

# Grazers, shredders and filtering carnivores—The evolution of feeding ecology in Drusinae (Trichoptera: Limnephilidae): Insights from a molecular phylogeny

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## Abstract

We examined the phylogenetic relationships between species and genera within the caddisfly subfamily Drusinae (Trichoptera: Limnephilidae) using sequence data from two mitochondrial loci (*cytochrome oxidase 1*, *large subunit rRNA*) and one nuclear gene (*wingless*). Sequence data were analysed for 28 species from five genera from the subfamily. We analysed individual and combined data sets using a Bayesian Markov Chain Monte Carlo and a maximum parsimony approach and compared the performance of each partition for resolving phylogenetic relationships at this level. In terms of resolution and phylogenetic utility *wingless* outperformed the two mitochondrial gene partitions.

Using both Shimodaira-Hasegawa and expected likelihood weights tests we tested several hypotheses of relationships previously inferred based on adult morphological characters. The data did not support the generic concept, or many previously proposed species groupings, based on adult morphology. In contrast, the molecular data correlated with the morphology and feeding ecology of larvae. Using Bayesian ancestral character state reconstructions we inferred the evolution of feeding ecology and relevant larval morphological characters. Our analyses showed that within the subfamily Drusinae two derived feeding types evolved. One of these—grazing epilithic algae—is otherwise unusual in the Limnephilidae and may have promoted the high degree of diversity in the Drusinae.

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## 1. Introduction

Trichoptera (caddisflies), is the 7th largest insect order with over 13,000 described species, and the largest order of insects whose members are almost exclusively aquatic. It is the sister taxon to Lepidoptera, the moths and butterflies, but among other characters differs in that most larvae are

aquatic. In contrast to the majority of other insects the larval stage of caddisflies is the most conspicuous and familiar to the non-entomologist because of the intricate portable cases and delicate silken nets the larvae construct (Wiggins, 2004). Like Lepidoptera caterpillars, Trichoptera larvae produce silk from the labium, and it is probably due to the diverse ways in which silk is used to exploit various aquatic niches that the order owes its evolutionary success (Mackay and Wiggins, 1979). The larvae of Trichoptera are found in all types of freshwater and even brackish aquatic habitats, but are especially abundant in rivers and streams. Caddisflies have a diverse feeding ecology. This includes shredding of

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leaf litter detritus, gathering fine organic particles, sucking algal cells, scraping periphyton off exposed surfaces, filtering the water of suspended food, preying on other aquatic invertebrates, or feeding on living green plants or algae. Through these diverse feeding strategies, caddisflies are fundamental participants in nutrient dynamics and energy flow in aquatic ecosystems (Resh and Rosenberg, 1984; Wallace and Webster, 1996). Despite their ecological importance and the diversity of feeding types, little is known about the evolution of their feeding ecology.

Biological monitoring of water quality depends heavily on caddisflies, especially in North America, Europe and Australia (Wright et al., 1984, 2000; Smith et al., 1999; Barbour and Yoder, 2000; Graf et al., 2002; Hering et al., 2006). The different sensitivity of caddisfly species is widely used for monitoring pollutants and other types of environmental disturbance (Rosenberg and Resh, 1993; Dohet, 2002), making caddisflies primary indicator taxa in monitoring water quality together with mayflies and stoneflies (Buffagni et al., 2006; Moog et al., 2004).

Despite the large number of species known, their unique and diverse life histories, and the important role caddisflies play in stream assessment, our knowledge about the evolutionary history of the group is limited (Morse, 1997). Few molecular phylogenies exist for caddisflies (Morse, 1997), and most of these studies have focussed on resolving the deeper level relationships between suborders or families (Kjer et al., 2001, 2002; Geerts et al., 2001; Dreesmann and Wichard, 2002). Few investigations have examined within family relationships (Myers and Sperling, 2002; Geraci et al., 2005) or intraspecific populations structure (Myers et al., 2001; Wilcock et al., 2001, 2003; Pauls et al., 2006). Thus, while the deep relationships and intraspecific population structure are becoming better understood, there is a significant lack of molecular based studies looking at interspecific relationships and genus level diversification in caddisflies.

The Drusinae Banks, 1916 is a subfamily of the Limnephilidae Kolenati, 1848 (Trichoptera). The group is restricted in its range to Eurasian mountain ranges from the Caucasus in the East to the Iberian Peninsula in the South-West. Most Drusinae are highland insects with a preference for cold running water. Despite its small range and the relatively narrow ecological niche, the group is highly diversified with 87 species known to date. Three quarters of the Drusinae are endemics limited to a single or very few mountain ranges, making the group an ideal model for studying recent evolution, diversification and speciation.

The last major treatment of the group was conducted by Schmid (1956). In his seminal work he described and characterised seven genera and six species groups within genus *Drusus* Stephens, 1837 based on adult morphology of the 42 Drusinae taxa known at the time. Based on character distribution in the group, Schmid (1956) proposed a phylogeny of the subfamily (Fig. 1). Since then a new genus and many new species have been described, more than dou-

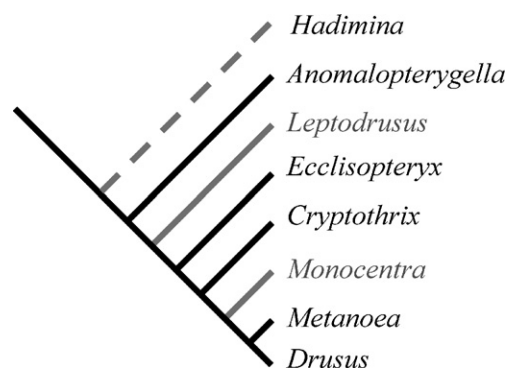


Fig. 1. Phylogenetic classification proposed by Schmid (1956) and Sipahiler (2002). Dotted branch shows inferred basal position of *Hadimina* following Sipahiler (2002). Grey branches indicate monotypic genera we could not sample for this study.

bling the total number of Drusinae known to 87 (Sipahiler, 2002; Malicky, 2005). Marinkovic-Gospodnetic (1976), Kumanski (1988) and Sipahiler (1999) have provided further summaries of species groupings, taking into account some newer species descriptions. Also, several recent studies have focussed on describing larval stages of Drusinae and identifying a variety of feeding strategies within the subfamily (Graf et al., 2005; Waringer et al., in press-a, 2007, in press-b). Taken together these studies provide an ideal basis for testing hypotheses on relationships within and between genera, and trait evolution in these organisms.

Our study has three main objectives. First, we want to provide the first multi-gene molecular phylogeny in caddisflies at the level of genera and species. Using a molecular phylogeny we will examine the evolutionary relationships within the Limnephilidae subfamily Drusinae and test existing hypotheses on the generic concept and specific relationships. We will also examine the phylogenetic utility of adult and larval morphology. Second, we want to reconstruct the evolution of feeding types in the subfamily using a coupled Bayesian/Maximum Likelihood approach (Pagel and Lutzoni, 2002) that allows more realistic reconstructions than maximum parsimony based methods. Third, we want to compare and evaluate the utility of three gene regions for reconstructing evolutionary relationships within caddisfly families and within and between genera to provide a basis for future phylogenetic studies of caddisflies. One of these regions (mitochondrial cytochrome oxidase I) was previously used for studying intraspecific population structure and within family relationships. To date, 16S rRNA has not been tested in an extensive framework, and the third gene (wingless) has not been used in previous studies of caddisflies.

## 2. Materials and methods

### 2.1. Taxon sampling

A data matrix containing 53 specimens from 28 species of Drusinae was constructed with sequences from

mitochondrial cytochrome oxidase I (mtCOI, 498 bp), 16S rRNA (mtLSU, 506 bp) and nuclear wingless (nuWG, 472 bp) genes (Table 1). The data for this study were generated at the Research Institute Senckenberg (RIS), at the Pritzker Laboratory for Molecular Systematics and Evolution at The Field Museum (FM-PL) and by the Nano+Bio Zentrum Kaiserslautern University (NBZ). Five of the currently eight recognised genera in the Drusinae (Schmid, 1956; Sipahiler, 2002; Table 2) were included. We have not been able to get fresh material of the monotypic genera *Hadimina*, *Leptodrusus*, and *Monocentra*. Three species of one other Limnephilidae subfamily (Chaetopterygini, Stenophylacini) were included as outgroups.

Most of the sequence data used in this study was generated from adult male specimens. Females or larvae were only used (a) when sequences were also available from adult males, or (b) in cases where no adult males were available, only for those taxa where female or larval stages are clearly recognised and easily delimited from other species (e.g., *Drusus chrysotus* larvae). The material for this study was collected by the authors and several other colleagues (Table 1) using water nets, sweeping nets or light traps. The nomenclature follows Malicky (2005, 2007).

## 2.2. Molecular techniques

Whole genomic DNA was extracted from the abdomen or 2 legs from adults or larvae using the DNEasy Tissue or QIAmp Micro Kits (both Qiagen) following the manufacturer's protocol. Cleared genitalia, remaining legs, head, thorax and wings were kept as specimen vouchers.

PCR mixes and procedures varied for each target region. PCR primers and procedures for mtCOI are described in Pauls et al. (2006). PCR primers were LR-J-12887 (5'-CGCCTGTTTATCAAAAACAT-3') and LR-N-13398 (5'-CCGGTCTGAACTCAGATCACGT-3') (both Simon et al., 1994) for mtLSU, and Wingnut1a (5'-GAAATGCGNCARGARTGYAA-3') and Wingnut3 (5'-ACYTCRCARCACCARTGRAA-3') (Goldstein, unpublished) for nuWG. PCR mixes for mtLSU (New England Biolabs) contained 2.5 µl 10× standard PCR buffer, 0.2 µM dNTPs, 0.8 µM of each PCR primer, 1 mM MgCl<sub>2</sub>, 5 µg BSA, 1 U *Taq*-polymerase and 4 µl undiluted DNA in 25 µl. The amplification program included 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 80 s. PCR mixes for nuWG (Roche) contained 2.5 µl 10× standard PCR buffer, 0.2 µM dNTPs, 1.6 µM of each PCR primer, 1.5 mM MgCl<sub>2</sub>, 2.5 µl BSA (New England Biolabs), 0.4 µl *Taq*-polymerase and 4 µl undiluted DNA in 25 µl. The amplification program included 35 cycles of 95 °C for 45 s, 60 °C for 45 s and 72 °C for 90 s.

Purified PCR products were sequenced using the PCR primers on a ABI 3730XL capillary sequencer (Applied Biosystems) at FM-PL or an ABI 3100 at NBZ. Sequences were edited in Seqman II 4.0 (DNAStar).

## 2.3. Phylogenetic analysis

Sequences were aligned using Clustal W (Thompson et al., 1994) as implemented in BioEdit (Hall, 1999) and manually edited. All individual and combined data sets were analysed using Bayesian (B/MCMC) and maximum parsimony (MP) methods. Analyses were performed in Paup\* 4.0b10 (Swofford, 2001) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with gaps treated as missing data.

For all MP analyses, a heuristic search with 100 random taxon addition replicates was conducted with TBR branch swapping and MULTREES option in effect, MAXTREES set to autoincrease, equally weighted characters and gaps treated as missing data. Robustness of individual branches was estimated by maximum parsimony bootstrap proportions (BP) (Felsenstein, 1985) following Sung et al. (2007). Non-parametric bootstrap support values were obtained with 100 bootstrap replicates, each with five replicates of random sequence addition, TBR branch swapping, MULTREES off and with a maximum of two trees saved per replicate. To assess homoplasy levels, consistency index (CI), retention index (RI) and rescaled consistency (RC) index (Farris, 1989) were calculated from each parsimony search.

Bayesian phylogenetic analyses were performed using the Markov chain Monte Carlo method (B/MCMC) and the model selected for each partition using Modeltest version 3.5 (Posada and Crandall, 1998) for single gene and combined data set analyses. Two parallel analyses with 12 chains each were run for  $2 \times 10^6$  generations for single gene and two-gene partition data sets and  $5 \times 10^6$  generations for the three gene combined data set. Trees were sampled every 100th generation. The first  $1 \times 10^6$  generations were discarded as burn-in. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (<http://tree.bio.ed.ac.uk/software/tracer/>) to ensure that stationarity was achieved after the first  $1 \times 10^6$  generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium plateau. From the remaining trees a majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade.

We used a Bayesian approach to examine the heterogeneity in phylogenetic signal among the data partitions (Buckley et al., 2002). For the separate genes and the concatenated analyses, the set of topologies reaching 0.95 posterior probabilities were estimated. The combined analysis topology was then examined for conflict with the 0.95 posterior intervals of the single gene analyses. If no conflict was evident, it was assumed that the two data sets were congruent and could be combined.

## 2.4. Hypothesis testing

We used the Shimodaira and Hasegawa (1999) (SH) test and expected likelihood weights test (ELW) (Strimmer and

Table 1  
Material used in this study

Taxon	Locality	Stage/ sex	mtCOI	mtLSU	nuWG	Collector
<i>Anomalopterygella chauiniana</i>	D, Spessart, Bieber above Roszbach, 13.07.2004	L	EU215079	EU215174	EU215121	Pauls & Sundermann
<i>Chaetopterygopsis maclachlani</i>	D, Black Forest, Brotenaubach, 04.05.2003	L	EU215081	EU215176	EU215123	Pauls
<i>Chaetopteryx rugulosa</i>	AT, Fischbacher Alps, Stiftingstalbach, 12.10.2006	M	EU215083	EU215178	EU215125	Graf
<i>Conisorophylax consors</i>	CH, Alps, Furkapass, 15.10.2006	M	EU215080	EU215175	EU215122	Graf
<i>Cryptothrix nebulicola</i>	I, Bergamask Alps, San Marco Pass, 14.08.2000	M	EU215082	EU215177	EU215124	Graf
<i>Drusus alpinus</i>	CH, Alps, Furkapass, Sidelen tributary, 17.07.2004	M	EU215084	EU215179	EU215126	Lubini, Pauls & Sundermann
<i>Drusus alpinus</i>	CH, Alps, St. Gotthardt Pass, 21.07.2006	M	EU215085	EU215180	EU215127	Graf
<i>Drusus annulatus</i>	SK, Muranska Planina, Havranik tributary, 22.05.2003	L	EU215086	EU215181	EU215128	Blonar & Pauls
<i>Drusus annulatus</i>	D, Black Forest, Brotenaubach, 11.05.2006	M	EU215087	EU215182	EU215129	Sundermann
<i>Drusus balcanicus</i>	BG, Balkan Range, Zavodna River, 24.08.2003	M	EU215088	EU215183	EU215130	Beskov, Kumanski & Pauls
<i>Drusus biguttatus</i>	AT, Nockberge, St. Oswald Stream, 27.07.2006	M	EU215089	EU215184	EU215131	Graf, Pauls & Schmidt-Kloiber
<i>Drusus botosaneanui</i>	BG, Pirin Mts, Demyanishka River, 19.08.2003	M	EU215090	EU215185	EU215132	Kumanski & Pauls
<i>Drusus brunneus</i>	RO, Caliman Mts, Toplita, Lomas river, 29.07.2003	L	EU215091	EU215186	EU215133	Pauls & Ujvarosi
<i>Drusus brunneus</i>	RO, Hășmașu Mare Mts., Voșlăbeni, Sugó Cave	M	EU215092	—	EU215134	Balint
<i>Drusus brunneus</i>	RO, Apuseni Mts, Buscat springs, 03.08.2003	L	—	EU215187	—	Pauls & Ujvarosi
<i>Drusus chrysotus</i>	AT, Soboth, Krumbach tributary, 18.05.2002	L	AY954395	EU215188	EU215135	Graf & Pauls
<i>Drusus chrysotus</i>	AT, Soboth, Krumbach tributary, 18.05.2002	L	—	—	EU215136	Graf & Pauls
<i>Drusus chrysotus</i>	AT, Saualpe, Springs near Ladinger Hütte, 30.06.2006	M	EU143739	EU215189	—	Graf, Pauls & Schmidt-Kloiber
<i>Drusus adustus</i>	AT, Soboth, Krumbach tributary, 18.05.2002	L	EU143738	EU215193	EU215140	Graf & Pauls
<i>Drusus adustus</i>	AT, Saualpe, Springs near Ladinger Hütte, 30.06.2006	M	EU215096	EU215194	EU215141	Graf, Pauls & Schmidt-Kloiber
<i>Drusus discolor</i>	RO, Retezat Mts, Rausor Valley, 08.08.2003	L	EU215095	EU215192	EU215139	Pauls & Ujvarosi
<i>Drusus discolor</i>	BG, Pirin Mts, Demyanishka River, 19.08.2003	M	EU215093	EU215190	EU215137	Beskov, Kumanski & Pauls
<i>Drusus discolor</i>	RO, Bucegi Mts, Pietra Alba, 05.08.2003	F	EU215094	EU215191	EU215138	Pauls & Ujvarosi
<i>Drusus discophorus pallidus</i>	BG, Pirin Mts, Banderishka River, 18.08.2003	M	EU215097	EU215195	EU215142	Beskov, Kumanski & Pauls
<i>Drusus discophorus pallidus</i>	BG, Pirin Mts, Banderishka River, 18.08.2003	M	EU215098	EU215196	EU215143	Beskov, Kumanski & Pauls
<i>Drusus franzi</i>	AT, Saualpe, 29.5.2006	M	—	EU215197	—	Graf
<i>Drusus franzi</i>	AT, Saualpe, 29.5.2006	M	EU215099	—	EU215144	Graf
<i>Drusus franzi</i>	AT, Koralpe, Weinebene, 27.5.2006	M	EU215100	EU215198	EU215145	Graf
<i>Drusus melanchaetes</i>	CH, Alps, Meienreuss tributary, Sustenpass, 18.07.2004	M	EU143740	EU215199	EU215146	Lubini, Pauls & Sundermann
<i>Drusus mixtus</i>	CH, Jura, Dou springs near Cormoret, 17.04.2006	L	EU215101	EU215200	EU215147	Stucki
<i>Drusus monticola</i>	AT, Soboth, Krumbach tributary, 18.05.2002	L	EF464556	EU215201	EU215148	Graf & Pauls
<i>Drusus monticola</i>	AT, Saualpe, Springs near Ladinger Hütte, 15.6.2006	F	EF464560	EU215202	EU215149	Graf
<i>Drusus muelleri</i>	CH, Alps, Meienreuss tributary, Sustenpass, 18.07.2004	M	AY954400	EU215203	EU215150	Lubini, Pauls & Sundermann
<i>Drusus muelleri</i>	CH, Alps, Furkapass, Springs of Mutt tributary, 17.07.2004	M	AY954398	EU215204	EU215151	Lubini, Pauls & Sundermann
<i>Drusus muelleri</i>	CH, Alps, Grimsensee Zulauf, 18.07.2004	M	AY954401	EU215205	EU215152	Lubini, Pauls & Sundermann
<i>Drusus nigrescens</i>	CH, Alps, Furkapass, Springs of Mutt tributary, 17.07.2004	M	EF464562	EU215206	EU215153	Lubini, Pauls & Sundermann
<i>Drusus nigrescens</i>	CH, Alps, Furkapass 21.7.2006	M	EF464565	EU215207	EU215154	Graf
<i>Drusus rectus</i>	F, Pyrenees, Breche de Roland, 13.7.1999	M	EU215105	—	EU215158	Lorenz
<i>Drusus rectus</i>	F, Pyrenees, Cirque de Govarine, 14.07.1999	F	—	EU215211	—	Lorenz
<i>Drusus romanicus</i>	RO, Apuseni Mts, Buscat springs, 03.08.2003	M	EU215102	EU215208	EU215155	Pauls & Ujvarosi
<i>Drusus romanicus</i>	RO, Retezat Mts, Rausor Valley, 08.08.2003	M	EU215103	EU215209	EU215156	Pauls & Ujvarosi
<i>Drusus romanicus</i>	BG, Pirin Mts, Banderishka River, 18.08.2003	M	EU215104	EU215210	—	Beskov, Kumanski & Pauls
<i>Drusus romanicus</i>	BG, Pirin Mts, Banderishka River, 18.08.2003	M	—	—	EU215157	Beskov, Kumanski & Pauls
<i>Drusus trifidus</i>	AT, Ennstaler Alps, Gesäuse, 02.07.2006	M	EU215108	EU215214	EU215161	Graf
<i>Drusus trifidus</i>	AT, Ennstaler Alps, Gesäuse, 02.07.2006	M	EU215109	EU215215	EU215162	Graf
<i>Eccisopteryx asterix</i>	AT, Soboth, Krumbach tributary, 18.05.2002	L	EU215111	EU215217	EU215164	Graf & Pauls
<i>Eccisopteryx asterix</i>	AT, Karawanken, Babniakgraben, 22.7.2006	L	EU215110	EU215216	EU215163	Graf
<i>Eccisopteryx dalecarlica</i>	RO, Țarcău, Poiana Mărului	F	EU215106	EU215212	EU215159	Balint
<i>Eccisopteryx dalecarlica</i>	RO, Țarcău, Poiana Mărului	F	EU215107	EU215213	EU215160	Balint
<i>Eccisopteryx dalecarlica</i>	D, Spessart, Jossa below Sahlensee, 10.03.2003	L	EU215112	EU215218	EU215165	Lohse

(continued on next page)

Table 1 (continued)

Taxon	Locality	Stage/sex	mtCOI	mtLSU	nuWG	Collector
<i>Ecclisopteryx dalecarlica</i>	D, Spessart, Jossa below Sahlensee, 10.03.2003	L	EU215113	EU215219	EU215166	Lohse
<i>Ecclisopteryx guttulata</i>	AT, Jogland, Lafnitz tributary, 24.05.2002	M	EU215114	EU215220	EU215167	Graf & Pauls
<i>Ecclisopteryx madida</i>	RO, Bucegi Mts, Pietra Alba, 05.08.2003	M	EU215115	EU215221	EU215168	Pauls & Ujvarosi
<i>Ecclisopteryx madida</i>	AT, Nockberge, St. Oswald Stream, 12.08.2006	M	EU215116	EU215222	—	Graf
<i>Ecclisopteryx madida</i>	AT, Nockberge, St. Oswald Stream, 17.08.2006	M	—	—	EU215169	Graf
<i>Ecclisopteryx malickyi</i>	IT, Alto-Adige, Campo Rosso, 15.10.2006,	F	EU215117	EU215223	EU215170	Graf
<i>Metanoea flavipennis</i>	CH, Val Müstair, 20.07.2006	M	EU215118	EU215224	EU215171	Graf
<i>Metanoea rhaetica</i>	AT, Nockberge, St. Oswald Stream, 01.07.2003	M	EU215119	EU215225	—	Graf
<i>Metanoea rhaetica</i>	AT, Saulalpe, Springs north of Offener Hütte, 30.06.2006	M	—	—	EU215172	Graf, Pauls & Schmidt-Kloiber
<i>Metanoea rhaetica</i>	AT, Nockberge, St. Oswald Stream, 01.07.2006	M	EU215120	EU215226	EU215173	Graf, Pauls & Schmidt-Kloiber

Localities are given with country code, mountain range, locality and collection date. Stage/sex refers to L: larva; M: adult male; F: adult female. GenBank accession codes are given for each taxon for each gene region used in the study.

Rambaut, 2002) to evaluate whether our data are sufficient to reject alternative topologies using the combined data set. Such topologies, which may not be significantly worse than the obtained topology, might be present in suboptimal trees not sampled or not present in the 50% majority-rule consensus tree of the MCMC sampling. The following hypotheses were tested if they were not supported by the phylogenetic topology: (1) monophyly of the genera *Ecclisopteryx* (H1), *Metanoea* (H2) and *Drusus* (H3); (2) species groupings within *Drusus* (H4–H10), which primarily reflect adult genital morphology based on Schmid (1956), Markinkovic-Gospodnetic (1976), Kumanski (1988) and Sipahiler (1999) (Tables 4 and 5); (3) the generic concept proposed by Schmid (1956) (H11–H14) (Fig. 1 and Tables 4, 5). The SH and ELW tests were performed using Garli (Zwickl, 2006) and Tree-PUZZLE 5.2 (Schmidt et al., 2002) with the combined data set. Unconstrained and constrained maximum likelihood (ML) analyses were performed using Garli employing the GTR+I+G nucleotide substitution model. A pair of trees including the best tree agreeing with each of the null hypotheses, i.e., the constrained ML tree, and the unconstrained ML tree were compared in SH and ELW tests using the “user tree evaluation” option with accurate parameter estimation assuming the GTR model in Tree-PUZZLE 5.2 for each hypothesis (H1–H14).

### 2.5. Ancestral character state reconstructions

We used BayesMultiState (Pagel et al., 2004) as implemented in BayesTraits (<http://www.evolution.rdg.ac.uk>) to reconstruct evolution of traits relevant to the feeding ecology of the Drusinae. This Bayesian approach estimates ML rates of character change and ancestral character states and incorporates uncertainty about the process of character change and the phylogeny by using a Bayesian tree sampling (Huelsenbeck et al., 2000; Pagel et al., 2004). Ancestral state posterior probabilities for a given node

were estimated by multiplying the mean ancestral character state probability at that node across all trees by the portion of the trees in which that node was found (Pagel et al., 2004). The analysis was performed on 2000 trees taken every 1000th generation from the last  $1 \times 10^6$  generations from both of the combined data B/MCMC runs to ensure independence of successive trees.

## 3. Results

### 3.1. Molecular data and utility of individual gene partitions

To evaluate the relationships within the Drusinae 151 new sequences were obtained for this study (45 mtCOI, 53 mtLSU, 53 nuWG) (Table 1). We summarised sequence and tree characteristics for single-gene and combined data sets in Table 3. Variability and number of parsimony informative characters ranged between 21.74–34.74% (110–173 sites) and was highest in mtCOI, followed by nuWG and mtLSU, respectively (Table 3). No significantly supported conflicts were observed between the three partitions when comparing the 95% majority-rule consensus trees of single gene analyses. This is consistent with the hypothesis that all data partitions evolved along the same underlying topology. We thus combined data partitions to 3 two-gene and 1 three-gene data sets.

We summarised maximum parsimony (MP) data and indices in Table 3. In single gene analyses, levels of homoplasy were highest in mtCOI and lowest in nuWG. Combined gene partition analyses exhibited lowest levels of homoplasy, when mtCOI was not included (RC: 0.57). In analyses of the combined three-gene data set RC was relatively low (0.28). The number of most parsimonious trees found in single gene analyses decreased with number of variable sites. Only two shortest trees were found in analyses of the combined three-gene data set. The number of polyspecific clades (i.e., clades with two or more species)

Table 2  
Morphological characters of Drusinae genera

Genus	Distinctive characters and selected synapomorphies in adults
<i>Anomalopterygella</i>	Sexual dimorphism Reduced forewings in adult males Extremely elongated lower appendix on genital armature Abdominal tracheal gills Prominent setae on wing venation
<i>Cryptothrix</i>	Abdominal tracheal gills Discoidal cell of anterior wings straight and 1.5× longer than its base Anastomosis of anterior and posterior wings interrupted
<i>Drusus</i>	Wing anastomosis is regular Forks are not stilted, except f5 Anterior tarsus not enlarged Intermediate appendices of male genitalia only show minor levels of reduction
<i>Ecclisopteryx</i>	Segment IX apically enlarged forming an obvious bulge Reduced genital cavity
<i>Hadimina</i>	Females with 4 segmented maxillary palps Abdominal tracheal gills Apical segments of antennae notched Preanal appendices sclerotized dorsally Short aedeagus, curved ventrally Aedeagus without paramers Oval lobes on tergite VIII Shape of segment IX
<i>Leptodrusus</i>	Antennae longer than anterior wings in males Elongated labial and maxillary palps in males Lack fold in the posterior wings Slim pointed anterior wing Xth segment fused with intermediate appendices
<i>Metanoea</i>	Lacks fold in the posterior wings All 3 appendices of genital armature equally extended Inferior appendage IX almost completely fused Intermediate appendices quite distant from one another
<i>Monocentra</i>	Scales on wings

Sources: Rambur (1842), McLachlan (1880), Schmid (1956), Sipahiler (2002).

supported by bootstrap values  $\geq 70$ , was highest for nuWG (11) and lowest for mtLSU (2) and increased in combined data sets. The highest number of highly supported clades was found in analyses of the combined three-gene data set (13).

We summarised likelihood parameters in the seven B/MCMC samples in Table 3. G/C content was highest in the nuWG partition (59.1%), while the mitochondrial genes showed a strong bias toward A/T (GC-content 29.1% for mtCOI, 17.3% for mtLSU). The gamma shape parameter  $\alpha$  was similar in mtLSU (0.129) and nuWG (0.119), but much higher in mtCOI (0.911). The number of clades with more than one species that receive posterior probabilities  $\geq 95\%$  was highest in nuWG for single gene analyses (14)

and lowest in mtLSU (4). In combined analyses, the lowest number of supported clades was found when combining the two mitochondrial genes (9), the highest number (18) was reached after combining all three partitions. After  $2 \times 10^6$  generations, the average standard deviation of split frequencies between parallel runs was considerably higher in mtLSU than in nuWG or mtCOI or whenever mtLSU was combined with one of the other partitions. In analyses of the combined three-gene data set, however the deviation was relatively low (0.005881 after  $2 \times 10^6$  generations).

### 3.2. Phylogenetic reconstructions

Phylogenetic relationships were investigated using single-gene, two-gene and a three-gene region data set (mtCOI+mtLSU+nuWG). The summary of support for individual clades using individual gene partitions and the four possible combinations is given in Table 4. The combined three-gene data set had an aligned length of 1476 bp with 428 variable positions (Table 3). Parsimony analysis of the three-gene data set yielded two most parsimonious trees (1460 steps, CI = 0.39, RI = 0.71, RC = 0.28, Table 3). The two trees only differed in the order of specimens within *Drusus muelleri*. The topology of the most parsimonious trees differed slightly from the 50% majority-rule consensus tree obtained from the B/MCMC tree sampling in the position of clades within the epilithic grazers, but the clades themselves are the same (Figs. 2 and 3). There were no differences in the monophyly of and relationships within genera, species groups, or feeding ecology.

Both the MP and B/MCMC trees from the three-gene combined analysis support monophyly of the subfamily Drusinae (pp = 1.0; bs = 100%) (clade 1, Figs. 2 and 3). All species where more than one specimen was analysed are monophyletic, although not always significantly supported (*D. romanicus* and *D. nigrescens* with pp < 0.95). Within the Drusinae, both topologies show a basal clade with *Cryptothrix nebulicola*, *Drusus muelleri*, *D. romanicus*, *D. chrysotus* and *D. discolor* (clade 2, Figs. 2 and 3). This clade, however, lacks strong support. Within this clade, *C. nebulicola* is basal to a highly supported clade (pp = 0.99, bs = 86) comprising members of the genus *Drusus*. Within this clade, *D. muelleri* and *D. chrysotus* are basal to *D. discolor* and *D. romanicus*.

The remaining species fall into two major clades. One clade (clade 4, Figs. 2 and 3) includes *Drusus alpinus* and *D. franzi* (pp = 1.0, bs = 100). Clade 4 is sister to a clade comprising the remaining Drusinae (clade 5, Figs. 2 and 3). Clade 5 falls into four supported smaller clades in the B/MCMC analysis, all of which are also recovered in the MP analysis, but not always supported (Figs. 2 and 3). Clade 5a (pp = 1.0, bs = n.a.) comprises two well-resolved sister groups with *Drusus balcanicus* and *D. discophorus pallidus* (pp = 1.0, bs = 100%) and *D. botosaneanui*, *Ecclisopteryx dalecarlica*, *E. guttulata* and *E. madida* (pp = 0.97, bs = 58%), respectively. Clade 5b (pp = 0.98,

Table 3  
Comparison of performance of data partitions under maximum parsimony and in a Bayesian framework

	mtCOI	mtLSU	wg	mtCOI+mtLSU	mtCOI+wg	mtLSU+wg	mtCOI+mtLSU+wg
Total characters	498	506	472	1004	970	978	1476
Variable (%)	173 (34.74)	110 (21.74)	145 (30.72)	283 (28.19)	328 (32.78)	255 (26.07)	428 (29.0)
<i>Maximum parsimony</i>							
Uninformative characters	12	55	41	67	53	96	108
Informative characters (%)	161 (32.33)	55 (10.87)	104 (22.03)	216 (21.51)	265 (27.32)	159 (16.26)	320 (21.68)
Consistency index (CI)	0.2819	0.6543	0.6822	0.3373	0.3553	0.6528	0.3911
Retention index (RI)	0.6246	0.7833	0.9085	0.6384	0.7035	0.8671	0.7078
Rescaled consistency index (RC)	0.1761	0.5125	0.6197	0.2153	0.2499	0.5660	0.2768
Tree length	965	188	258	1171	1261	458	1460
No MP trees	16	416	64	16	25	128	2
Polyspecific nodes bootstrap $\geq 70$	6	2	11	7	9	11	13
<i>Bayesian/MCMC</i>							
Selected model under hLRT	GTR+I+G	K81uf+I+G	TrN+G	GTR+I+G/ K81uf+I+G	GTR+I+G/ TrN+G	K81uf+I+G/ TrN+G	GTR+I+G/ K81uf+I+G/TrN+G
Polyspecific nodes pp $\geq 0.95$	6	4	14	9	16	15	18
Average distance between runs	0.007708	0.026674	0.007544	0.016278	0.004448	0.011423	0.002051
Log-likelihood	-4635.113	-1912.363	-2346.681	-6627.26	-7158.902	-4417.382	-9156.271
Tree length	6.369	6.734	9.43	2.995	3.252	6.224	2.303
Model parameter rate of change A/C	0.0192	0.02339	0.04757	0.02699	0.03718	0.03693	0.03958
Model parameter rate of change A/G	0.448	0.731	0.312	0.412	0.238	0.381	0.273
Model parameter rate of change A/T	0.03424	0.03627	0.07971	0.04833	0.07508	0.102	0.06585
Model parameter rate of change G/C	0.03807	0.0803	0.07486	0.03345	0.04837	0.114	0.06314
Model parameter rate of change C/T	0.449	0.113	0.413	0.463	0.584	0.315	0.538
Model parameter rate of change G/T	0.01256	0.01591	0.07246	0.01619	0.01704	0.05166	0.0203
Stationary nucleotide frequency of A	0.355	0.411	0.234	0.356	0.279	0.322	0.32
Stationary nucleotide frequency of C	0.203	0.076	0.323	0.165	0.253	0.2	0.195
Stationary nucleotide frequency of G	0.082	0.097	0.268	0.104	0.189	0.193	0.164
Stationary nucleotide frequency of T	0.361	0.416	0.175	0.374	0.279	0.284	0.321
Shape parameter $\alpha$ for $\gamma$ distribution of rate variation	0.911	0.129	0.119	0.169	0.499	0.144	0.229
Proportion of invariable sites	0.579	0.783	0.312	0.146	0.476	0.324	0.291

Table 4  
Summary of interspecific nodes identified in single gene and combined analyses

Clade	Species included in analyses	Hypothesis	mtCOI	mtLSU	wg	mtCOI+mtLSU	mtCOI+wg	mtLSU+wg	mtCOI+mtLSU+wg
<i>Taxonomic groupings</i>									
Drusinae			1.00/77	1.00/100	1.00/100	1.00/99	1.00/100	1.00/100	1.00/100
Chaetopterygini			–/50	0.97/65	1.00/100	0.74/–	0.98/83	1.00/100	1.00/83
<i>Ecclisopteryx dalecarlica</i> + <i>E. guttulata</i>		H1	1.00/100	0.53/–	0.68/–	1.00/93	1.00/91	0.86/61	1.00/93
<i>Ecclisopteryx dalecarlica</i> + <i>E. guttulata</i> + <i>E. madida</i>		H1	–/57	0.58/53	0.68/–	0.88/84	–/–	0.80/59	–/64
<i>Ecclisopteryx asterix</i> + <i>E. malickyi</i>		H1	–/–	–/–	0.98/66	–/–	1.00/–	0.97/69	1.00/–
<i>Metanoea</i> spp.	<i>rhaetica</i>	H2	0.71/–	–/–	0.95/71	–/–	1.00/69	0.74/64	1.00/80
	<i>flavipennis</i>								
<i>Drusus</i> spp.	All	H3	–/–	–/–	–/–	–/–	–/–	–/–	–/–
<i>D. discolor</i> -group	<i>discolor</i>	H4	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	<i>chrysotus</i>								
	<i>adustus</i>								
<i>D. muelleri</i> -group	<i>muelleri</i>	H5	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	<i>romanicus</i>								
<i>D. annulatus</i> -group	<i>annulatus</i>	H6	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	<i>rectus</i>								
	<i>botosaneanui</i>								
<i>D. mixtus</i> -group	<i>mixtus</i>	H7	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	<i>biguttatus</i>								
	<i>brunneus</i>								
	<i>trifidus</i>								
<i>D. alpinus</i> -group	<i>alpinus</i>	H8	–/–	0.56/–	1.00/100	–/–	1.00/87	1.00/100	1.00/94
	<i>franzi</i>								
<i>D. discophorus</i> -group	<i>D. pallidus</i>	H9	1.00/100	1.00/ 89	1.00/100	1.00/100	1.00/100	1.00/100	1.00/100
	<i>balcanicus</i>								
<i>D. bosnicus</i> group	<i>monticola</i>	H10	1.00/100	0.71/–	1.00/90	1.00/100	1.00/100	1.00/98	1.00/100
	<i>nigrescens</i>								
<i>Ecclisopteryx</i> + <i>Cryptothrix</i> + <i>Drusus</i> + <i>Metanoea</i>	All	H11	–/–	–/–	–/–	–/–	–/–	–/–	–/–
<i>Cryptothrix</i> + <i>Drusus</i> + <i>Metanoea</i>	All	H12	–/–	–/–	–/–	–/–	–/–	–/–	–/–
<i>Drusus</i> + <i>Metanoea</i>	All	H13	–/–	–/–	–/–	–/–	–/–	–/–	–/–
<i>Metanoea</i> outside <i>Drusus</i>	All	H14	–/–	–/–	–/–	–/–	–/–	–/–	–/–
<i>Feeding ecology groupings</i>									
Filtering carnivores			–/–	–/–	–/–	–/–	0.91/55	–/–	0.88/75
Filtering carnivores within genus <i>Drusus</i>			–/–	–/–	0.75/82	–/52	0.99/55	0.64/73	0.99/86
Omnivorous generalist shredders			–/–	0.56/–	1.00/100	–/–	1.00/87	1.00/100	1.00/94
Epilithic grazers			–/–	–/–	1.00/100	–/–	1.00/94	1.00/100	1.00/100

Shown are B/MCMC posterior probabilities (before slash) and parsimony bootstrap support values (after slash). Nodes not present or unresolved in a certain data set are indicated by “–”.



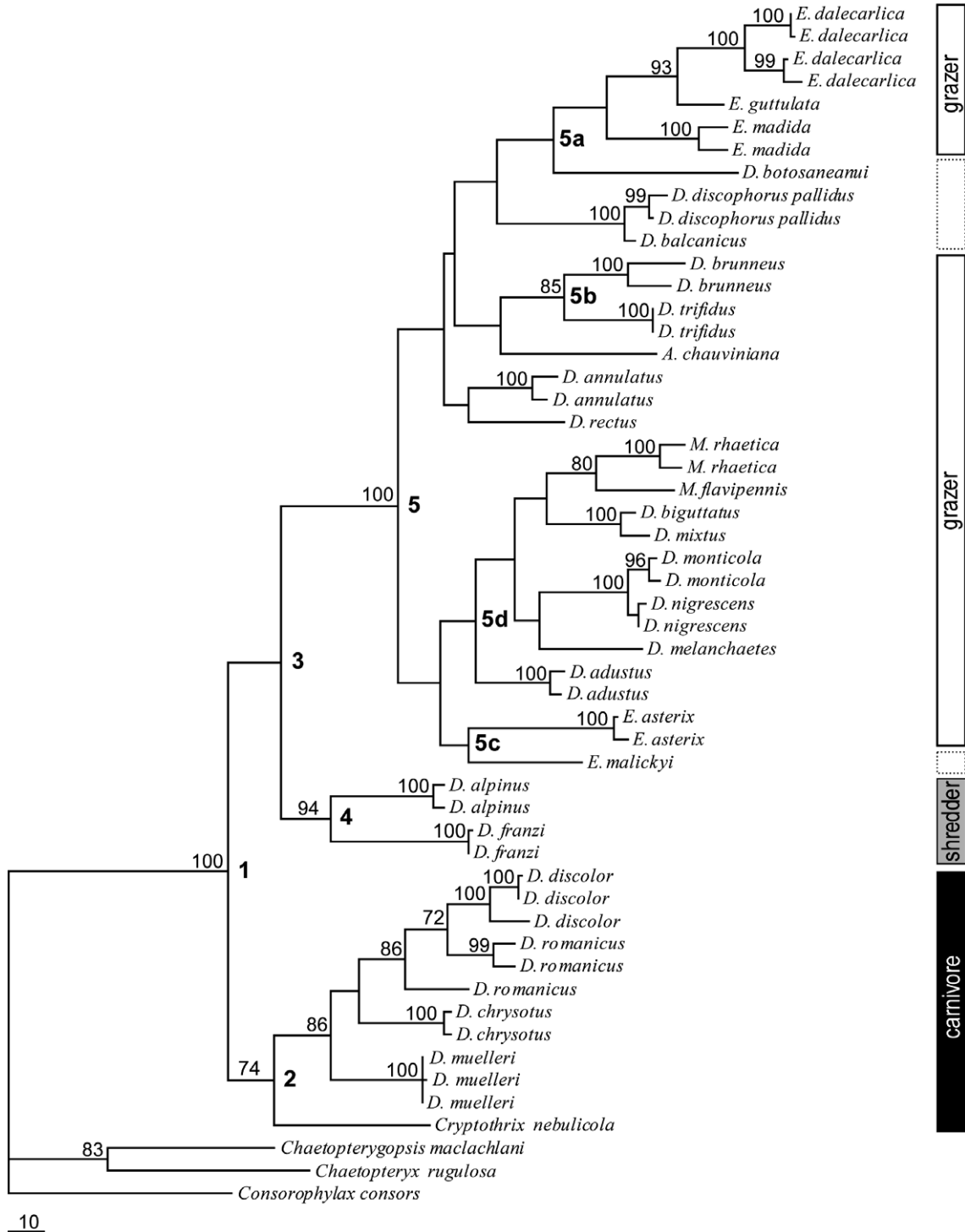


Fig. 3. One of two most parsimonious trees recovered in the MP analysis. Above nodes are bootstrap values above 70%. Bold numbers indicate the nodes referred to in the text. Other annotations as in Fig. 2.

The current generic concept is not supported in our analysis. The genera *Anomalopterygella*, *Cryptothrix*, *Ecclisopteryx* and *Metanoea* are nested in *Drusus*. *Ecclisopteryx* is polyphyletic and falls into two separate clades. Within *Drusus* three groups distinguished by adult genital morphology were supported in our study: the *Drusus alpinus*-group, *D. discophorus*-group and *D. bosnicus*-group. The remaining species groups within *Drusus* are not supported

in our study (*D. discolor*-group, *D. muelleri*-group, *D. annulatus*-group, *D. mixtus*-group).

### 3.3. Hypothesis testing

The results of the SH and ELW tests for probabilities of alternative topologies are shown in Table 5. Monophyly of the genera *Drusus* (H3) and *Ecclisopteryx* (H1) is signifi-

Table 5  
Alternative topology hypotheses tested with ELW-test and SH-test

Hypothesis		ELW		SH	
		Result	C	Result	p
	Monophyly of...		0.185		0.979
H1	<i>Ecclisopteryx</i>	Rejected	<0.01	Rejected	<0.01
H2	<i>Metanoea</i>				
H3	<i>Drusus</i>	Rejected	<0.01	Rejected	<0.01
H4	<i>D. discolor</i> -group	Rejected	<0.01	Rejected	<0.01
H5	<i>D. muelleri</i> -group	Rejected	0.015		0.518
H6	<i>D. annulatus</i> -group	Rejected	<0.01		0.531
H7	<i>D. mixtus</i> -group	Rejected	<0.01		0.178
H8	<i>D. alpinus</i> -group		0.199		0.995
H9	<i>D. discophorus</i> -group		0.199		0.995
H10	<i>D. bosnicus</i> -group		0.199		1.0
H11	<i>Ecclisopteryx</i> + <i>Cryptothrix</i> + <i>Drusus</i> + <i>Metanoea</i>	Rejected	<0.01		0.073
H12	<i>Cryptothrix</i> + <i>Drusus</i> + <i>Metanoea</i>	Rejected	0.019	Rejected	<0.01
H13	<i>Drusus</i> + <i>Metanoea</i>	Rejected	<0.01	Rejected	<0.01
H14	<i>Metanoea</i> outside <i>Drusus</i>	Rejected	<0.01	Rejected	<0.01

cantly rejected in both, as was the placement of *Metanoea* outside *Drusus* (H14). The original classification proposed by Schmid, 1956 (Fig. 1) is rejected with both tests (H12, H13). The basal position of *Anomalopterygella* (H11) is not rejected significantly by both tests (SH,  $p = 0.073$ ). Within *Drusus*, monophyly of the *discolor*-group (H4) is significantly rejected in both tests. Following the ELW test the *muelleri*-group (H5), *annulatus*-group (H6), and *mixtus*-group (H7) are significantly rejected. However, in the SH test these three groups cannot be significantly rejected with the data at hand. Hypotheses H2 and H8–H10 could not be rejected in ELW or SH tests based on the data at hand.

### 3.4. Feeding ecology and ancestral character state reconstruction

With respect to larval mouthpart anatomy, three distinct species groupings exist in known Drusinae larvae: (1) carnivorous filterers, with teeth around the mandible edges, modifications of head capsules, additional spines on the legs and long filtering bristles at the first abdominal sternum; (2) omnivorous generalists, with shredder mandibles with teeth around the edges but lacking additional spines and long bristles; (3) epilithic grazers, with spoon-shaped mandibles without teeth along the edges and additional fine setae on faces of femora (Waringer et al., in press-a). These morphologies and feeding types were found to be characteristic for each of the three major clades (clades 2, 4 and 5) found in the Drusinae (Figs. 2 and 3).

We reconstructed ancestral character states for the 5 nodes described in Section 3.2, to investigate the evolution of mandible type and presence of filtering bristles within the Drusinae. Fig. 4 shows the results of this ancestral character reconstruction analysis for the five nodes over the 2000 examined trees. Only the probabilities of a single character state are shown, since the two alternative states are complementary. Shown in the plots are presence of teeth on the mandible edge (left) and presence of filtering

bristles on the thorax and legs (right). The mean  $p$ -values over all 2000 examined trees ( $\emptyset_{ML}$ ) are given for each node. At node 1, which includes all Drusinae, the analysis suggested significantly the presence of a mandible with teeth ( $\emptyset_{MLT} = 0.969$ ). The presence or absence of filtering bristles is not resolved ( $\emptyset_{MLB} = 0.593$ ). At node 2, the analysis infers that both teeth on mandibles ( $\emptyset_{MLT} > 0.999$ ) and filtering bristles ( $\emptyset_{MLB} > 0.999$ ) are present. At node 3 the state of teeth on mandibles is not resolved by the analysis ( $\emptyset_{MLT} = 0.812$ ), while filtering bristles are evaluated to be significantly absent ( $\emptyset_{MLT} = 0.014$ ). At node 4 filtering bristles are inferred absent ( $\emptyset_{MLB} = 0.003$ ), but the analysis significantly suggests that there are teeth on the mandible edges ( $\emptyset_{MLT} > 0.999$ ). At node 5, the analysis significantly infers that both teeth on mandibles and filtering bristles are absent (both  $\emptyset_{ML} < 0.001$ ).

## 4. Discussion

### 4.1. Phylogenetic relationships in Drusinae

The phylogenetic relationships recovered in our study do not support the current generic classification, which is based on adult morphology (Schmid, 1956). Our study supports monophyly of the subfamily, with respect to outgroups from subfamily Limnephilinae. Two currently recognised genera, *Drusus* and *Ecclisopteryx*, are not monophyletic. *Drusus* is polyphyletic with *Anomalopterygella*, *Ecclisopteryx* and *Metanoea* nested within. Schmid (1956) considered *Anomalopterygella* as the basal taxon in the Drusinae. Our phylogeny does not support this position and ELW tests rejects it. *Anomalopterygella* clusters in *Drusus* (sister to *D. brunneus* and *D. trifidus*) but its placement within the group is not supported by our statistical tests. Its position within *Drusus* is somewhat surprising considering the large number of characteristic and distinctive features of *A. chawiniana* (Table 2).

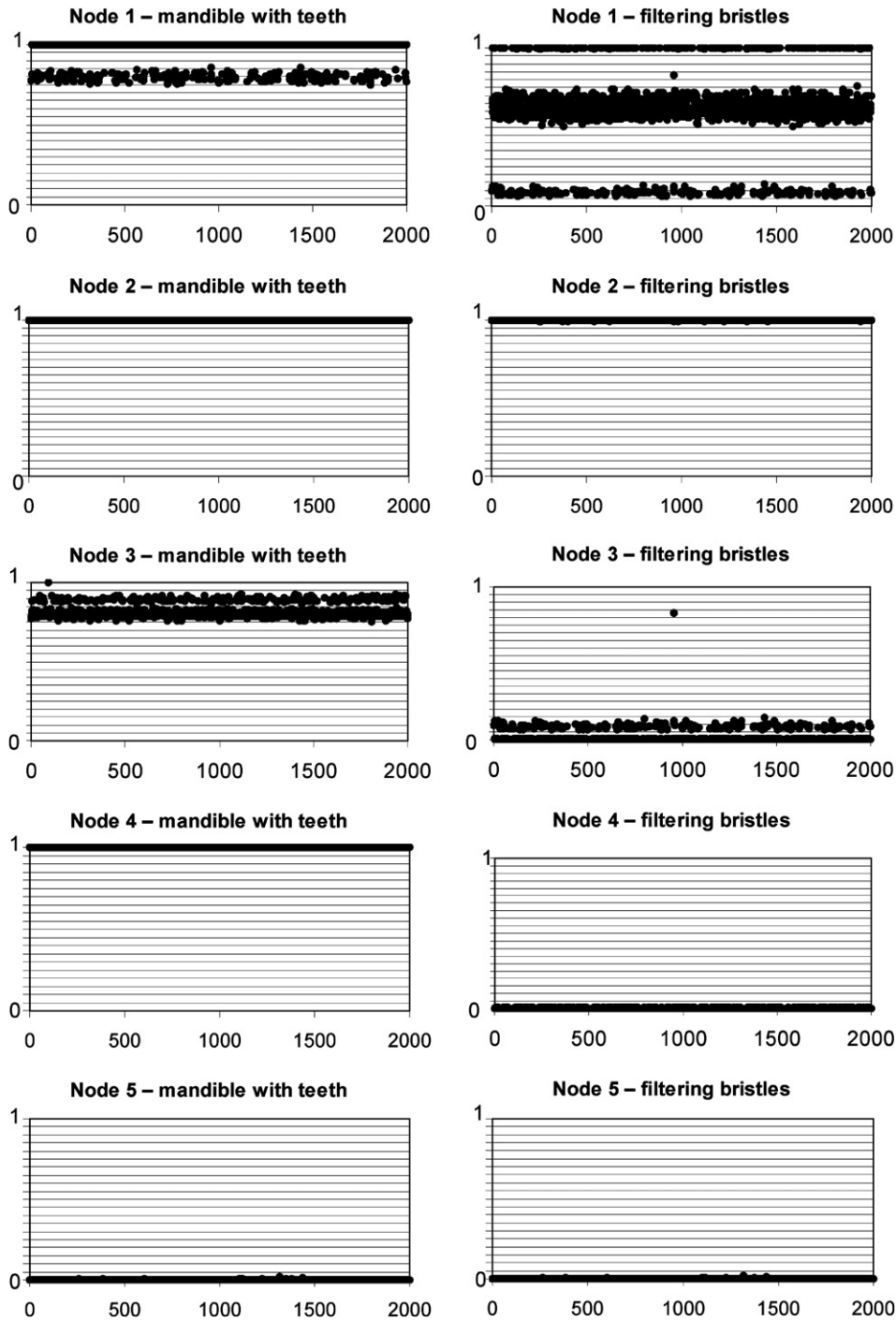


Fig. 4. Ancestral character reconstruction of mandible type and presence of filtering bristles on thorax and legs for five specific clades (as shown in Figs. 2 and 3). The probability of presence of teeth on mandible edges (left column) and presence of filtering bristles on first abdominal sternum and legs (right column) for each node was reconstructed on 2000 trees generated by Markov chain Monte Carlo sampling.

In our analyses *Ecclisopteryx* falls into two well-supported clades within *Drusus*. Monophyly of the genus is also rejected with our hypothesis testing approaches. In one clade we find *E. asterix* and *E. malickyi*. The second clade of *Ecclisopteryx* groups *E. dalecarlica*, *E. guttulata* and *E. madida* with *Drusus balcanicus*, *D. botosaneanui* and *D. discophorus pallidus*. This relationship is well-supported by the Bayesian inference, and was resolved in the

maximum parsimony tree, but not recovered in the bootstrap analysis. Schmid (1956) stated that the morphological differences between *Ecclisopteryx* and *Drusus* are limited to the genital armature, but consistent and stable enough to support two genera. In contrast, our study suggests that *Ecclisopteryx* and *Drusus* are not true evolutionary units.

Monophyly of the genus *Metanoea* is supported in our analyses. However, the genus is nested within and not sister

to *Drusus*. Schmid (1956) accepted *Metanoea* but raised doubts of its distinction based on the known differential characters (Table 2). In this study we could only include two of the five recognised species in the genus. Further investigations including more species of *Metanoea* are needed to fully resolve its status.

Four of the seven species groupings proposed by Schmid (1956), Marinkovic-Gospodnetic (1976), Kumanski (1988) and Sipahiler (1999) we tested are not supported by our analyses. For those species groups that are observed in our topologies, we were only able to sample two sister species. It seems that very close adult similarities result from close relationships and could represent natural evolutionary units. Some of these species pairs comprise more or less vicariant species. *D. alpinus* and *D. franzi* are endemics of the western-central and south-eastern Alps, respectively. *D. nigrescens* is a local endemic of the western-central Alps, while *D. monticola* mainly occurs further east. Both species pairs may be examples of refugial lineage divergence of a common ancestor which was forced to retreat to south-eastern and south-western refugia during the early or middle Pleistocene (Hewitt, 2004; Pauls et al., 2006). The divergence between the sister taxa is less strong in *D. nigrescens* and *D. monticola*. This could result from a later split of the lineage or recurring introgression in the two species that do occasionally occur in the same region.

#### 4.2. Larval morphology and feeding type evolution

##### 4.2.1. Larval morphology and phylogenetic grouping

With exception of a few species groups, the three major clades within Drusinae (clades 2, 4, 5) do not correspond to the adult genitalia-based generic and species group classifications proposed by Schmid (1956), Marinkovic-Gospodnetic (1976) or Kumanski (1988). Instead, they correspond to the three distinct species groupings based on mouthpart anatomy and feeding ecology (Waringer et al., in press-a) (Figs. 2 and 3). The larvae of the subfamily Drusinae all have single filament abdominal gills, a fully sclerotised pronotum and mesonotum, and build a cylindrical, slightly curved and slightly conical sand case (Szczesny, 1978; Ulmer, 1909; Waringer, 1985; Waringer and Graf, 1997, 2004; Waringer et al., 2000). In *Cryptothrix nebulicola*, *Drusus chrysotus*, *D. discolor*, *D. muelleri* and *D. romanicus*, mandibles with teeth around edges are present; this, together with additional setae on the legs and with long filtering bristles at the first abdominal sternum, identifies this group as carnivorous filterers. Gut content analysis of several species within this group (*D. discolor*, *D. romanicus*, *D. muelleri*) confirms their carnivorous filtering feeding ecology (Bohle, 1983; Graf and Pauls, unpublished data). These species also share a unique synapomorphy: all species in this group have concave cavities or major indentations in the head capsule. In all other species of Drusinae, the head capsule is rounded without cavities or indentations. In our analyses these species group together in clade 2. Although the inclusion of *C. nebulicola* in this

group is not significant in the Bayesian inference (pp < 0.95), the relatively high bootstrap value supports inclusion (bs = 74%). Also all other Drusinae belong to one of two other significantly supported clades (pp > 0.95; bs ≥ 94%).

A second larval feeding type (omnivorous generalist shredders) was recently identified using DNA-based associations with adult specimens (Graf et al., in preparation; Waringer et al., in press-a). *D. franzi* and *D. alpinus* have mandibles with teeth around edges, but additional filtering spines on legs and bristles on the first abdominal sternum are lacking, characterising them as omnivorous generalist shredders. These two species are sister taxa in the highly supported clade 4 (pp > 0.95, bs = 94%). To date only *Drusus alpinus* and *D. franzi* are known to have the morphological characteristics of omnivorous generalist shredders as described above.

Of the remaining 21 species in our analyses 17 have a spoon-shaped mandible without teeth and additional setae and bristles are lacking (Szczesny, 1978; Waringer et al., 2000, 2007, in press-b). This identifies these species as grazers, which feed mainly on epilithic algae. For the four other species included in our analyses (*D. balcanicus*, *D. botosaneanui*, *D. discophorus pallidus*, *E. malickyi*), the larval stages are unknown. However, based on their position in the phylogeny, we predict that these larvae are epilithic grazers with a spoon-shaped mandible.

##### 4.2.2. Feeding type evolution

Based on our study, two alternative scenarios of the evolution of feeding ecology are possible: (1) progression from ancestral omnivorous shredders to both filtering carnivores and epilithic grazers or (2) evolution from filtering carnivores to omnivorous shredders and epilithic grazers. The first alternative is more likely based on the fact that all other Limnephilids are known to be shredders, but the latter alternative cannot be ruled out with the data at hand. The mandible in the shredders *D. alpinus* and *D. franzi* is of the ancestral type with teeth along the edges (Graf et al., in preparation). Based on our ancestral character state reconstructions the mandible with teeth appears to be the ancestral state, which is maintained in the carnivorous filterers and omnivore generalist shredders (Nodes 1–4). The spoon-shaped grazer mandible seems to be derived (Node 5), having reduced or lost the teeth on the mandible edge. The acquisition of filtering bristles seems to be a derived character unique to a single clade in our study (Node 2).

With few exceptions, all Limnephilidae are shredders (Graf et al., 2002). Other feeding types are only found in the Drusinae and sporadically among other genera (*Melampophylax* and *Micropterna*). *Melampophylax mucoreus*, *M. nepos* and *Micropterna testacea*, for example, are Limnephilinae grazers with spoon-shaped mandibles. Whether the feeding type evolved only once or independently several times within Limnephilidae requires further phylogenetic analysis with a larger sampling of Limnephilidae taxa. The evolution of feeding types in the Drusinae

follows the ontogeny of individuals. Nielsen (1942) studied the larval development of *Ecclisopteryx guttulata* and observed that in first instar larvae both mandibles have a ventral tooth. Additionally, two or three dorsal teeth are present on the left and right mandible, respectively. From the second instar larvae onward, the mandibles are spoon-shaped without any teeth on the mandible edges.

Most of the extant Drusinae species whose larvae are known are grazers or carnivorous filterers. Weaver and Morse (1986) hypothesised generally for caddisflies that feeding specialisation may have opened opportunities to colonise new ecological niches and could have promoted diversification in these organisms significantly. Considering the high number of derived grazers, such changes in feeding ecology may be responsible for much of the diversification within Drusinae. Dietary shifts have also been made responsible for high levels of diversity in other groups including beetles (e.g., Leschen and Buckley, 2007; Farrell, 1998) and fish (e.g., Horstkotte and Strecker, 2005).

#### 4.2.3. Importance of larval morphology for phylogenetic studies

Larval features correspond well with our phylogenetic results. In Drusinae larval morphology outperforms adult characters as phylogenetic discriminators. While most classifications of caddisfly species within genera are based on adult characters (Schmid, 1956; Flint, 1989; Holzenthal and Andersen, 2007), the use of larval characters in systematics and caddisfly phylogeny has been recognised for some time (Scott, 1975; Wiggins, 1981; Weaver and Morse, 1986; Kjer et al., 2001, 2002; DeMoor, 2002; Kjaerandsen, 2004). In previous studies larval and adult characters have been incorporated into a joint matrix for phylogenetic studies. Studies using a molecular phylogeny to examine utility of adult and larval characters, however, are lacking. In their subordinal molecular phylogeny of Trichoptera, Kjer et al. (2001, 2002) were unable to fully resolve the basal relationships in Trichoptera, which traditionally consists of three suborders and infraorders based on larval morphology and behaviour: Annulipalpia, Integripalpia and Spicipalpia (Martynov, 1924; Weaver, 1984).

Our study explicitly shows that at the level of genera and species groupings adult genital morphology does not recover the same relationships we find with an independent, molecular phylogeny, while our species are all recovered as monophyletic entities. While adult genital morphology clearly delimits individual species of Drusinae, relationships between these species are better understood if we also incorporate larval characteristics. Our results suggest that larval features and characters may be more useful in resolving evolutionary relationships between species within families or subfamilies than previously recognised. Utility of larval characters or characters of immature, life stages is widely recognised in other insect groups including beetles (Michat, 2006; Solodovnikov, 2007), and butterflies and moths (Hebert et al., 2004; Wagner et al., 2006). Additional lower level molecular studies on caddisfly families

and genera are needed to better judge the use of adult and larval characters in Trichoptera.

#### 4.3. Utility of mtLSU, mtCOI and nuWG for lower level phylogenetic reconstruction of Drusinae

All three gene partitions we used are suitable for phylogenetic inference at the level examined in this study. Based on CI, RI, variability and resolution, nuWG performs best as a single marker for resolving relationships among species within this subfamily. Both mitochondrial genes are also useful, but variability and resolution are limited in mtLSU, while problems of homoplasy seem to limit the utility of mtCOI. Despite shortcomings in individual partitions, the best resolution is obtained using a combined data set, a result that is consistent with those observed in other studies of insects (Kjer et al., 2001, 2002; Hughes and Vogler, 2004; Nazari et al., 2007). Previous molecular studies on caddisflies have used a common set of molecular markers including various fragments of mitochondrial DNA (COI, 16S rRNA) and nuclear loci including (EF1- $\alpha$ , rRNA) (Kjer et al., 2001, 2002; Myers et al., 2001; Myers and Sperling, 2002; Geraci et al., 2005; Leese, 2004; Pauls et al., 2006). The utility of some of these genes for studying subordinal relationships in caddisflies was rigorously tested by Kjer et al. (2001). These authors concluded that at subordinal levels, rRNA data was very useful, while taken alone, both mtCOI and EF1- $\alpha$  are of little use for resolving subordinal phylogenetic relationships due to saturation and homoplasy issues. However, combined with other data sets, they still proved useful in finding the best estimate of phylogenetic hypotheses (Kjer et al., 2001), especially at lower phylogenetic levels. For example, Geraci et al. (2005) used EF1- $\alpha$  in combination with mtCOI and nu rRNA, to examine relationships between subfamilies in Hydropsychidae, but its utility was not evaluated. Based on our results, mtCOI is valuable in combination with other genes as it increases resolution. The present study is the first that uses and evaluates the utility of mtLSU and nuWG in a larger phylogenetic context in caddisflies. Both genes, especially nuWG, appear to be useful in multi-gene analyses which aim to resolve phylogenetic relationships at lower taxonomic rank in caddisflies.

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