



A hairy case: The evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera) [☆]



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ABSTRACT

The caddisfly subfamily Drusinae BANKS comprises roughly 100 species inhabiting mountain ranges in Europe, Asia Minor and the Caucasus. A 3-gene phylogeny of the subfamily previously identified three major clades that were corroborated by larval morphology and feeding ecologies: scraping grazers, omnivorous shredders and filtering carnivores. Larvae of filtering carnivores exhibit unique head capsule complexities, unknown from other caddisfly larvae. Here we assess the species-level relationships within filtering carnivores, hypothesizing that head capsule complexity is derived from simple shapes observed in the other feeding groups. We summarize the current systematics and taxonomy of the group, clarify the systematic position of *Cryptothrix nebulicola*, and present a larval key to filtering carnivorous Drusinae.

We infer relationships of all known filtering carnivorous Drusinae and 34 additional Drusinae species using Bayesian species tree analysis and concatenated Bayesian phylogenetic analysis of 3805 bp of sequence data from six gene regions (mtCOI5-P, mtCOI3-P, 16S rDNA, CADH, WG, 28S rDNA), morphological cladistics from 308 characters, and a total evidence analysis.

All analyses support monophyly of the three feeding ecology groups but fail to fully resolve internal relationships. Within filtering carnivores, variation in head setation and frontoclypeus structure may be associated with progressive niche adaptation, with less complex species recovered at a basal position. We propose that diversification of complex setation and frontoclypeus shape represents a recent evolutionary development, hypothetically enforcing speciation and niche specificity within filtering carnivorous Drusinae.

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1. Introduction

Aquatic invertebrates have evolved a staggering diversity of different feeding types (e.g., grazers, shredders, scrapers, gatherers, filter feeders, predators, and piercers) (Cummins and Klug, 1979; Mecom, 1972; Wallace and Merritt, 1980). In particular, feeding ecology of caddisfly larvae is highly diverse (Graf et al., 2008; Mackay and Wiggins, 1979), rendering them fundamental

participants in nutrient and energy fluxes in aquatic and the adjacent terrestrial ecosystems (Covich et al., 1999; Wallace and Webster, 1996).

In benthic insects, passive filter-feeding has evolved in Ephemeroptera, Trichoptera and Diptera. Most filter-feeding Diptera and all filter-feeding Ephemeroptera develop specialized structures, whereas the majority of Trichoptera employ silken-nets for filter-feeding (McCafferty and Bae, 1992; Merritt and Wallace, 1984). Within Trichoptera, most families of Annulipalpia *sensu* Malm et al. (2013) are net-spinning filter-feeders that construct specialized nets, befitting their ecological niche (Holzenthal et al., 2007; Malm et al., 2013). Despite the heterogeneity of behavioral adaptations in net-spinning European Trichoptera, only few genera of Annulipalpia develop specialized

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mouthparts to exploit fine particulate organic matter (FPOM), whereas the majority develops shredder-like mouthparts (W. Graf, J. Waringer, unpubl. data). Filter-feeding Integripalpia do not construct filtering nets, but rather employ their whole body as a filtering structure. Members of the European genera *Allogamus*, *Brachycentrus*, and *Drusus* (Graf et al., 2008) and the South-East Asian genus *Limnacentropus* (Wiggins, 1969; Wallace and Merritt, 1980; Graf unpubl. data) exhibit filtering bristles on legs and sometimes on sterna, as well as modifications of the legs (e.g. elongation of femora in *Brachycentrus*) or heads (as in Drusinae) as morphological adaptations to filter-feeding. Drifting macrozoobenthos and large organic particles were found to constitute the primary food particles foraged for in *B. subnubilus* (Majecski et al., 1997) and some species of filtering carnivorous Drusinae (Bohle, 1983, 1987; Graf unpubl. data). Particularly larvae of Chironomidae and Ephemeroptera are preyed upon by filtering carnivorous Drusinae (Bohle, 1983, 1987; Graf unpubl. data), whereas FPOM seems to be of lesser importance.

Understanding evolution of feeding modes and associated traits can help us to peruse evolutionary ecology of Trichoptera, particularly with respect to evolutionary constraints on niche exploitation (cf. Dijkstra et al., 2014). To this end, the subfamily Drusinae represents an ideal model, as it exhibits unusually high feeding diversity including unique and complex feeding strategies among caddisflies (Graf et al., 2009; Pauls et al., 2008).

The limnephilid subfamily Drusinae comprises 8 currently recognized genera: *Anomalopterygella* Fischer, 1966; *Cryptothrix* McLachlan, 1867; *Drusus* Stephens, 1835; *Ecclisopteryx* Kolenati, 1848; *Hadimina* Sipahiler, 2002; *Leptodrusus* Schmid, 1955; *Metanoea* McLachlan, 1880; *Monocentra* Rambur, 1842. Most Drusinae are cold-stenotopic species restricted to Eurasian mountain ranges. The highly disjunct distribution patterns of some Drusinae species reflect the complex evolutionary history of the group characterized by long-time persistence of distinct lineages (e.g. through multiple glacial cycles) and high levels of small scale allopatric speciation (Graf et al., 2008; Pauls et al., 2006; Previšić et al., 2009, 2014b). Thus, the subfamily features many endemic species (Graf et al., 2008; Malicky, 2005; Kučinić et al., 2011; Oláh, 2010, 2011).

The last exhaustive treatment of this group was conducted by Schmid (1956), who identified 7 genera of Drusinae and 6 species groups within the largest genus *Drusus*. He defined the subfamily mainly by venation characteristics of the male wings, and the presence of specialized A2 and A3 veins in the male hindwings, which form a pouch that harbors long vein bristles of unknown function (Schmid, 1956). Schmid (1956) examined 37 out of a total of 41 then known species, of which 35 are still valid. Since then, 1 new genus and many new species have been described, resulting in roughly 100 known extant species (Graf et al., 2008; Malicky, 2004, 2005; Kučinić et al., 2011; Oláh, 2010, 2011; Olah and Kovács, 2013; Previšić et al., 2014a; Sipahiler, 2002).

A first molecular phylogenetic study on the subfamily Drusinae revealed the prevalence of three distinct evolutionary lineages, reflecting feeding ecology of larvae (Graf et al., 2009; Pauls et al., 2008): (1) carnivorous filterers (developing toothed shredder-like mandible edges, modifications of head capsules, and additional filtering spines on legs and the first abdominal sternum), (2) omnivorous shredders (exhibiting toothed shredder mandibles, but lacking additional spines and filtering bristles), (3) epilithic grazers (developing spoon-shaped mandibles without teeth and lacking additional spines and filtering bristles). Further, Pauls et al. (2008) rejected most of the generic concepts and species groupings that Schmid (1956) had proposed based primarily on the morphology of adult male terminalia. In particular, the monotypic genus *Cryptothrix* was found to be closely related to the filtering carnivorous Drusinae, whereas the genus *Drusus* was

identified as paraphyletic with *Anomalopterygella*, *Metanoea*, polyphyletic *Ecclisopteryx*, and *Cryptothrix* nested within (Pauls et al., 2008). The genus *Drusus* was found to comprise members of all three larval feeding types (Graf et al., 2009; Pauls et al., 2008).

Taxonomic knowledge enabling larval identification were hitherto presented for 47 Drusinae species only (references in Previšić et al., 2014a; Vitecek et al., 2015a; Waringer et al., 2013a,b). Larval stages of Ephemeroptera, Plecoptera and Trichoptera are crucial quality elements for contemporary biological water quality monitoring approaches (AQEM consortium, 2002; Barbour et al., 1999; Barbour and Yoder, 2000; Graf et al., 2002). Particularly the differential sensitivity of caddisfly species to minute environmental changes renders them ideal to monitor pollution or anthropogenic disturbance (Graf et al., 2002), particularly at species level (Waringer et al., 2013c). As all identifiable members of Drusinae are used as bio-indicators and sensitive species in biological monitoring (Moog et al., 2002), larval keys to this highly diversified group are crucial. Currently available larval keys cover roughly 50% of all known species, and need to be extended in order to exploit the full bioindicative potential of Drusinae.

In this study, we examine whether adult apomorphies exist for the feeding groups *sensu* Pauls et al. (2008). Further, to resolve phylogenetic relationships within filtering carnivorous Drusinae we infer phylogenetic relationships in a Bayesian framework using sequence data from 6 loci (mtCOI5-P, mtCOI3-P, 16SnrDNA, CADH, WG, 28SnrDNA) and 308 morphological characters in separate species tree and concatenated Bayesian inference on the molecular data set, cladistic analysis on the morphological dataset, and total evidence analysis based on the combined molecular and morphological dataset. We aim to identify potential evolutionary trends in larval head setation and head capsule complexity, and clarify the systematic position of two recently described species (Vitecek et al., 2015b), *Cryptothrix* and an unassociated new larva of filtering carnivorous Drusinae displaying remarkable, potentially transitional characters. Additionally, we provide re-descriptions of all known filtering carnivorous Drusinae larvae and present descriptions and larval keys to identify the hitherto unidentifiable larvae of the *Drusus discolor* group *sensu* Pauls et al. (2008).

2. Materials and methods

2.1. Taxon sampling

Adult specimens were collected using sweep nets and light traps; larvae were collected by handpicking (Table A1). Specimens were stored in 70% and 96% EtOH for morphological and molecular analysis, respectively.

2.2. Comparative morphological analysis and coding of adult and larval characters

Morphological characteristics of male terminalia were examined in cleared specimens. Specimens were cleared using either the Qiagen Blood and Tissue Kit for DNA-extraction according to the manufacturer's recommendation and subsequent KOH-treatment (Böhm et al., 2011), or KOH-treatment. Nomenclature of male terminalia follows Nielsen (1957, for *Limnephilus flavicornis* Fabricius) using the simplifying terms “superior appendages” for the lateral processes of segment X (cerci *sensu* Snodgrass, 1935), and “intermediate appendages” for the sclerite and the anterior process of segment X (paraproct *sensu* Snodgrass, 1935). Larval morphological features were examined following Wiggins (1998) and Waringer and Graf (2011). Nomenclature of primary setae and setal areas follows Wiggins (1998).

Illustrations were prepared according to [Holzenthal \(2008\)](#) and [Thomson and Holzenthal \(2010\)](#) in which pencil drawings made with a camera lucida are digitized, edited and inked in Adobe Illustrator (v. 16.0.4, Adobe Systems Inc.). We used a Nikon SMZU-1000 stereomicroscope (larval heads, male terminalia) or a Wild M20 compound microscope (details of male terminalia) for comparative morphological examination of specimens.

All morphological characters used in the present study have been newly defined. Each character was evaluated for scoring consistency, shared presence in 2 or more taxa, and unique presence in 1 taxon. Characters were included if they could be clearly identified and delimited. Non-additive binary presence/absence coding of characters was used, based on the following assumptions: (1) the subfamily Drusinae was demonstrated to represent a monophyletic entity ([Pauls et al., 2008](#)); (2) assuming (1) to be true, we hypothesize a primordial set of characters that was present in the Drusinae ancestor; (3) presence of a certain character in a taxon is a derived homologous state shared with all other taxa that develop this character, and, vice versa, shared absence of a certain character in several taxa represents a homologous plesiomorphic state, as this putatively was the character state in a hypothetical Drusinae ancestor. If a character could not be assessed, it was scored as ‘missing’. However, states of binary coding do not imply assumptions regarding the plesiomorphic or derived nature of characters. Definitions of morphological characters and the morphological matrix used in phylogenetic analysis are provided in the [supplementary material \(Appendix A, B\)](#).

2.3. DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from the abdomen or the thorax of adult or larval specimens using the DNEasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. Standard PCR procedures and primers were used ([Table 1](#)). PCR reactions were setup in 10 µl reactions. Unpurified PCR products were sequenced on an ABI 3177XL capillary sequencer at BIK-F

using the PCR primers and two additional internal primers for D2 (D2UP-4 and D2DN-B, [Zhou et al., 2007](#)).

2.4. Datasets, sequence alignment and phylogenetic analysis

Phylogeny of Drusinae was inferred using a 42 species (40 ingroup species, 107 terminal taxa), 6 loci (mtCOI5-P, mtCOI3-P, 16SsrDNA, CADH, WG, 28SnrDNA), 3805 bp molecular dataset ([Table A1](#)). Additionally, phylogenetic relationships of Drusinae were estimated using a 41 terminal taxa (39 ingroup species), 308 character morphological dataset ([Appendix B](#)) and a 41 terminal taxa (39 ingroup species), 4113 character combined morphological and molecular dataset.

Sequences were edited in Geneious R6 ([Kearse et al., 2012](#)) and aligned using MAFFT v7 ([Katoh and Standley, 2013](#)) as implemented in Geneious R6. Nucleotide substitution models for each partition were selected according to the Bayesian Information Criterion in the model test module of Mega v5.1 ([Tamura et al., 2011](#)) ([Table 2](#)). For phylogenetic analysis, the 16SsrDNA and 28SnrDNA fragments were not partitioned.

Phylogenetic relationships were inferred using Bayesian Inference (BI) through BEAST v1.8 ([Drummond et al., 2012](#)) and MrBayes 3.2 ([Ronquist et al., 2012](#)). To examine heterogeneity of phylogenetic signal among data partitions, ≥ 0.95 posterior probability topologies of B/MCMCMC single gene and combined data analyses were examined. Single gene analyses were performed for each partition in MrBayes 3.2, implementing the respective substitution models. Four parallel runs with 6 chains each were carried out (10×10^6 generations, sampling every 1000th generation). Stationary distribution of runs in the same optimal tree space was assumed if the average standard deviation of split frequencies reached values below 0.01. Additionally, MrBayes parameter files were examined in Tracer v1.6 ([Rambaut et al., 2014](#)) to assess if runs had reached a stationary phase and converged on model parameters. A maximum clade credibility tree was estimated based on trees sampled by MrBayes after discarding the first 2,500 trees of each run as burn-in using TreeAnnotator v1.8

Table 1
PCR primers and PCR cycling conditions.

Fragment	Primers & primer concentration	PCR cycling conditions	Taq kit	Additional reagents
mtCOI5-P	HCO2198 & LCO1490 (Folmer et al., 1994)	0.25 µM 5'95 °C, 5 × (30"95 °C, 1'44 °C, 1'72 °C), 15 × (30"95 °C, 30"48 °C, 1'72 °C), 20 × (30"95 °C, 30"50 °C, 1' + (10" * n) 72 °C)	peqGOLD HotTaq	–
mtCOI3-P	Jerry & S20 (Pauls et al., 2006)	0.25 µM 5'95 °C, 35 × (45"95 °C, 30"45 °C, 45"72 °C), 5'72 °C	peqGOLD HotTaq	–
16SrDNA	Lepto-F & Lepto-R (Malm and Johanson, 2008)	0.75 µM 3'95 °C, 35 × (30"95 °C, 30"52 °C, 40"72 °C), 5'72 °C	peqGOLD HotTaq	4 mg BSA
WG	WGbDrrev (5'-ACCCTCTCCCGCARCACTTGAG) & WGbDrfwd (5'-CTTGCTGGATGCGTCTGCC) ^a	0.5 µM 5'95 °C, 35 × (45"95 °C, 45"60 °C, 90"72 °C), 7'72 °C	Qiagen Hotstar Taq plus Master mix	–
CADH	1028r-ino & 743nF-ino (Johanson and Malm, 2010)	0.25 µM 5'95 °C, 35 × (45"95 °C, 30"50 °C, 45"72 °C), 5'72 °C	peqGOLD HotTaq	–
D2 (28SnrDNA)	D1-3up1 (5'-CGAGTAGCGGCGAGCGAACGGA) & D3-TRIC-DN (5'-ATTCCTGACTTCGACCTGA) ^b	0.25 µM 3'95 °C, 35 × (45"95 °C, 45"60 °C, 60"72 °C), 5' 72 °C	peqGOLD HotTaq	2 mg BSA, 5% DMSO

^a Unpublished primer sequence by M. Bálint.

^b Unpublished primer sequence by K. Kjer.

Table 2
Substitution models used in phylogenetic analysis.

Fragment	Unpartitioned	Codon position 1	Codon position 2	Codon position 3
mtCOI5-P	GTR+G+I	TN93+G	TN93+G	HKY
mtCOI3-P	GTR+G+I	TN93+G+I	K2+G	HKY
16SrDNA	T92+G	–	–	–
WG	T92+G	T92	JC+G	JC
CADH	T92+G+I	HKY+G	TN93	T92
D2 (28SnrDNA)	T92+G+I	–	–	–

(Rambaut and Drummond, 2013). Lack of incongruencies among individual data partitions was assumed to indicate homogeneity of phylogenetic signal from each respective partition. Data sets were concatenated for BI if there were no incongruencies.

Bayesian inference of the concatenated dataset (mtCOI5-P+mtCOI3-P+16SmtDNA+CADH+28S) was performed (10×10^6 generations, sampling every 1000th generation) in 4 independent runs with 6 chains each to obtain gene trees. Performance of the B/MCMCMC analyses was scrutinized as stated above.

In addition to a concatenated analysis, a species tree analysis was performed to infer a species tree from separate gene trees using *BEAST (Heled and Drummond, 2010) as implemented in BEAST. Species identity (as determined by classical taxonomy) was used as species trait. Genealogical relationships between species were inferred assuming a Yule speciation tree prior, and running a species tree analysis of 60×10^6 generations, sampling every 10,000th generation. The analysis was run 4× independently to assure topological convergence among runs. *BEAST log files were examined in Tracer v1.6 to assess when runs had reached a stationary phase. Support for tree topologies estimated by *BEAST analysis was assessed by constructing a maximum clade credibility tree running TreeAnnotator v1.8 after discarding the first 2500 trees as burn-in.

Phylogenetic analysis of a purely morphological and a combined dataset was performed in MrBayes 3.2 following Wright and Hillis (2014) to assess adequacy of morphological character coding in species-level phylogenetic analysis of limnephilid Trichoptera. For the morphological data partition, rate of variation was assumed to follow a gamma-shaped distribution. Tree space was sampled every 1000th generation for 5×10^6 generations in 4 parallel runs with 6 chains each for both the morphological and the combined dataset.

Systematic suggestions concerning the subfamily Drusinae will be made based on the results of the species tree analysis only, as this method provides more accurate estimations of phylogenetic trees (Heled and Drummond, 2010).

3. Results

3.1. Phylogenetic inference

3.1.1. Molecular dataset

In all analyses monophyly of Drusinae, and monophyly of larval feeding groups within Drusinae were highly supported.

In the species tree analysis of our data set (Fig. 1) omnivorous shredders (Clade S), filtering carnivores (Clade F) and scraping grazers (Clade G) were well delineated and highly supported ($\geq 95\%$ posterior probability). The topology suggests a dichotomous diversification within Drusinae, with a split between highly supported scraping grazers (Clade G, Fig. 1) and (omnivorous shredders + filtering carnivores) (Clades C and S, Fig. 1). Within the highly supported filtering carnivores, *Cryptothrix nebulicola* is returned as basal sister to a highly supported clade (Clade 1, Fig. 1A) comprising filtering carnivorous *Drusus* spp. Within filtering carnivorous *Drusus* spp., (*D. muelleri* + *D. sp. Valchiusella* I) form a highly supported sister species pair to all other members of the group. In the latter group the tree topology suggests *D. chrysotus* as basal to a highly supported clade (Clade 2, Fig. 1) comprising *D. sp. nov. Valchiusella* II + *D. discolor* + *D. sp. Bucegi* + *D. siveci* + *D. romanicus* + *D. meridionalis* stat. nov. + *D. macedonicus* + *D. krpachi* + *D. malickyi*. Relationships between species are not resolved (i.e., lack strong support) in the latter clade except for (((*D. discolor* + *D. sp. Bucegi*) + *D. siveci*) + *D. romanicus*) and ((*D. macedonicus* + *D. krpachi*) + *D. malickyi*) (Clades 3 and 4, Fig. 1).

A B/MCMCMC gene tree analysis (Fig. A1) based on concatenating the same dataset procured a similar topology with the following differences: (1) Gene tree analysis suggests filtering carnivores (Clade C, Fig. A1) opposed to (scraping grazers + omnivorous shredders) (Clades G and S, Fig. A1). This relationship is, however, not strongly supported; (2) Within the filtering carnivore clade, gene tree analysis suggests an unsupported basal position of *C. nebulicola* as sister to the other, highly supported, carnivorous Drusinae (Clade 1, Fig. A1); (3) the gene tree analysis suggests (*D. sp. nov. Valchiusella* II + *D. chrysotus*) (Clade 2, Fig. A1) as sister to the other filtering carnivorous *Drusus* spp. (Clade 3, Fig. A1); (4) relationships between species were not resolved, except for ((*D. discolor* + *D. sp. Bucegi*) + *D. siveci*) and ((*D. macedonicus* + *D. krpachi*) + *D. malickyi*) (Clades 4 and 5, Fig. A1); (5) gene tree analysis returns *D. meridionalis* stat. nov. unsupported sister to *D. romanicus* (Fig. A1).

3.1.2. Hypothesis testing

We performed hypothesis testing to assess if alternative topologies not displayed in the consensus or maximum credibility trees allow for alternative placements of *C. nebulicola* as well as *D. sp. nov. Valchiusella* II. Alternative composition of clades was assessed from the last 15,000 trees from the species tree analysis using functions from the package 'ape' (Paradis et al., 2004) in a specialized R script (R Core Team, 2013). Alternative topologies concerning the placement of *D. sp. nov. Valchiusella* II and their frequencies are given in Table 3. No alternative topologies concerning the placement of *C. nebulicola* were found in the Bayesian species tree sample.

3.1.3. Morphological and combined datasets

The analysis of a 41 taxa morphological dataset comprising 308 male, female, and larval characters suggested monophyletic Drusinae and monophyletic omnivorous shredders as sister to (filtering carnivores + scraping grazers) (Fig. A2A). However, this relationship is not supported. The phylogenetic analysis of the morphological dataset did not support any of the deeper nodes, and only terminal splits between morphologically highly similar sister species are supported. Filtering carnivores are not supported. Also, internal nodes within filtering carnivores are not supported. The topology suggests the following strongly supported sister species groups: (*D. muelleri* + *D. sp. Valchiusella* I), (*D. krpachi* + *D. malickyi*), and (*D. discolor* + *D. siveci*). Relationships between the other taxa, and the sister species groups are not resolved.

A B/MCMCMC analysis of a concatenated data set comprising 3805 bp molecular and 308 morphological characters suggests strongly supported monophyletic Drusinae, and monophyletic feeding groups within Drusinae (Fig. A2B). Scraping grazers are opposed to an unsupported clade comprising omnivorous shredders + filtering carnivores. Regarding the filtering carnivores, tree topology suggests *C. nebulicola* as basal sister to all other filtering carnivorous *Drusus* spp. Within these (*D. muelleri* + *D. sp. Valchiusella* I) is basal to a highly supported clade comprising ((*D. chrysotus* + *D. sp. nov. Valchiusella* II) + ((*D. discolor* + *D. siveci*) + ((*D. romanicus* + *D. meridionalis* stat. nov.) + ((*D. macedonicus* + *D. krpachi*) + *D. malickyi*))). Internal nodes of the latter clade are highly supported except for the relationship between (*D. romanicus* + *D. meridionalis* stat. nov.) and ((*D. macedonicus* + *D. krpachi*) + *D. malickyi*).

3.2. Taxonomic differentiation of Drusinae feeding groups

3.2.1. Morphological diagnosis of filtering carnivorous Drusinae

The three feeding groups are well defined by unique characters in larval and adult morphology. Filtering carnivores within Drusinae develop a unique combination of morphological

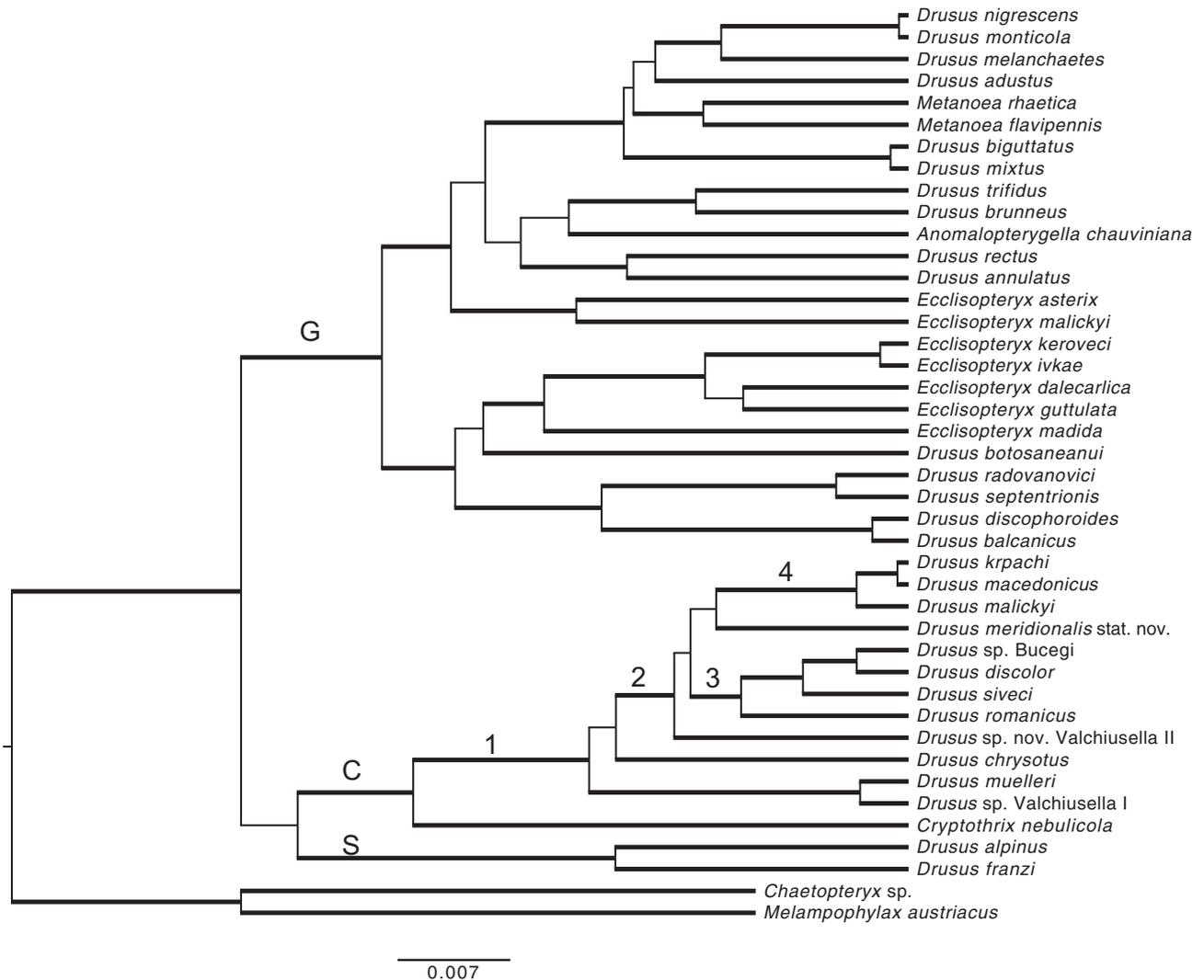


Fig. 1. Results of phylogenetic inference. B/MCMC species tree analysis for 42 species (107 terminal taxa) based on 3805 bp-long sequence from 6 loci (mtCOI5-P + mtCOI3-P + 16SmtDNA + CADH + 28S). Majuscles and numbers at branches indicate clades referred to in the text. For a detailed description of the analytical processes, see Materials and Methods, section *Molecular dataset and phylogenetic inference*. Bold branches indicates posterior probabilities $\geq 95\%$.

Table 3
Alternative topologies concerning the placement of *D. sp. nov. Valchiusella* II.

Hypothesis	Alternative topology	Relative frequency
T1	<i>Drusus chrysotus</i> + <i>D. sp. nov. Valchiusella</i> II	0.01
T2	<i>D. chrysotus</i> + <i>D. sp. nov. Valchiusella</i> II + <i>D. muelleri</i> + <i>D. sp. Valchiusella</i> I	0.01
T3	<i>D. chrysotus</i> + <i>D. malickyi</i> + <i>D. krpachi</i> + <i>D. macedonicus</i> + <i>D. siveci</i> + <i>D. sp. Bucegi</i> + <i>D. romanicus</i> + <i>D. discolor</i> + <i>D. meridionalis</i> stat. nov.	0.19
T4	<i>D. sp. nov. Valchiusella</i> II + <i>D. malickyi</i> + <i>D. krpachi</i> + <i>D. macedonicus</i> + <i>D. meridionalis</i> stat. nov.	8.24
T5	<i>D. sp. nov. Valchiusella</i> II + <i>D. siveci</i> + <i>D. sp. Bucegi</i> + <i>D. romanicus</i> + <i>D. discolor</i>	38.84
T6	<i>D. sp. nov. Valchiusella</i> II + <i>D. siveci</i> + <i>D. sp. Bucegi</i> + <i>D. discolor</i>	2.01
T8	<i>D. sp. nov. Valchiusella</i> II + <i>D. romanicus</i>	7.73
T9	<i>D. sp. nov. Valchiusella</i> II + <i>D. meridionalis</i> stat. nov.	3.15
T10	<i>D. sp. nov. Valchiusella</i> II + <i>D. malickyi</i> + <i>D. krpachi</i> + <i>D. macedonicus</i>	4.49

synapomorphies that define this group: (1) straight hindwing A2 and A3 in males (Fig. 2a) [opposed to differentially modified hindwing venations of omnivorous shredders (Fig. 2b), or scraping grazers (Fig. 2d)]; (2) a posterolateral position of the duct opening of the male abdominal sternite V gland (Fig. 2c) [a character shared with omnivorous shredders, but distinct from the duct opening position in scraping grazers (Fig. 2e)]; (3) an incomplete, i.e., ventrally unfused, anal tube lacking modifications of the tips in females; (4) toothed larval mandibles; (5) absence of carinae, (6) presence of forked lamellae (*sensu* Wiggins, 1998) dorsal the lateral

line, (7) presence of filtering bristles on the first abdominal sternite, and (8) a modified head capsule of larvae (Fig. 3). This combination of adult and larval characters separates the filtering carnivores from representatives of epilithic grazers and omnivorous shredders within Drusinae.

3.2.2. Morphological diagnosis of larvae and adults of filtering carnivorous Drusinae

Larvae of carnivorous Drusinae are easily recognized and differentiated from all other Drusinae larvae by the following

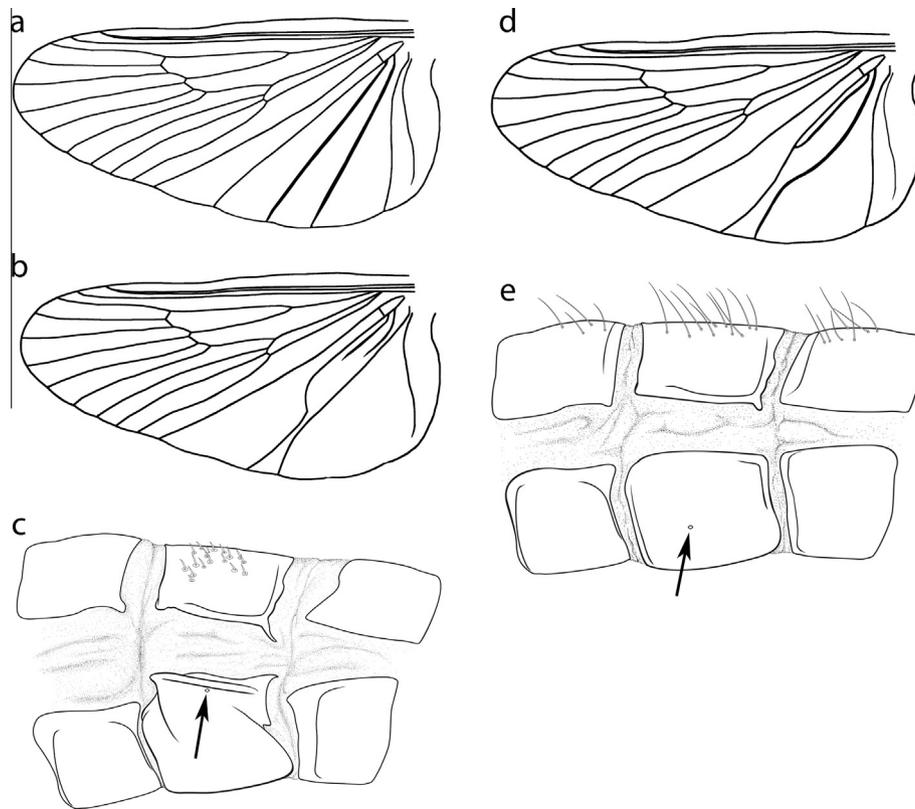


Fig. 2. Adult male apomorphies of Drusinae feeding types: a, generic male hind wing of a filtering carnivorous *Drusus* spp. and *Cryptothrix nebulicola*; b, generic male hindwing of an omnivorous shredder; c, position of the abdominal sternite V gland duct opening in filtering carnivorous and omnivorous shredder Drusinae (arrow), and formation of the male abdominal sternite V in *D. chrysotus* of *C. nebulicola*; d, generic male hindwing of a scraping grazer; e, position of the male abdominal sternite V gland duct opening and formation of male abdominal sternite V in scraping grazer Drusinae (*D. biguttatus*). Venation patterns depicted in A, and the lateral position of the male abdominal sternite V gland as illustrated in C are autapomorphic characters of filtering carnivorous Drusinae. Del. Vitecek.

combination of characters: (1) larval mandibles with terminal teeth; (2) absence of carinae; (3) presence of filtering bristles on legs and the first abdominal sternite; (4) a modified head capsule that is characterized by a sharp bend of the frontoclypeus between the eye and the anteclypeus in lateral view, optionally with modified setae or a woolly layer of hair (Table 4).

Species-specific formations of the head capsule allow grouping of larvae of filtering carnivorous Drusinae. Based on the presence or absence of flocculent hair or setae, three distinct groups can be distinguished: (1) *C. nebulicola*, *D. muelleri*, *D. sp.* Valchiusella I, *D. chrysotus*; (2) *D. sp. nov.* Valchiusella II; (3) *D. discolor*, *D. siveci*, *D. meridionalis* stat. nov., *D. romanicus*, *D. macedonicus*, *D. krpachi*. Members of the first group exhibit a hair- and setaeless, moderately modified head capsule, that is either flattened or concave in frontal view (Fig. 4a–c). The only member of the second group exhibits a distinct station, a unique morphological feature among Drusinae (Fig. 3i). Members of the third group exhibit flocculent hair that covers head and pronotum, and a protruding median bulge of the frontoclypeus with species-specific ramifications in frontal view (Fig. 4d–i). Within this group, ramification complexity is lowest in *D. macedonicus*, which exhibits only 2 ventrolateral protrusions of the median bulge (Fig. 4i). *Drusus krpachi* and *D. meridionalis* stat. nov. each exhibit 6 protrusions of the median bulge, all of which extend to the border of the frontoclypeus in *D. krpachi*, but only two (ventrolateral and dorsal) in *D. meridionalis* stat. nov. (Fig. 4f,h). The other species of group (3) exhibit 8 protrusions of the median bulge (Fig. 4c,e,g). Additionally, *D. romanicus* and *D. meridionalis* stat. nov. exhibit indentations of the parietalia in frontal view (Fig. 4f and g).

Larvae of *D. malickyi* and *D. sp.* Bucegi are not known, but putatively also exhibit flocculent hair on heads and pronota as well as species-specific formations of the frontoclypeus.

Morphologically, *D. krpachi* and *D. malickyi* males are most similar to *D. macedonicus*, but differ in the shapes of the superior, intermediate and inferior appendages (Vitecek et al., 2015b). Further, all filtering carnivorous Drusinae with the exception of *D. discolor* exhibit discrete distribution patterns (Fig. 5).

Detailed (re-)descriptions of filtering carnivorous Drusinae larvae, including a key to the last larval stages, are provided in the supplementary material (Appendix C).

4. Discussion

4.1. Phylogenetic relationships and taxonomy of the filtering carnivores

Larval feeding groups were returned as monophyletic in the current analysis, confirming prior results from Pauls et al. (2008). We also found the same pattern of paraphyly concerning the genera *Drusus* and *Ecclisopteryx*: both genera were found in several clades in all phylogenetic trees. Interestingly, morphological similarities of larvae in the grazer clade were again found to be predictive value for phylogenetic grouping of clades (Waringer et al., 2015).

All analyses support a monophyletic filtering carnivore clade with *C. nebulicola* as a basal member of the filtering carnivores. While not significantly supported in all cases, all analyses suggest a close relationship of *C. nebulicola* to the other filtering

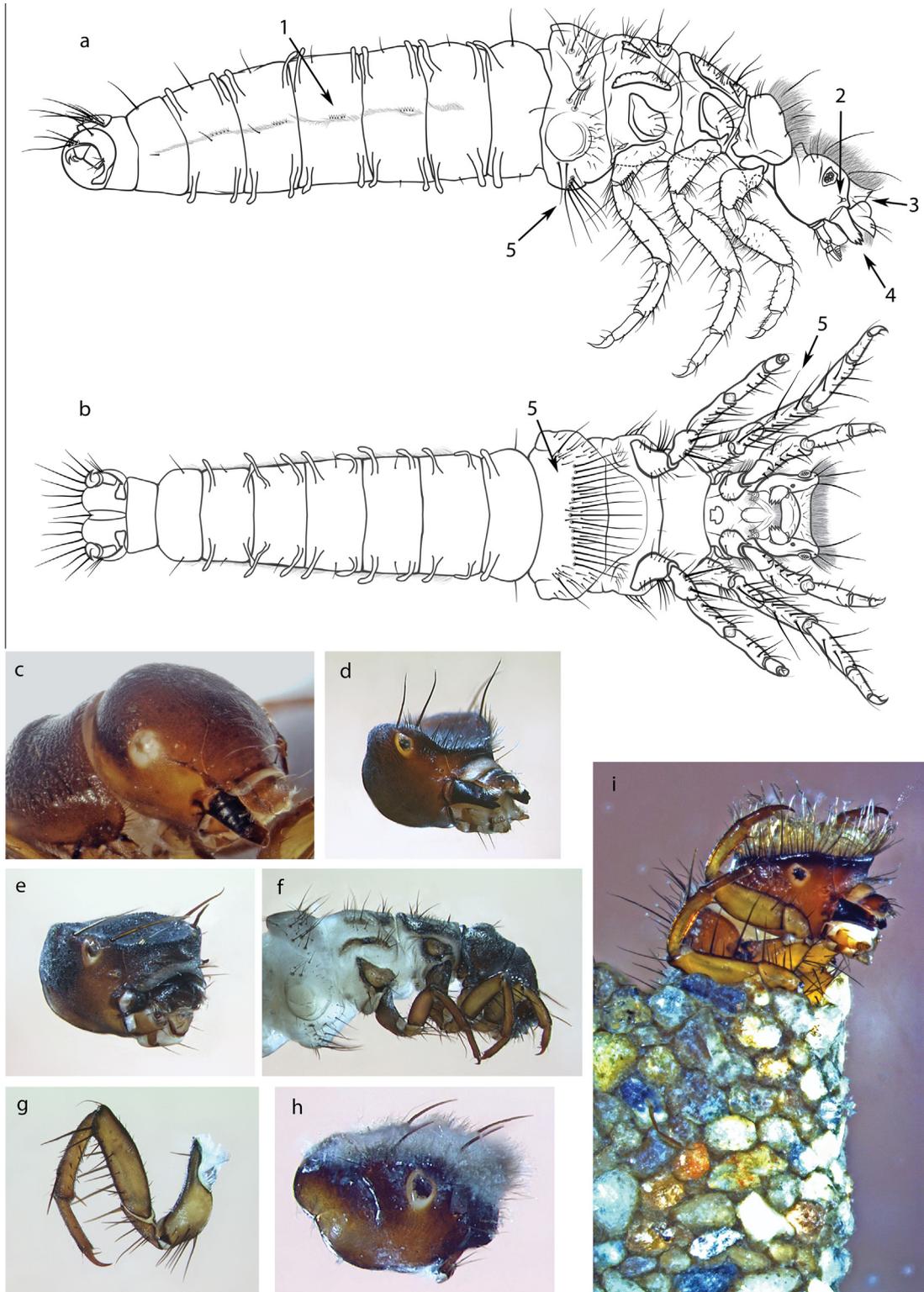


Fig. 3. Characteristics of Drusinae larvae. a, generic habitus (lateral view) of a filtering carnivorous *Drusus* spp. larva developing flocculent hair on head and prothorax (e.g., *D. discolor*), arrows 1–5 indicate larval autapomorphies of this group (1, forked setae dorsal the lateral line; 2, antennae not based on carinae; 3, a modified head capsule with a sharp bend of the frontoclypeus; 4, toothed mandibles; 5, filtering bristles on the 1st abdominal sternite and legs); b, generic habitus (ventral view) of a filtering carnivorous *Drusus* spp. larva developing flocculent hair on head and prothorax, arrows 5 indicate filtering bristles on the 1st abdominal sternite and legs; c, head and prothorax of a scraping grazer *Drusus* spp. (*D. serbicus*) showing the typical rounded head capsule, carinae and toothless mandibles; d, head of *D. chrysotus* showing the typical modification of the head capsule of this species; e, head of *D. muelleri* showing the typical modification of the head capsule of this species; f, lateral view of the thorax and head of *D. muelleri* showing the typical filtering bristles on the first abdominal sternite; g, Mid-leg of *D. muelleri* showing the typical filtering bristles at legs of filtering carnivorous Drusinae; h, lateral view of the head capsule of *D. discolor*, showing the flocculent hair as typical for some filtering carnivore *Drusus* spp.; i, lateral view of *Drusus* sp. nov. *Valchiusella* II, showing the typical cephalic setation of this taxon. Illustration a–b del. Vitecek; Photographs d–h by W. Lechthaler, Vienna.

Table 4
Synopsis of characters separating 5th instar Drusinae larvae according to feeding types.

Mandibles with terminal teeth	Legs & first abdominal sternite filter bristles	Forked lamellae dorsal the lateral line	Antennae located on carinae	Head	Head covered in setae	Head covered in flocculent hair	
No	Absent	Absent	Yes (Fig. 2c)	Round (Fig. 2c)			Drusinae grazer
Yes (Fig. 2a, c)	Absent	Present (Fig. 3a, arrow 1)	No	Round			<i>Drusus franzi</i> / <i>D. alpinus</i> ^b
Yes	Present (Fig. 3a, b, and j)	Present	No	Modified ^a (Fig. 3a, c, d, e, and i)	x (Fig. 3i)		<i>Drusus</i> sp. nov.
Yes	Present	Present	No	Modified			Valchiusellae II
Yes	Present	Present	No	Modified		x (Fig. 3h)	Carnivorous Drusinae 1 Carnivorous Drusinae 2

^a In lateral view with a distinct step between the labrum and the frontoclypeus surface.

^b Omnivorous shredders.

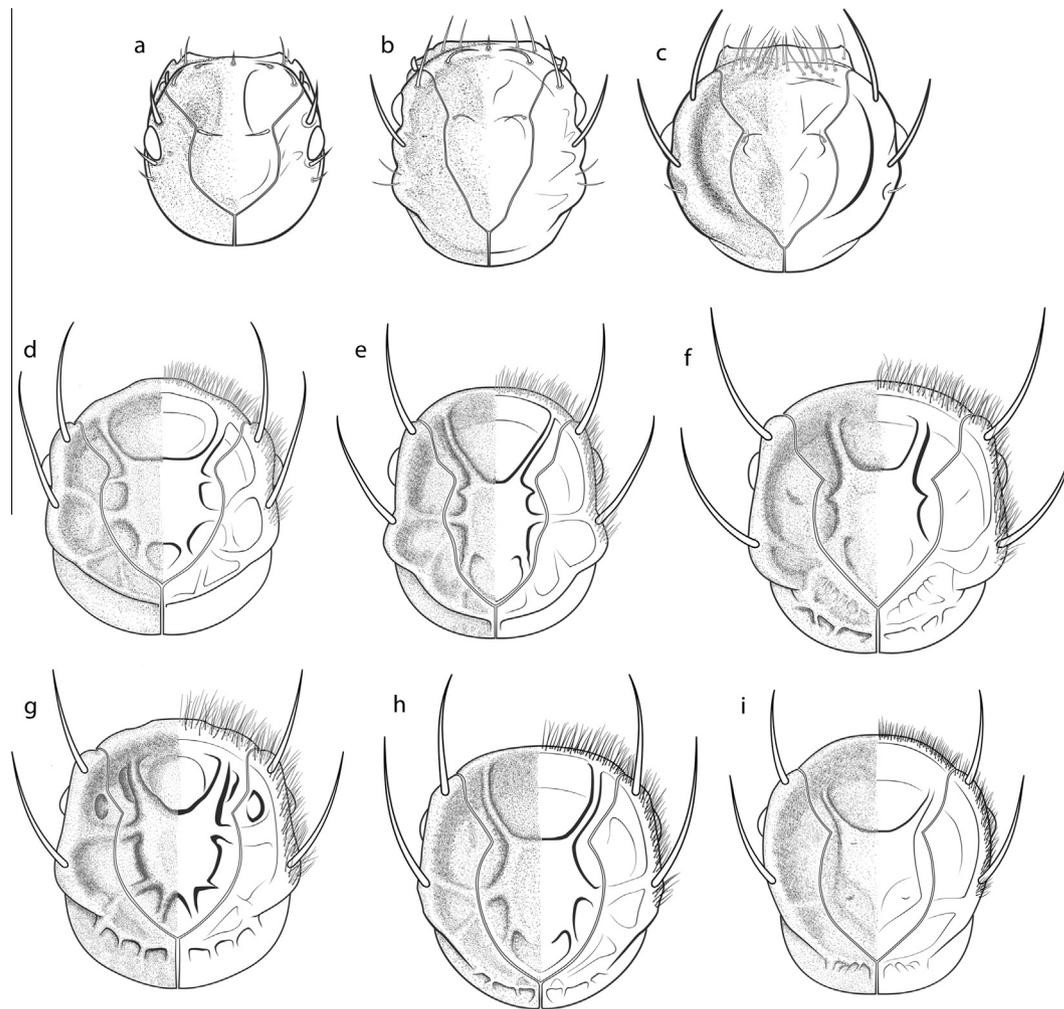


Fig. 4. Frontal view of larval heads of filtering carnivorous Drusinae illustrating the species-specific head and frontoclypeus morphology. a, *Cryptothrix nebulicola*; b, *Drusus muelleri* and *D. sp. Valchiusella* I; c, *D. chrysotus*; d, *D. discolor*; e, *D. siveci*; f, *D. meridionalis* stat. nov.; g, *D. romanicus*; h, *D. krpachi*; i, *D. macedonicus*. Heads rotated, vertex to bottom. Left halves of heads stippled to provide a slightly more realistic view, right halves simplified line drawings. Del. Vitecek.

carnivorous Drusinae, and exclusion of *C. nebulicola* from the other main clades (shredders and grazers). Larvae of *C. nebulicola* were found to behave as carnivores via gut content analysis (Bohle, 1987) and to develop a setation pattern on legs and the first abdominal sternite remarkably similar to all other filtering carnivores. Male *C. nebulicola* share hind wing and abdominal sternite V characters with all members of the filtering carnivores. Thus, morphological, phylogenetic and ecological data suggests to group *C. nebulicola* with the other filtering carnivores within Drusinae.

Therefore, the validity of the genus is questionable. The logical consequence would be to omit *Cryptothrix* and to include the species in *Drusus*, applying the principle of priority. However, the genus *Drusus* is paraphyletic (Pauls et al., 2008; this study). Thus, nomenclatural steps regarding generic status should only be undertaken upon revision of the entire subfamily.

Species tree analysis suggests *D. meridionalis* stat. nov. as sister to *D. macedonicus* + *D. krpachi* + *D. malickyi*, whereas gene tree analysis suggests this species as sister to *D. romanicus*. However,

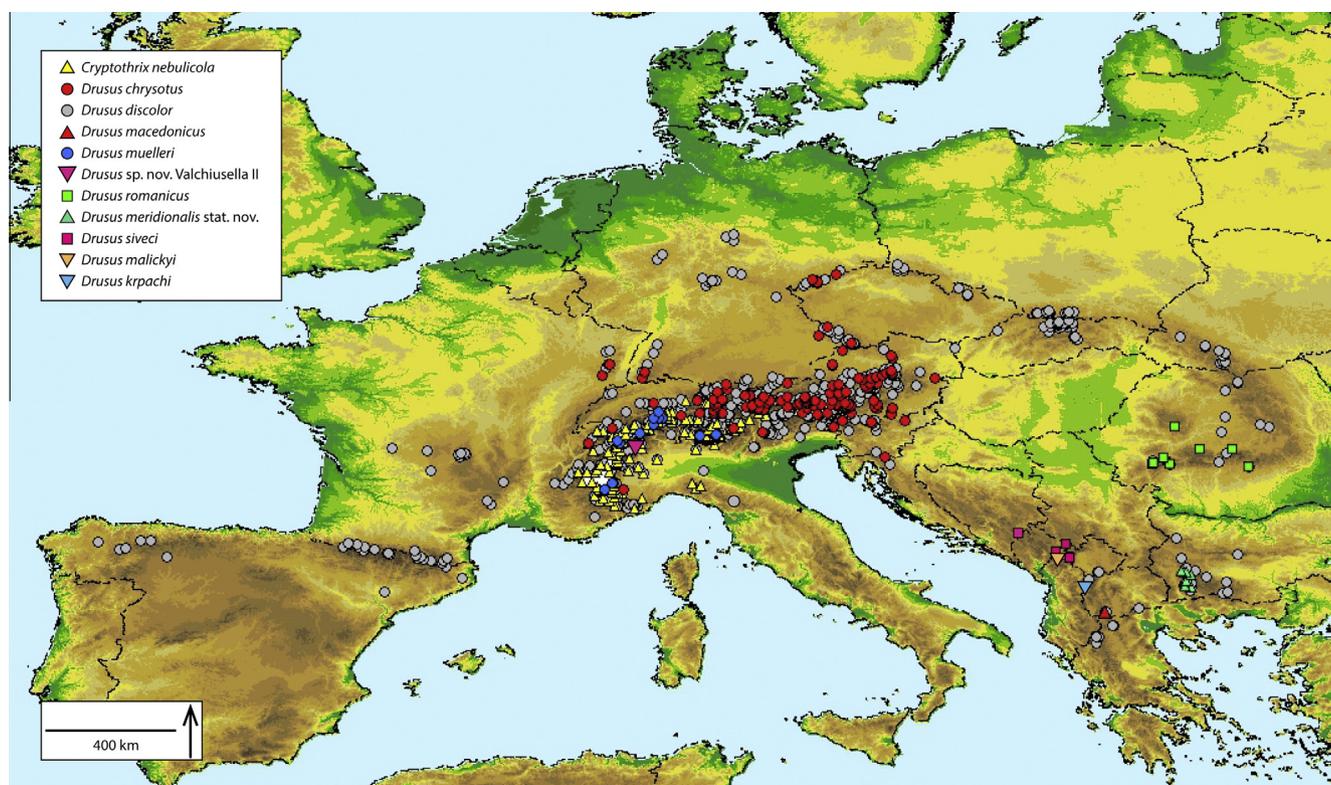


Fig. 5. Distribution of filtering carnivorous Drusinae. Within filtering carnivorous Drusinae, several regional and micro-endemics occur concentrated in the Carpathians and the Balkans (*D. muelleri*, *D. romanicus*, *D. siveci*, *D. macedonicus*, *D. meridionalis* stat. nov., *D. krpachi*, *D. malickyi*), opposed to widespread species (*D. discolor*, *D. chrysotus*, and *C. nebulicola*). *Drusus* sp. nov. Valchiusella II is a micro-endemic of the Western Alps.

in both analyses *D. meridionalis* stat. nov. is well delineated from *D. romanicus*. The two taxa are also clearly distinguishable by morphological characteristics of the male terminalia and the larval frontoclypeus. Together our results clearly support raising *D. meridionalis* stat. nov. to species rank.

Concerning the status of the putative new species, phylogenetic inference strongly supported all potentially new species (*D. sp. Valchiusella* I, *D. sp. nov. Valchiusella* II, *D. sp. Bucegi*), as well as the most recently described species *D. krpachi* and *D. malickyi* (Vitecek et al., 2015b). The other potential new species are difficult to address, as they either exhibit only minute differences in the male terminalia to already known species, or do not differ in larval morphology (Appendix C). We therefore refrain from describing them based on the current knowledge.

Of particular interest is *D. sp. Bucegi*, as this putative new species is represented by a single individual that is morphologically close to *D. romanicus*, which is also corroborated by nuclear markers, whereas mitochondrial markers indicate a close relationship to *D. discolor*. Potentially, this individual might be the result of a hybridization event, as both *D. discolor* and *D. romanicus* were present at the collection location and historic introgression was previously inferred among the species (Pauls et al., 2009). Hybridization in caddisflies was demonstrated to produce adult males with terminalia sharing characters of both parent species (Malicky and Pauls, 2012). Alternatively, infection with *Wolbachia* sp. (cf. Hurst and Jiggins, 2005; Kondo et al., 2002) or incomplete lineage sorting (cf. Degnan and Rosenberg, 2006; Pollard et al., 2006) might result in an individual exhibiting similar characteristics. To clarify the situation, we would need to analyze more specimens, assess infestation levels of *Wolbachia* sp., and ideally perform hybridization experiments in order to identify factors that could lead to a specimen exhibiting such characteristics. This, however, is beyond the scope of this study and we therefore refrain from a formal description of this form at this time.

Both *D. sp. Valchiusella* I and *D. sp. nov. Valchiusella* II are well delineated and highly supported by both gene tree and species tree analysis. However, differences of specimens between *D. sp. Valchiusella* I and *D. muelleri* are minute or absent (cf. Vitecek et al., 2015b; Appendix C). Also, the type locality of the ‘lost’ species *D. chapmani* is in the vicinity of Locarno, Switzerland (Malicky, 2005; McLachlan, 1901) close to where we found *D. sp. Valchiusella* I. This further complicates taxonomic decisions. Currently, we refrain from describing this species without more comparative material from the whole range of *D. muelleri*.

The outstanding larval habitus of *D. sp. nov. Valchiusella* II would justify the description of this taxon. However, we consider it odd to describe a species based on the larva alone, and thus refrain from the description of this species.

4.2. Performance of morphological data in phylogenetic reconstruction of Drusinae

Phylogenetic reconstruction of Drusinae based exclusively on morphological data allows species delimitation and identification of sister species but fails to resolve deeper nodes and the relationships in the group. This is most likely because we attempted to reconstruct phylogenetic relationships within Drusinae on species level. A reduced dataset encompassing exclusively characters that distinguish morphologically similar lineages will most likely produce supported deeper nodes, but fail to resolve final taxa. Further, selection and coding of species-level characters for the morphological dataset is delicate, as species (following the morphological species concept (Wheeler and Meier, 2000)) are defined by the stability of their unique combination of morphological characters. Larval characters in particular might be misleading, as the risk of assessing adaptations to certain ecological constraints rather than phylogenetic signal is prevalent. Adult genital characters might not be susceptible to ecological constraints, but are difficult to assess

as they have been shown to vary over populations (Kučinić et al., 2013; Malicky, 2004; but see Oláh et al., 2012, 2013, 2014). Overall, this demonstrates the significance of molecular data and inaptitude of a purely morphological approach for species-level reconstruction of Drusinae phylogeny.

4.3. Significance of larval head modifications in filtering carnivorous Drusinae

Larval heads seem to follow a certain pattern of development within the filtering carnivore clade. Tree topology of species tree analysis suggests a basal position of *C. nebulicola*, (*D. muelleri* + *D. sp. Valchiusella I*) and *D. chrysotus* in the carnivore clade, species which share a hairless, and moderately modified (either flattened or concave) larval head capsule. These species are followed by a strongly supported clade comprising all other carnivorous Drusinae in which larvae develop a modified setation or flocculent hair on their strongly modified head capsule. Of particular interest is *D. sp. nov. Valchiusella II*, as this taxon seemingly is basal to all species in which larvae develop flocculent head hair in species tree analysis. Therefore, this taxon might represent a 'missing link' concerning hair development in carnivorous Drusinae, given the intriguing head setation and absence of flocculent hair. At present, however, although our testing of alternative topologies did not find evidence to convincingly reject the basal position (Table 3), the topologies are not sufficiently well resolved to be certain.

Interestingly, within the clade where species that develop cephalic hairs, a pattern of increasing diversification of frontoclypeus structures is evident. However, no assumption on the direction of diversification can be made, since structural complexity increases in a seemingly random manner within the clade. Nevertheless, species-specificity of frontoclypeus structures allows identification of larval stages of *D. discolor*, *D. siveci*, *D. romanicus*, *D. meridionalis* stat. nov., *D. macedonicus*, and *D. krpachi* that hitherto were indistinguishable from another, extending the number of identifiable Drusinae larvae from 48 to 53 (Botosaneanu, 1959; Previšić et al., 2014a; Vitecek et al., 2015a; Waringer et al., 2010; Waringer and Graf, 2011). Considering the feeding habits of filtering carnivorous Drusinae (Bohle, 1983, 1987; Graf et al., 2008), we assume an ecological significance of the distinct and diverse larval head morphology that developed in this group. The flocculent hair observed in this group might, e.g. mimic a certain type of substrate, thus attracting potential prey organisms (Bohle, 1983). Additionally or alternatively, the anatomy of the larval heads may aid in maintaining the stereotypical filtering posture first described in *D. discolor* (Bohle, 1983; Graf, unpubl. data). Interestingly, the most simple frontoclypeus observed in filtering carnivorous Drusinae is developed by *D. macedonicus* larvae which live in streamlets under rocks and within moss-mats, whereas species with highly ramified frontoclypeus structures (such as *D. discolor*, *D. romanicus*) inhabit fast flowing microhabitats (Graf, unpubl. data). Also, all filtering carnivores (except *C. nebulicola*) develop 4 distinct cephalic setae of presumably sensory function that might aid in exploration and detection of an ideal hydrological niche. Discreteness of hydraulic microhabitats was demonstrated in *Brachycentrus occidentalis*, a species that employs a similar filtering mode as the carnivorous Drusinae (Wetmore and Mackay, 1990). Therefore, we assume that the degree of ramification represents adaptation to a certain niche, and is directionally proportional to hydraulics at the most often used microhabitat in carnivorous Drusinae. The modification of the head capsule and frontoclypeus may thus allow these taxa to optimize their foraging strategies under specific flow conditions (Wallace and Merritt, 1980). The preference for optimal hydrological conditions may explain why some of these species like *D. discolor* (Graf, 1997; Lavandier, 1992) or *D. muelleri* (Graf et al., 2005) accumulate in high densities similar to other non-annulipalpan

Trichoptera (e.g., *Allogamus auricollis*: Alp, 2006; Geddes, 1981; Graf et al., 1992; Reichholf, 1995; *Brachycentrus maculatus*: Nielsen, 1943; *Brachycentrus subnubilus*: Burmeister, 1991; Gunn, 1985; Majecki et al., 1997; *Limnacentropus himalayanus*: Wiggins, 1969).

4.4. Ecology, endemism and potential threats of filtering carnivorous Drusinae

The findings of the present study highlight the significance of European mountain ranges as centres of freshwater biodiversity. Ecologically, larvae of all filtering carnivores exploit similar microhabitats: fast-flowing stretches in hypocrenal to metarhithral regions of cold mountain brooks (Graf et al., 2008; Graf and Schmidt-Kloiber, 2011). The disjunct distribution of adequate habitats due, e.g. to their prevalence at higher elevations (e.g. Pauls et al., 2006, 2009) or as a consequence of regional karstification (Previšić et al., 2009, 2014b)), and a putatively low dispersal capacity (e.g. Müller-Peddinghaus, 2011) presumably facilitated allopatric speciation of filtering carnivorous Drusinae even at small geographic scale leading to the present distribution patterns. Distribution patterns of filtering carnivorous Drusinae can be grouped into widespread species (*D. discolor*, *D. chrysotus*, *C. nebulicola*), regional endemics (*D. muelleri*, *D. romanicus*), and micro-endemics (*D. siveci*, *D. macedonicus*, *D. meridionalis* stat. nov., *D. krpachi*, *D. malickyi*, *D. sp. nov. Valchiusella II*) (Fig. 5). The collection of the micro-endemic *D. sp. nov. Valchiusella II* is remarkable as it demonstrates the presence of yet unknown species in a supposedly well-surveyed area, the Western Alps. The recent description of *D. krpachi* and *D. malickyi* increases the number of endemic Western Balkan Drusinae from 30 to 32, out of a total of now 40 Drusinae species known from this region (Graf et al., 2008; Graf and Schmidt-Kloiber, 2011; Oláh, 2010, 2011; Oláh and Kovács, 2013; Previšić et al., 2014a; Vitecek et al., 2015a, 2015b; Ibrahim, pers. comm.).

Endemic and micro-endemic freshwater species are particularly vulnerable to climate change and (anthropogenic) habitat alteration (Bálint et al., 2011; Conti et al., 2014; Hering et al., 2009; Tierno de Figueroa et al., 2010). Currently, construction of hydropower dams poses one of the greatest threats to freshwater biodiversity, especially in emerging economies (Zarfl et al., 2014). Many regional and micro-endemic Drusinae occur on the territory of such emerging economies, and are particularly threatened by increasing hydropower plant construction in the Western Balkans (Freyhof, 2012; Schwarz, 2012). Small hydropower plants fed by damming of small cold-water mountain streams are especially problematic for highly diverse highland caddisflies such as Drusinae (Previšić et al., 2014a; Vitecek et al., 2015b) or *Chaetopteryx* species (Kučinić et al., 2013). The recent description of *D. krpachi* and *D. malickyi* (Vitecek et al., 2015b) thus accentuates the exigency of biodiversity research in Eastern and Southern Europe in combination with the instigation of adequate conservation measures. Also, continuation of the presently prevailing energy policy will likely result in the loss of known and unknown biodiversity.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.07.019>.

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