills are present on the second and third segment (postsegmental position). The lateral fringe is present on the last third of the second to the beginning of the eighth abdominal segment. Setae are present at the anterior and posterior faces of all femora. The row of dorsal setae at the mid- and hindleg tibiae extend over the whole length of the segment (Fig. 5).

Morphological separation of *Drusus melanchaetes* from other European Trichoptera

A summary of morphological features for the identification of limnephilid and Drusinae larvae is given in Waringer (1985). Within the framework of the limnephilid key by Waringer & Graf (1997, 2004), *Drusus melanchaetes* is separated from other species by the following features:

- metanotum covered by three pairs of small sclerites (Fig. 6, m);
- head and pronotum without a thick layer of woolly hairs (Fig. 1);
- head capsule without groups of additional spines, without central concavity and rims surrounding the frontoclypeus (Fig. 1);
- first abdominal sternum without a large median sclerotized patch;
- pronotum without ridge; in profile, dorsal outline evenly rounded in its posterior third, thereby creating a small dorsal hump (Fig. 7);
- Mandibles lacking terminal teeth along edges as well as ridges in the central concavity;
- Middle and hindleg femora faces with additional setae (Fig. 5, arrow);
- Anteromedian metanotal sclerites large, ovoidal, their median separation being distinctly smaller than their maximum extension along the body axis (Fig. 6);
- Row of setae at anterior border of pronotum extending as far as the pronotal midline;
- Row of dorsal setae at mid- and hind tibiae extending over the whole length of the segment (Fig. 5).

At this position *Drusus melanchaetes* keys out together with *Drusus destitutus*. The species are readily separated by the shape of the lateral head profile: in *D. melanchaetes* this profile from the anterior border of the frontoclypeus to the eyes is almost straight and shows a small step at the height of the antenna (Fig. 3, arrow), whereas in *D. destitutus* the lateral head profile is evenly rounded (Fig. 4). In addition, in frontal view, the shape of the lateral head outline between eyes and anterior border of the frontoclypeus is straight in *D. melanchaetes* (Fig. 1) and bent in *D. destititus* (Fig. 2). In *D. melanchaetes*, the pronotum has a distinct ventro-lateral bulge most easily seen in frontal view (Figs. 7, 9) which is lacking in *D. destitutus* (Figs. 8, 10). The number of posterodorsal setae (pds) at the eighth abdominal dorsum is 4-8 in *D. destitutus* (2 long, 2 (very rare) -6 short setae) and 2-4 in *D. melanchaetes* (2 long, 0-2 short setae) (Figs. 11, 12). In *D. destitutus* the pronotum is covered by prostrate white setae (Graf 1993), which are much shorter and also scarcer in *D. melanchaetes*.

Phenology, habitat, and distribution

Last instar larvae of *D. melanchaetes* were collected on 27 June 2001 at the Munt da San Franzesc, Poschiavo (2150 m a.s.l.), on 8 September 2004 at the Valetta di S. Gottardo (2270 m a.s.l.) and on 13 October 2006 at the Furkapaß (2386 m a.s.l.). *D. melanchaetes* is known to be on the wing from April, in higher altitudes from June to August (Schmid 1956; Graf, unpublished data). Observing last instar larvae late in the summer and early autumn suggests the species survives winter in a late larval stage, or that the species might have a semivoltine life cycle. This could result from the short growth period in high altitudes. In a study in the high altitudes of the Pyrenees, Lavandier (1992) measured head capsule widths to reconstruct the larval growth cycle of *Drusus discolor* and observed a two year development.

At the Furkapaß numerous adults of *Allogamus mendax* and *Consorophylax consors* as well as larvae of *D. muelleri, D. nigrescens* and *Rhyacophila intermedia* were found in October. At this location, the small, spring-fed, 50 m long, first order tributary is part of the Mutt watershed; it is a clean, fast-flowing, summer-cold mountain brook bordered by meadows. At this location, *D. melanchaetes* was sympatric with *D. muelleri, Lithax niger* and Plecoptera such as Dyctiogenus fontium, Protonemura lateralis, Leuctra ravizza, L. rosinae, Nemoura mortoni and *N. sinuata.* The larvae of *D. melanchaetes* are found also in small brooklets, fed only by snowmelt water and therefore are dry in summer; one location is an outlet of a high alpine lake. Ephemeroptera species found together at these localities with *D. melanchaetes* are *Rhithrogena loyolaea, Baetis alpinus* and *Ecdyonurus helveticus.*

According to Malicky (2004), *D. melanchaetes* is a west-alpine species; records exist from Switzerland (Lubini-Ferlin & Vicentini 2005), Italy (Piemonte, Valle d'Aosta, Lombardia and Trentino – Alto Adige; Cianficconi 2002) and France (Barnard 2005) but the species is lacking in Germany (Robert 2001, 2004). In Austria, *D. melanchaetes* is reported from Carinthia, the Tyrol and Vorarlberg (Malicky 1999). The altitudinal range in Switzerland is between 1960 m and 2560 m a.s.l..

Discussion

According to Schmid (1956) *D. melanchaetes* is an isolated species within subfamily Drusinae. The species is characterised by its black or black-brown colour, its large wings and the large lower appendices which are dinstinctly pointed upwards. The superior and intermediate appendices are medium-sized with intermediate appendices being pointed and divergent. Schmid (1956) considers *D. melanchaetes* an isolated species intermediate between the *mixtus* group (*D. mixtus*, *D. biguttatus*, *D. improvisus*, *D. spelaeus*, *D. brunneus*, *D. trifidus*, *D. bolivari*) and *Drusus cantabricus*, another isolated species which, in turn, is adjacent to the *annulatus* group (*D. annulatus, D. rectus, D. tenellus, D. simplex*). Since we are lacking the closest relatives according to Schmid (1956), we cannot explicitly test his hypotheses with the current data set. However, in a preliminary phylogeny of the group (Pauls 2004, Pauls in prep.), *D. melanchaetes* is situated close to the species pair *D. monticola – D. nigrescens* and *D. destitutus*. We also observed this close relationship in our morphological and genetic analyses (Fig. 13). In the larval stage, these species and their immediate relatives are characterised by spoon-shaped mandibles without teeth along the anterior edges,which identifies them as scrapers feeding mainly on epilithic algae. Generally, epilithic algal growth is much higher at lotic stream sections and midstream than in lenitic sections or near the banks (Gessner 1955). This is why scraper species within the subfamily Drusinae are forced to expose themselves much more during feeding than omnivorous generalists feeding near the banks. In addition, in order to feed effectively, last and penultimate instars of scraping Drusinae species do not fix their cases at the substrate as the filter-feeding Drusinae do. This results in a significant over-represention of scraper Drusinae species in the drift when compared with their relative abundance on the stream bed (Bacher & Waringer 1996). The grouping of *D. melanchaetes* and the other species of epilithic grazers with smooth mandible edges were significantly different from the group of carnivorous filtering larvae with serrated mandible edges and filtering setae and bristles, such as *D. muelleri, D. chrysotus, D. discolour* and *D. romanicus* (Fig. 13). The groupings observed in our genetic analyses reflect, therefore, mouthpart morphology and feeding ecology very well.

The current study presents the third example of a larval description based on molecular association with adult caddisflies of the Drusinae (e.g. Graf, Lubini & Pauls 2005; Waringer, Graf, Pauls & Lubini 2007). Molecular associations between sexes or life stages are becoming more commonplace in caddisflies (Shan,Yang & Wang 2004) and other insect groups (e.g. Miller, Alarie, Wolfe & Whiting 2005; Willassen 2005), exemplifying how nucleotide sequence markers can facilitate and provide supportive evidence in taxonomic and systematic research. mtCOI is often propagated as a suitable gene for “barcoding” studies (Hebert, Cywinska, Ball & deWaard 2003). Our study shows that his region is indeed suitable for designating and delimiting species, but also shows the markers’ limits for DNA based-taxonomy concerning extremely closely related species, especially if lineage sorting is still incomplete (Pamilo & Nei 1988, Morando, Avila, Baker & Sites 2004). In our study this appears to be the case between the closely related species pairs *D. discolor* - *D. romanicus* and *D. nigrescens* - *D. monticola*. The use of a single mitochondrial gene region as proposed for “DNA barcoding” may not be sufficient to resolve such situations, and the choice of the marker of utmost importance (Mueller 2006).

Summary

To support species affiliation between known adults and unknown larvae, specimens were genetically analysed by means of DNA nucleotide sequence analysis of a 498bp long fragment of the mitochondrial cytochrome oxidase I gene (mtCOI). To further elucidate the association of specimens a phylogeny was estimated with the putative closest relatives of *D. melanchaetes* (*D. monticola* and *D. nigrescens*) and four other species of *Drusus* (*D. chrysotus, discolor, muelleri* and *romanicus*), yielding a highly supported monophyletic clade with *D. melanchaetes*.

Based on larval morphology, *D. melanchaetes* is separated from the very similar *D. destitutus* by the shape of the lateral head profile, the shape of the lateral head outline and differences in the shape of the pronotum and in the number of posterodorsal setae at the eighth abdominal dorsum.

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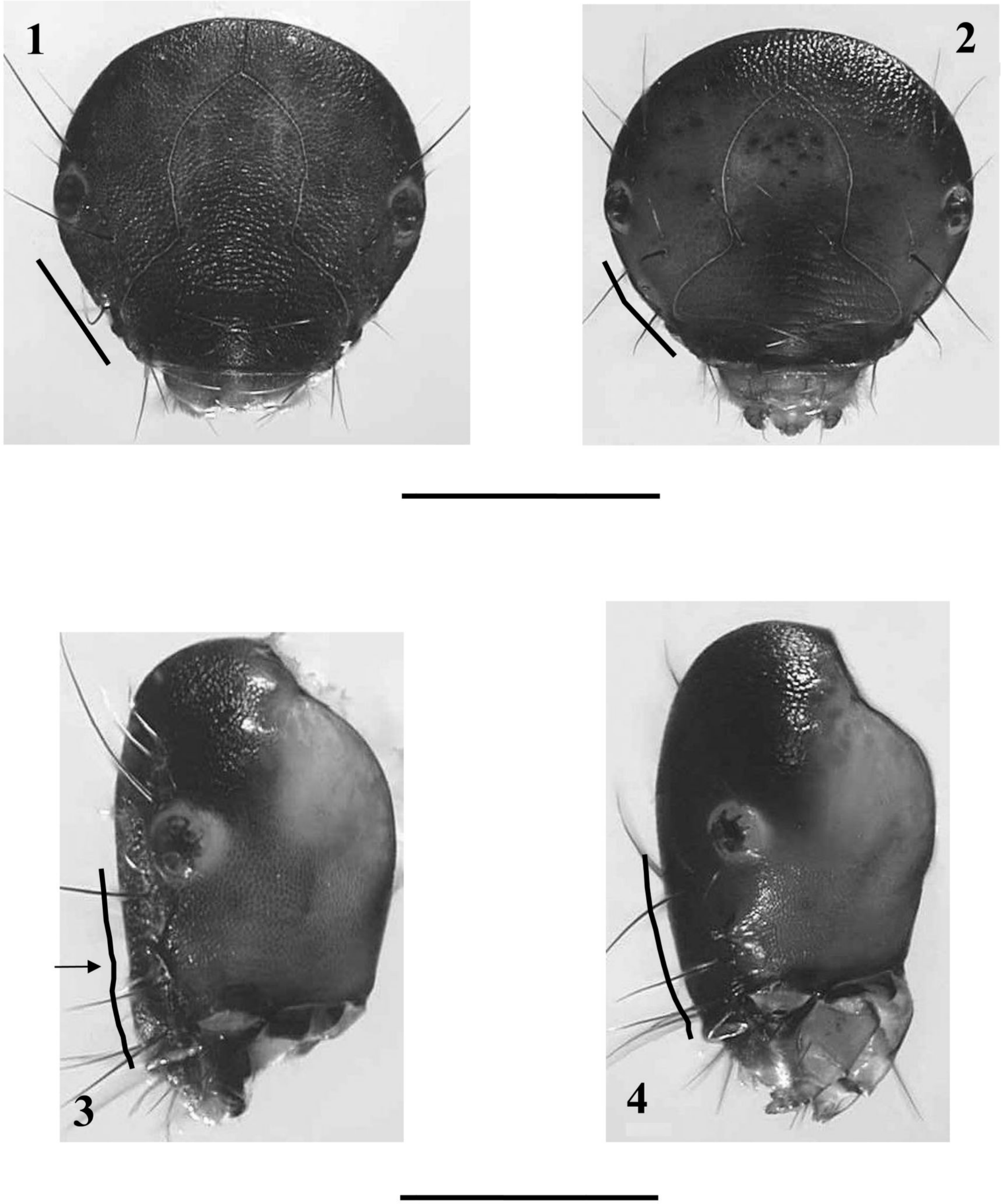
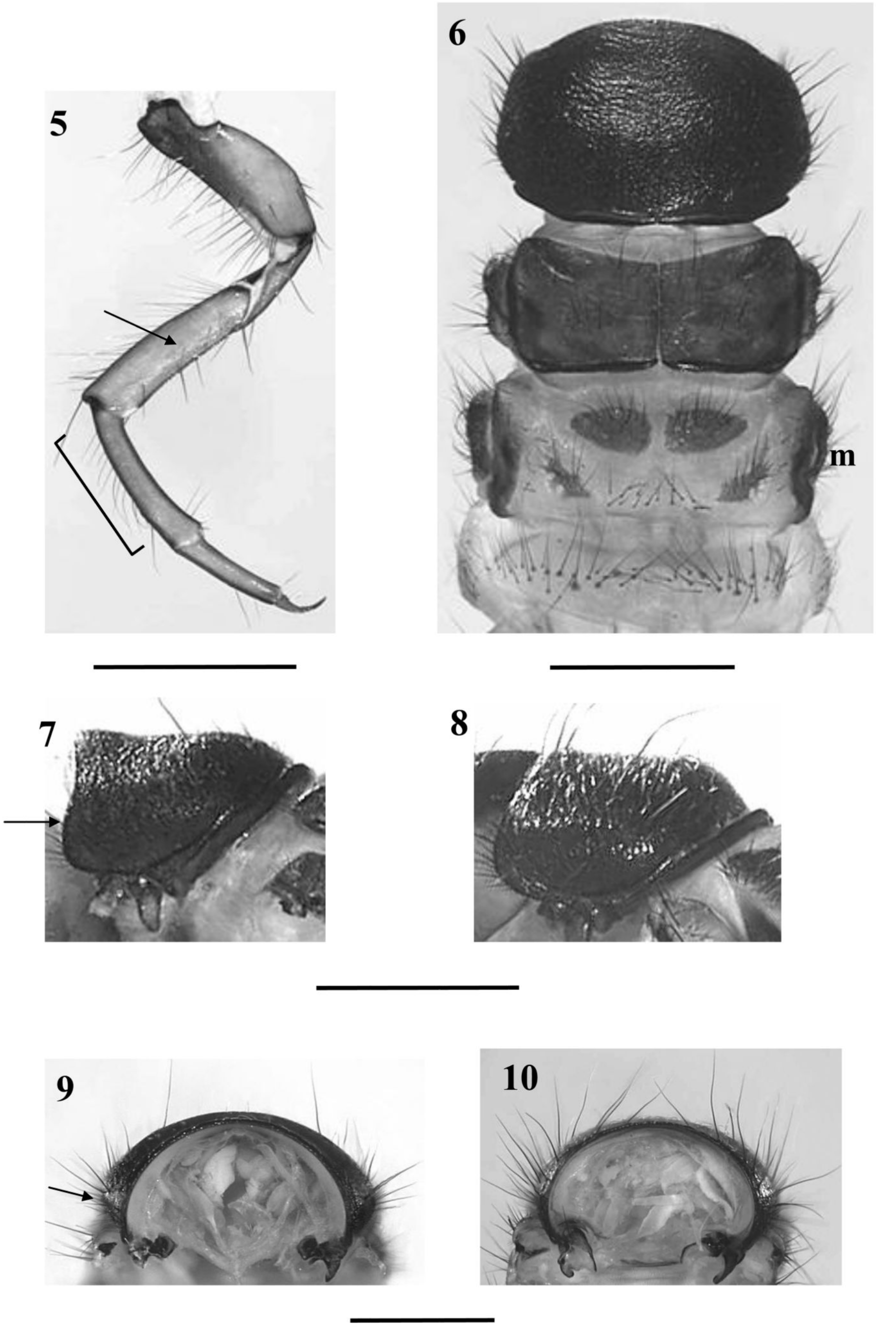


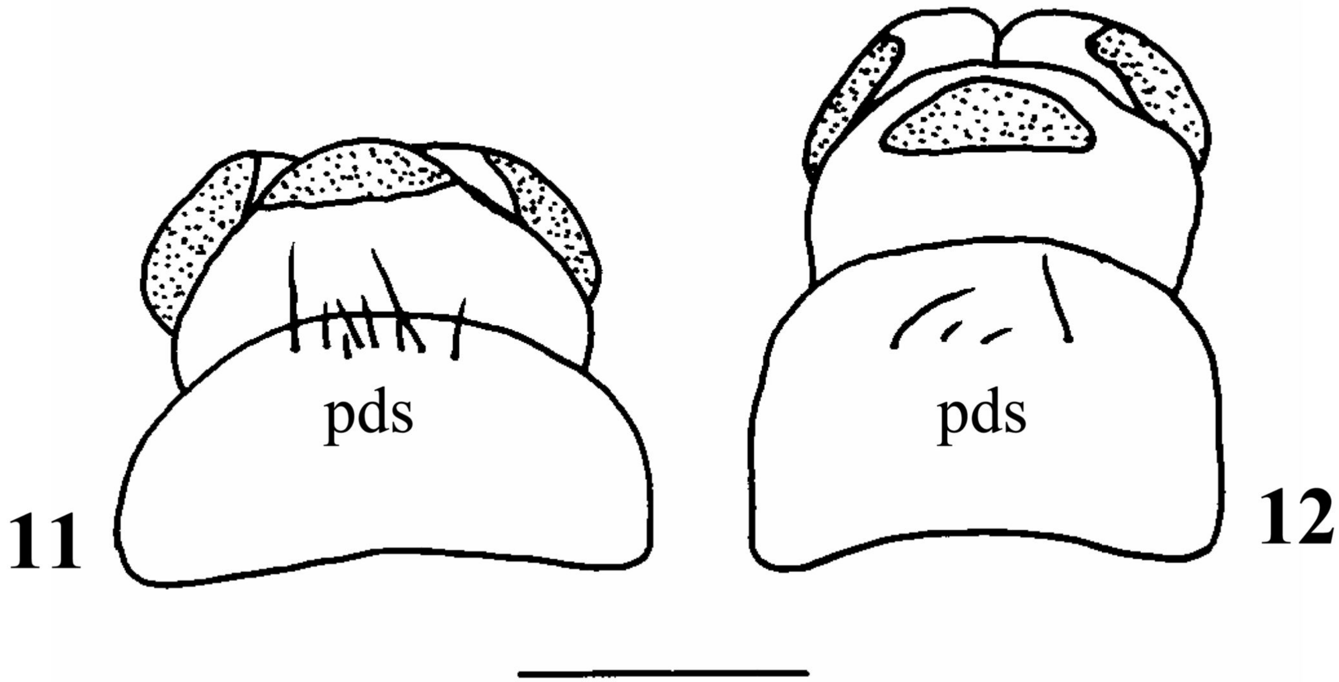
Figs.1 – 4.

Figs.1 – 2 Head, frontal view of fifth instar larvae (with frontal profile lines): 1: *Drusus melanchaetes*, 2: *D. destitutus*. Figs. 3 - 4: Head, left lateral view of fifth instar larvae (with lateral profile lines; arrow: step in profile): 3: *D. melanchaetes*, 4: *D. destitutus*. Scale bars: 1mm.



Figs. 5 - 10.

Figs. 5 - 8 *Drusus melanchaetes*, fifth instar larva; 5: left hindleg, anterior view; arrow=additional setae on face of femur; 6: thorax and first abdominal segment, dorsal view; m=metanotum; Fig. 7: pronotum, left lateral view; arrow: lateral bulge; Fig. 8: *Drusus destitutus*, pronotum, left lateral view; Fig. 9: *D. melanchaetes*, fifth instar larva: pronotum, frontal view; arrow: lateral bulge; Fig. 10: *D. destitutus*, pronotum, frontal view. Scale bars: 1mm.



Figs. 11 - 12.

Dorsal view of eighth and ninth abdominal dorsum of 11: *Drusus destitutus*, 12: *D. melanchaetes*. Setae are not shown; sclerites indicated by dotted areas; pds= posterodorsal setae at eighth abdominal dorsum. Scale bar: 1 mm.

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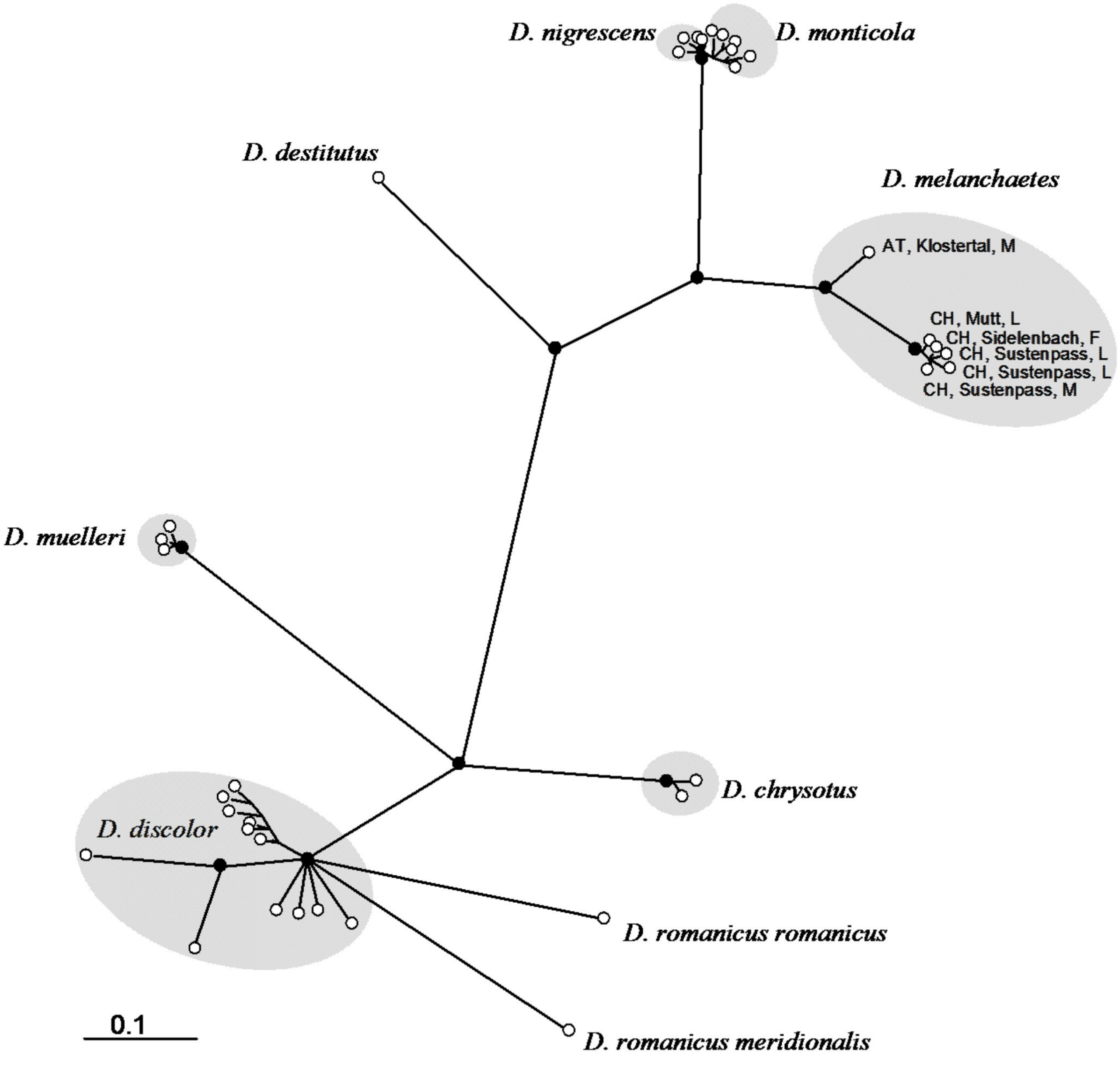

Fig. 13.

B/MCMC inference of haplotype phylogeny of *D. melanchaetes* and seven *Drusus* species. Unrooted 50% majority rule consensus tree based on a Bayesian sampling of 5000 trees. White circles depict terminal taxa; black circles indicate nodes with significant posterior probabilities (>0.94).

Table 1

Material used in this study. Sequences were generated in [A] this study; [B] Waringer et al. 2007, or [C] Graf et al. 2005

Drusus	Stage[*]	Locality	GenBank Accession
melanchaetes	M	CH, Meienreuss, East of Sustenpass, 16.07.2004	EU143740[A]
	F	CH, Sidelenbach, 16.07.2004	EF464555[B]
	L[°]	CH, Meienreuss, East of Sustenpass, 16.07.2004	EU143742[A]
	L[°]	CH, Meienreuss, East of Sustenpass, 16.07.2004	EU143743[A]
	L[°]	CH, Mutt and left tributaries, 17.07.2004	EU143744[A]
	M	AT, Vorarlberg, Klostertaler Bach, 27.07.1999	EU143741[A]
chrysotus	L	AT, Soboth, Krumbachquelle, 18.05.2002	AY954395[C]
	M	AT, Saualpe, Quellbäche bei Ladinger Hütte, 30.06.2006	EU143739[A]
destitutus	L	AT, Soboth, Krumbach, 18.05.2002	EU143738[A]
discolor	L	F, Auvergne, nameless brook	DQ351158[C]
	L	D, Ammer Mts., Kühbach, Soyermühle	DQ351160[C]
	L	D, Erzgebirge, Große Mittweida	DQ351162[C]
	L	D, Rothaargebirge, Hoppecke	DQ351165[C]
	L	F, Vosges Mts., La Meurthe	DQ351166[C]
	L	SK, Muranska Planina, Hronec at Patina	DQ351168[C]
	L	F, Pyrenees, Pec de Moli	DQ351183[C]
	M	CH, Jura Mts., Bouvier	DQ351189[C]
	L	F, Alpes maritimes, Via Ferrate l'Aguilette	DQ351193[C]
	M	RO, Rodna Mts., Lala Valley	DQ351199[C]
	M	BG, Rila Mts., Gyolska	DQ351204[C]
	M	BG, Rila Mts., Malyovishka	DQ351205[C]
monticola	L	AT, Nockberge, St. Oswald Bach B, 01.07.2006	EF464559[B]
	F	AT, Saualpe, Ladinger Hütte, 16.06.2006	EF464560[B]
	F	AT, Saualpe, Ladinger Hütte,16.06.2006	EF464561[B]
	L	AT, Saualpe, Offner Hütte, 30.06.2006	EF464558[B]
	L	AT, Soboth, Krumbach, 18.05.2002	EF464556[B]
	L	AT, Soboth, Krumbach, 18.05.2002	EF464557[B]
muelleri	M	CH, Tributary to Grimselsee, Grimselpass	AY954401[C]
	M	CH, Meienreuss, East of Sustenpass, 16.07.2004	AY954400[C]
	M	CH, Mutt and left tributaries, 17.07.2004	AY954398[C]
nigrescens	L	CH, Furka Pass 21.7. 2006	EF464567[B]
	M	CH, Furka Pass 21.7. 2006	EF464565[B]
	M	CH, Furka Pass 21.7. 2006	EF464566[B]
	F	CH, Mutt and left tributaries, 17.07.2004	EF464563[B]
	L	CH, Mutt and left tributaries, 17.07.2004	EF464568[B]

Drusus	**Stage**[*]	**Locality**	**GenBank Accession**
	L	CH, Mutt and left tributaries, 17.07.2004	EF464569[B]
	M	CH, Mutt and left tributaries, 17.07.2004	EF464562[B]
	M	CH, Mutt and left tributaries, 17.07.2004	EF464564[B]
romanicus meridionalis	M	BG, Banderishka River, above Vihren Chalet, 18.08.2003	AY954402[C]
romanicus romanicus	L	RO, Faearas Mts., Valea Buda, 07.08.2003	AY954403[C]

[o] Larva assigned in this study

[*] Stage: L: larva, M: male, F: female