

Cryptic diversity of caddisflies in the Balkans: the curious case of *Ecclisopteryx* species (Trichoptera: Limnephilidae)

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Abstract

Adults and larvae of two new cryptic, endemic caddisflies, *Ecclisopteryx keroveci* sp.n. and *Ecclisopteryx ivkae* sp.n., are described and illustrated from the Western Balkans. Phylogenetic analysis (Bayesian MCMCMC) and association of different life history stages in both cryptic species were achieved through comparison of morphological characters and mitochondrial (mtCOI and mtLSU) and nuclear (nuWG) gene sequence data. The new species form a sister clade to the widely distributed *E. dalecarlica* and *E. guttulata*, with which they were formerly misidentified. Adults differ from each other and other species in the genus by the uniquely shaped inferior appendages in males and segment X in females. The larvae differ from each other and their congeners in the shape of the pronotum, and presence and constitution of additional spines on the parietalia. Larvae of both species are grazers and prefer stony substrate. *Ecclisopteryx keroveci* sp.n. has a wide distribution in the Western Balkans, while *E. ivkae* sp.n. is endemic to Dalmatia. Our findings demonstrate the significance of the Western Balkans as a freshwater biodiversity hotspot, and accentuate the importance of research focused on freshwater biodiversity and biogeography in southern Europe.

Key words

Biodiversity, Drusinae, larval morphology, adult morphology, phylogeny.

1. Introduction

Cryptic species are typically defined as two or more distinct species that are classified as a single nominal species, mostly due to seemingly identical morphology (BICKFORD et al. 2007). A broader definition describes cryptic species as “those that cannot be identified by conventional means” (ROSS 1974). “Conventional

means” have changed historically, and numerous methods examining ecology, behaviour and genetics have been used to discover cryptic species (e.g. recognition of distinctive mating signals in various animals; review in HOWARD & BERLOCHER 1998, variability in allozyme markers; review in AVISE 2004, etc.). However, the dis-

covery of cryptic species has increased in recent years in different taxa, habitats, and regions, facilitated by the use of DNA barcoding among other methods (e.g. PFENNINGER & SCHWENK 2007; ZAKŠEK et al. 2009; PAULS et al. 2010; JACKSON et al. 2014; WEISS et al. 2014). Expectably, high cryptic diversity occurs in poorly surveyed and geographically isolated regions, such as the Balkan Peninsula (e.g. FRANCUSKI et al. 2011; KLOBUČAR et al. 2013; TSUOMANI et al. 2013). In fact, many cryptic species have been discovered recently in different freshwater taxa in the Balkans, including fishes (TSUOMANI et al. 2013), crayfishes (KLOBUČAR et al. 2013), amphipods (WEISS et al. 2014), and caddisflies (OLÁH et al. 2012).

Within Europe, highest species diversity and endemism of Trichoptera (caddisflies) fauna are recorded in ecoregions (sensu ILLIES 1978) covering diverse mountain areas (e.g. Iberic-Macaronesian Region [ER1], Italy, Corsica and Malta [ER3], Hellenic Western Balkan [ER6], The Carpathians [ER10], Alps [ER4] etc.; GRAF et al. 2008; GRAF & SCHMIDT-KLOIBER 2011). In particular, high numbers of endemic species are common in cold adapted montane groups, such as Drusinae (Limnephilidae) (MALICKY 2005; GRAF et al. 2008; OLÁH 2010, 2011; KUČINIĆ et al. 2011). Considering the remarkable distribution patterns of Drusinae (GRAF et al. 2008) and their complex evolutionary history (PAULS et al. 2006; PREVIŠIĆ et al. 2009, 2014), more endemics and cryptic species are likely to be discovered, particularly in poorly explored areas, such as the Balkans.

The subfamily Drusinae comprises eight genera, including *Ecclisopteryx* Kolenati, 1848, with five species (SCHMID 1956; PAULS et al. 2008). The genus is restricted to Europe. Three species have relatively wide and partially overlapping ranges: *Ecclisopteryx dalecarlica* Kolenati, 1848, *Ecclisopteryx guttulata* (Pictet, 1834) and *Ecclisopteryx madida* (McLachlan, 1867) (GRAF et al. 2008; GRAF & SCHMIDT-KLOIBER 2011; Fig. 1A). *Ecclisopteryx dalecarlica* was previously reported from throughout the Western Balkans (e.g., Slovenia: URBANIĆ 2004; Croatia: PREVIŠIĆ & POPIJAČ 2010; VUČKOVIĆ 2011; VUČKOVIĆ et al. 2011; Bosnia and Herzegovina: STANIĆ-KOŠTROMAN 2009; Kosovo: IBRAHIMI 2011). In older literature, however, *E. guttulata* was listed from only part of the Western Balkan region (e.g., Bosnia: RADOVANOVIĆ 1935; MARINKOVIĆ-GOSPODNETIĆ 1970).

The remaining two *Ecclisopteryx* species are endemic to the Karawanken and Julian Alps (*Ecclisopteryx asterix* Malicky, 1979) and the Lessinian Alps (*Ecclisopteryx malickyi* Moretti, 1991) (GRAF et al. 2008, 2011; GRAF & SCHMIDT-KLOIBER 2011; Fig. 1B). Monophyly of the genus *Ecclisopteryx* within Drusinae was rejected by a multigene phylogenetic study on the subfamily (PAULS et al. 2008). *Ecclisopteryx* species formed two distant lineages consisting of closely related species (*E. madida* + *E. guttulata* + *E. dalecarlica* and *E. malickyi* + *E. asterix*) that were corroborated by larval morphology and geographic distribution (PAULS et al. 2008; GRAF et al. 2011).

Ecclisopteryx specimens similar to *E. dalecarlica* were collected in eastern Bosnia and Herzegovina and

Dalmatia (southern Croatia). Comparison of morphological characters and molecular genetic sequence data [mitochondrial cytochrome oxidase *c* subunit I (mtCOI) and ribosomal large subunit (mtLSU = 16S) and nuclear wingless (nuWG)] with *Ecclisopteryx* specimens from other parts of the Western Balkan region and central Europe enabled us to distinguish two new *Ecclisopteryx* species that were previously overlooked and/or misidentified. Thus, in the current paper we describe the morphological features of males and females as well as the larvae of two new species. We also summarise the most important morphological characteristics enabling their identification and successful separation from each other and the other Drusinae. Moreover, we define distribution ranges of these cryptic species in the Western Balkans and discuss distribution patterns of the more widespread *E. dalecarlica* and *E. guttulata*.

2. Material and methods

2.1. Material

Specimens of *Ecclisopteryx keroveci* sp.n. were collected at the mouth of the Jabučica River where it joins the Sutjeska River (Table 1). Adults were collected using a UV light trap and larvae were collected by handpicking. Adults of *Ecclisopteryx ivkae* sp.n. were collected in the Glavaš spring and 2 sites in the upper reach of the Cetina River using a sweeping net (Table 1). Larvae were collected in the Glavaš spring by handpicking.

Collected specimens were stored in 70 and 96% EtOH for morphological and molecular analysis, respectively. All collected specimens are deposited in the Faculty of Science, University of Zagreb (Croatia), the Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and Applied Life Sciences, Vienna (Austria), and the Biology Centre, Oberösterreichisches Landesmuseum, Linz (Austria). Terminology for larval morphological features follows WIGGINS (1998) and WARINGER & GRAF (2011). Nomenclature of primary setae and setal areas follows WIGGINS (1998). Nomenclature of male terminalia follows NIELSEN (1957).

To delineate the two new species from remaining *Ecclisopteryx* species and maximise geographic coverage, we compared *Ecclisopteryx* specimens from the entire Western Balkan region in the current study (Table 1). For both morphological characters and mtCOI, mtLSU and nuWG sequence data, we also compared *E. dalecarlica* specimens from Northern, Central and Eastern Europe and *E. guttulata* from Central Europe (Table 1). Sequence data were taken from PAULS et al. (2008) and PREVIŠIĆ et al. (2014); additional specimens used for comparative morphology were provided by the many colleagues listed in Table 1 and the Acknowledgements.

Adult male and female of *E. dalecarlica* were redrawn based on material collected in Norway (Table 1).

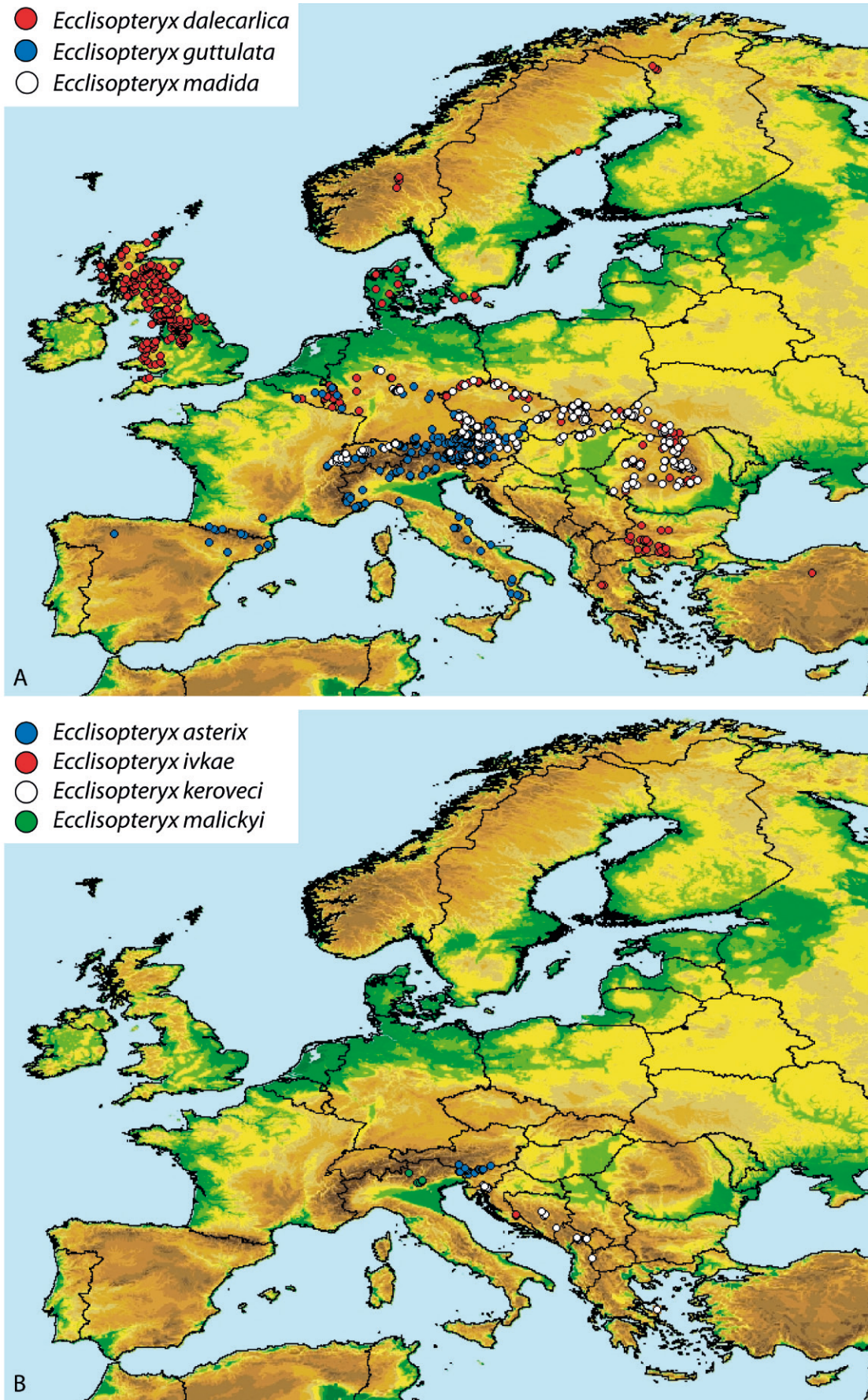


Fig. 1. Distribution of *Ecclisopteryx* species; **A:** widespread and **B:** endemic species (based on data from the Distribution Atlas of European Trichoptera [DAET; the BioFresh EU project–Biodiversity of Freshwater Ecosystems: Status, Trends, Pressures and Conservation Priorities]).

Table 1. Information on specimens used in this study. *Historical collection, geographic reference is provided for the closest town, Pazarić, as the exact location is not known; **Outgroup taxa in phylogenetic analysis. LT = locus typicus, IM = imagines, M = male, F = female, LV = larvae.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
<i>Ecclosopteryx dalecarlica</i> Kolenati, 1848											
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560	1 IM/M						Graf	this study
AT, Ritterkamp, Kamp Aqem	N 48.52887	E 15.10722	581	4 Lv						Graf	this study
SK, Vysoké Tatry (high Tatra Mts.), Podbanské, Béla river	N 49.14017	E 19.90247	934	5 IM (3M + 2F)						Graf	this study
D, Spessart, Jossa below Sahlensee	N 50.218548	E 9.484726	290		Lv	ED001	EU215112	EU215165	EU215218	Lohse	PAULS et al. 2008
D, Spessart, Jossa below Sahlensee	N 50.218548	E 9.484726	290		Lv	ED002	EU215113	EU215166	EU215219	Lohse	PAULS et al. 2008
N, Hedmark, Folidal, Streitlie	N 62.09520	E 9.96412	804		IM/M	fEca0801M	KM001830	KM001819	KM001825	Andersen	this study
N, Hedmark, Folidal, Streitlie	N 62.09520	E 9.96412	804	2 IM (1M + 1F)		fEca0802F	KM001829	KM001820	KM001826	Andersen	this study
RO, Jarcu Mts., Poiana Mărului	N 45.403056	E 22.540556	638		IM/F	Dsp023	EU215106	EU215159	EU215212	Ballint	PAULS et al. 2008
RO, Jarcu Mts., Poiana Mărului	N 45.403056	E 22.540556	638		IM/F	Dsp031	EU215107	EU215160	EU215213	Ballint	PAULS et al. 2008
RO, Caribaba, Tibau Valley	N 47.464228	E 24.842512	980		IM/F	fEca0701F	KM001831	KM001818	KM001824	Neu	this study
BG, Rhodope Mts., stream close to Teshel, Devin	N 41.666389	E 24.365556	870		IM/F	fEca0901F	KM001828	KM001818	KM001827	Neu	this study
RO, Făgăra Mts., Bălea Valley	N 45.665299	E 24.554063	800		IM/F	fEca0601F					this study
RO, Făgăra Mts., Bălea Valley	N 45.665299	E 24.554063	800		IM/F	fEca0602F				Balint, Theissinger & Taubmann	this study
RO, Sibiului Mts (Cindrei), Râu Sadu	N 45.624	E 24.033	770		IM/M	fEca0401M					this study
RO, Sibiului Mts (Cindrei), Râu Sadu	N 45.624	E 24.033	770		IM/F	fEca0402F					this study
RO, Muntii Ciucas, Sacele, stream close to Babarunca	N 45.513889	E 25.848333	960		IM/M	fEca0501M	KM001833		KM001822	Neu	this study
RO, Muntii Ciucas, Sacele, stream close to Babarunca	N 45.513889	E 25.848333	960		IM/F	fEca0502F	KM001832		KM001823	Neu	this study
<i>Ecclosopteryx ivkai</i> Previšić, Graf & Vitecek, sp.n.											
HR, Cetina River, Cveni most	N 43.960347	E 16.429489	370		IM/M	fEca0101M	KM001813	KM001799	KM001806	Previšić	this study
HR, Cetina River, Cveni most	N 43.960347	E 16.429489	370	12 IM (5M + 7F)	IM/M	fEca0102M	KM001815	KM001801	KM001808	Previšić	this study
HR, Cetina River, Cveni most	N 43.960347	E 16.429489	370		IM/F	fEca0101F	KM001812	KM001798	KM001805	Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386	12 IM (6M + 6F)	IM/F	fEca0102F	KM001814	KM001800	KM001807	Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386		Lv	fDsp3301L	KM001811	KM001797	KM001804	Kučinić & Previšić	this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386	8 Lv	Lv	fEca1201L	KM001816	KM001802	KM001809		this study
HR, Cetina River, Glavaš spring (LT)	N 43.976697	E 16.430150	386		Lv	fEca1202L	KM001817	KM001803	KM001810		this study
HR, Cetina River, Vrnalić	N 43.936253	E 16.443441	375	1 IM/M						Previšić	this study

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtL.SU		
<i>Ecclosipteryx keroveci</i> Previšić, Graf & Vitecek, sp.n.											
*BIH, Pazaric, SW of Sarajevo, valley of Krupa River	N 43.786	E 18.166	630	1 IM/M						Winneguth	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30101M	KM001785	KM001761	KM001773		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30102M	KM001786	KM001762	KM001774		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30103M	KM001787	KM001763	KM001775		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30105M	KM001788	KM001764	KM001776		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fdS30106F	KM001789	KM001765	KM001777	Ivković, Mihaljević,	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765	40 IM (20M + 20F)	IM/F	fdS30109F	KM001790	KM001766	KM001778	Miliša & Previšić	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30113M	KM001851				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/M	fdS30114M	KM001852				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fdS30115F	KM001853				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		IM/F	fdS30116F	KM001854				this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E1JAL1	KM001847	KM001836	KM001842		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765	10 Lv	Lv	E1JAL2	KM001848	KM001837	KM001843	Graf & Previšić	this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E2JAL1	KM001849	KM001838	KM001844		this study
BIH, Sutjeska NP, mouth of the Jabučica River (LT)	N 43.29022	E 18.61733	765		Lv	E2JAL2	KM001850	KM001839	KM001845		this study
BIH, Željznica River, upper reach	N 43.898666	E 17.952901	650	2 IM/M						Stanić-Koštroman	STANIĆ-KOŠTROMAN 2009
HR, Čabranka, spring reach	N 45.60126	E 14.64043	589	4 IM (3M + 1F); 2 Lv	IM/M	EdIM1	FJ002686		FJ002818	Bokan, Kučinić, Popijač & Previšić	PREVIŠIĆ et al. 2009; this study

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
<i>Ecclisopteryx keroveci</i> Previšić, Graf & Vitecek, sp.n.											
HR, Kupa, bridge before Čabranka mouth	N 45.524417	E 14.700383	292	2 IM (1M + 1F)						Popijač	PREVIŠIĆ & POPIJAČ 2010
HR, Velika Belica, bridge at Kuželj	N 45.475514	E 14.805144	242	1 IM/M		fEda1001M	KM001794	KM001770	KM001782	Popijač	PREVIŠIĆ & POPIJAČ 2010
KS, Pejë, Lumbardhi and Pejë's rivers	N 42.66128	E 20.25958	587	2 IM (1M + 1F)						Ibrahimi	IBRAHIMI 2011
MK, Radika river, Monastery St. Jovan Bigorski NE of Debar	N 41.623611	E 20.606111	694	2 IM/M						Chvojka	this study
MN, Brodovac, right tributary of Peručica	N 42.68587	E 19.73636	960	2 IM (1M + 1F)	IM/M	fEda0201M	KM001791	KM001767	KM001779	Miliša	this study
MN, Brodovac, right tributary of Peručica	N 42.68587	E 19.73636	960		IM/F	fEda0202F	KM001792	KM001768	KM001780	Miliša	this study
MN, Grncar, Gusinje	N 42.565944	E 19.833389	922	2 IM (1M + 1F)	IM/F	fEda1101F	KM001795	KM001771	KM001783	Graf	this study
MN, Grncar, Gusinje	N 42.565944	E 19.833389	922		IM/M	fEns0101M	KM001796	KM001772	KM001784	Graf	this study
MN, Peručica	N 42.69472	E 19.75661	884	5 IM (4M + 1F)	IM/M	fEda0301M	KM001793	KM001769	KM001781	Previšić	this study
<i>Ecclisopteryx asterix</i> Maicky, 1979											
AT, Soboth, Krumbach tributary	N 46.716667	E 15.066667	1130		Lv	EastDDes002	EU215111	EU215164	EU215217	Graf & Pauls	PAULS et al. 2008
AT, Karawanken, Babniakgraben	N 46.5201	E 14.2345	683		Lv	East003	EU215110	EU215163	EU215216	Graf	PAULS et al. 2008
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717		IM/M	fEas0101M	KM001760	KM001757	KM001754	Olah	this study
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717	4 IM (3M + 1F)	IM/M	fEas0102M		KM001758	KM001755	Olah	this study
SLO, Julian Alps, Radovna stream	N 46.4303	E 13.963	717		IM/M	fEas0103M		KM001759	KM001756	Olah	this study
<i>Ecclisopteryx guttulata</i> (Pictet, 1834)											
AT, Ybbs at Lunz	N 47.856	E 15.023	600	12 Lv						Graf	this study
AT, Jogland, Lafnitz tributary	N 47.43	E 15.48	1170		IM/M	Egut009	EU215114	EU215167	EU215220	Graf & Pauls	PAULS et al. 2008
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/M	fEgu0101M	KM001750	KM001742	KM001746	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220	4 IM (3M + 1F)	IM/F	fEgu0102F	KM001751	KM001743	KM001747	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/M	fEgu0103M	KM001752	KM001744	KM001748	Graf	this study
ES, Pyrenees, Val d'Aran, Salardu	N 42.706361	E 0.896944	1220		IM/M	fEgu0104M	KM001753	KM001745	KM001749	Graf	this study
<i>Ecclisopteryx madida</i> (McLachlan, 1867)											
RO, Bucegi Mts., Valea Dobresti, Cariera Lespezi, Pietrele Albe	N 45.287451	E 25.405600	889		IM/M	EM001	EU215115	EU215168	EU215221	Pauls & Ujvarosi	PAULS et al. 2008
SVK, Hronec	N 48.8	E 19.6	550		Lv	EM002		KM001727	KM001733	Graf	this study
SVK, Hronec	N 48.8	E 19.6	550		Lv	EM003		KM001728	KM001734	Graf	this study
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM004	EU215116	KM001729	EU215222	Graf	PAULS et al. 2008
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM005		KM001730	KM001735	Graf	this study
AT, Nockberge, St. Oswald stream	N 46.864432	E 13.787671	1570		IM/M	EM006	KM001739	EU215169	KM001736	Graf	PAULS et al. 2009

Table 1. continued.

Locality (country, name)	Longitude dec.	Latitude dec.	Altitude (m)	No of specimens and stage/sex		Specimen Code	GenBank Accession Nos			Collector	Publication
				Morphology	Molecular genetic analyses		mtCOI	nuWG	mtLSU		
<i>Ecclisopteryx madida</i> (McLachlan, 1867)											
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560		IM/M	fEma0101M	KM001740	KM001731	KM001737	Graf	this study
AT, Bruck/Lafnitz	N 47.4396	E 15.9138	560		IM/M	fEma0102M	KM001741	KM001732	KM001738	Graf	this study
<i>Ecclisopteryx malickyi</i> Moretti, 1991											
IT, springbrook near Camposilvano SE of Rovereto, Monti Lessini, Trentino	N 45.748231	E 11.151095	1171		IM/F	Ema001	EU215223	EU215170	EU2015117	Graf	PAULS et al. 2008
IT, springbrook near Camposilvano SE of Rovereto, Monti Lessini, Trentino	N 45.748231	E 11.151095	1171		Lv	Ema002	KM001726		KM001725	Graf	this study
** <i>Drusus discolor</i> (Rambur, 1842)											
HR, Čabranka, spring reach	N 45.60126	E 14.64043	589		IM/M	DdCAIM1	KC881331	KM001835	KM001841	Sivec	PREVIŠIĆ et al. 2014, this study
** <i>Allogamus uncatius</i> (Brauer, 1857)											
AT, Gampadelsbach, Voralberg	N 47.03638	E 9.89972	1555		IM/M	AUn003	KM001846	KM001834	KM001840	Graf	this study

Illustrations were prepared as described by THOMSON & HOLZENTHAL (2010). Briefly, pencil drawings were produced using a camera lucida mounted on a compound microscope, and digitally edited and inked.

2.2. DNA extraction and PCR amplification

DNA extraction and amplification were performed as outlined by PAULS et al. (2008) and PREVIŠIĆ et al. (2009) for the 541-bp-long fragment of the mitochondrial cytochrome oxidase *c* subunit I (mtCOI) using primers S20 and Jerry (SIMON et al. 1994; PAULS et al. 2006), a 346-bp-long fragment of the nuclear wingless gene (nuWG) using primers WGbDrrev (5'-ACCCTCTCC-CGCARCACATTGAG) and WGbDrfwd 5'-CTTGCTG-GATGCGTCTGCC), and a 362-bp-long fragment of the mitochondrial large ribosomal subunit gene (mtLSU) using primers LeptoF and LeptoR (MALM & JOHANSON 2008). Sequences were edited manually using the program Geneious R7 (Biomatters Ltd., New Zealand) and aligned using MAFFT v.7 (KATOH & STANDLEY 2013). Sequences were deposited in GenBank under accession nos: KM001724–KM001854. In addition, published sequences of all *Ecclisopteryx* species (PAULS et al. 2008; Table 1) were included in the alignment and intra- and interspecific uncorrected *p*-distances were calculated in Mega 4.0.1 (TAMURA et al. 2007) based on the 541-bp-long fragment of the mtCOI. For *p*-distances a colour heat map was drawn using the package ‘pheatmap’ in R (version 3.0.2, R CORE TEAM 2013).

2.3. Phylogenetic reconstruction

To examine *Ecclisopteryx* species delineation and association of specimens from the Western Balkans, we inferred a phylogeny using all available mtCOI, mtLSU and nuWG sequences of *Ecclisopteryx* species (Table 1). As outgroups taxa we used *Drusus discolor* (Limnephilidae: Drusinae) and *Allogamus uncatius* (Limnephilidae: Stenophylacini) (Table 1). According to the Akaike Information Criterion (AIC) test implemented in MrModeltest 2.2 (NYLANDER 2004) the following models of DNA substitution were identified as best-fit for particular data sets: mtCOI: Hasegawa–Kishino–Yano + Invariant + Gamma (HKY + I + G), mtLSU: General time reversible + Gamma (GTR + G), and nuWG; Hasegawa–Kishino–Yano + Gamma (HKY + G). The phylogeny was estimated using a Bayesian Metropolis-coupled Monte Carlo Markov Chain (MCMCMC) method with the program MrBayes 3.2. (RONQUIST & HUELSENBECK 2003) using concatenated sequences of the three genes. The matrix contained 6.5% missing data with 9 individuals missing one and 4 individuals missing two gene regions, respectively (Table 1). Two parallel runs were performed with four chains each (10 million generations, sampling every 1000th generation). The likelihood scores were plotted against generation time using Tracer 1.4 (DRUMMOND &

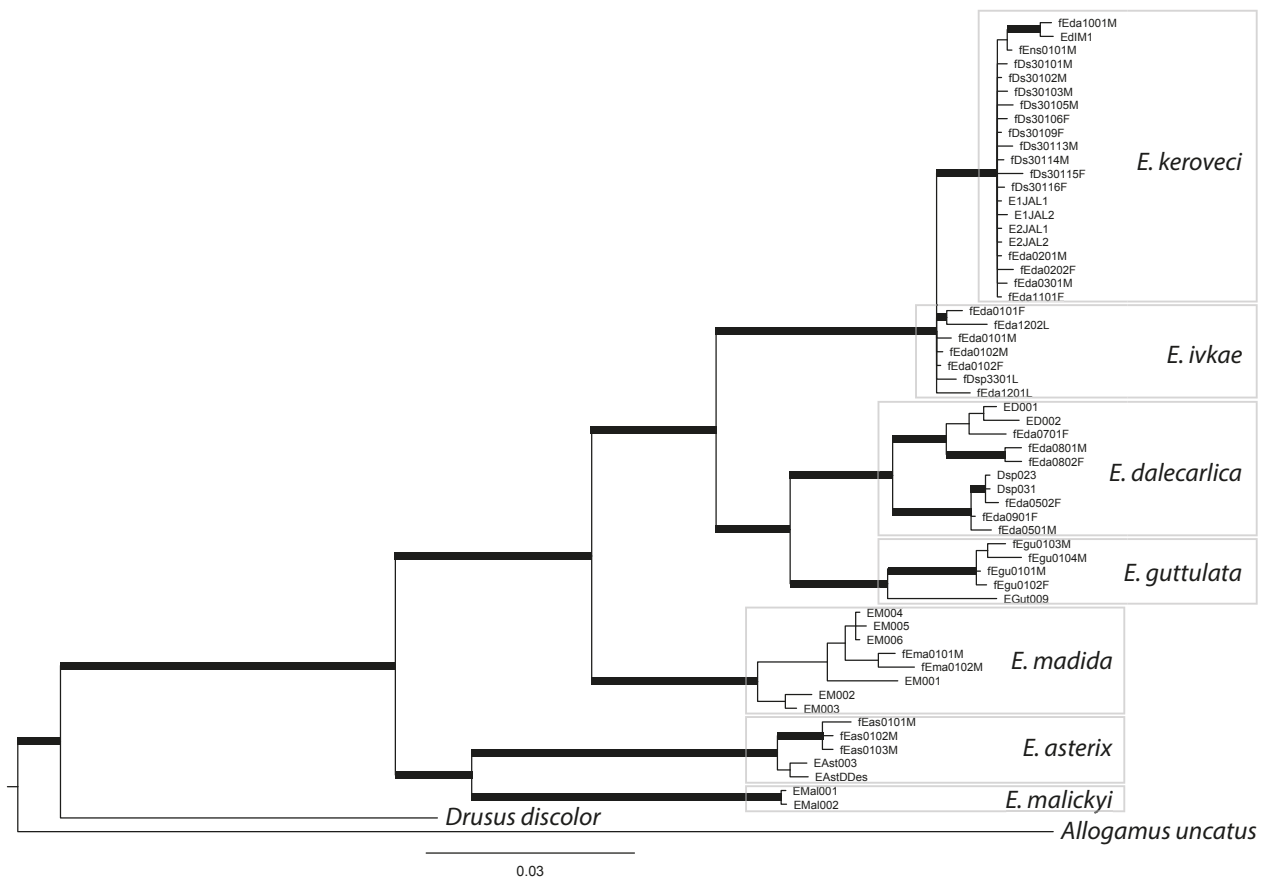


Fig. 2. Rooted Bayesian phylogenetic tree of seven *Ecclisopteryx* species based on the partial mitochondrial (mtCOI, mtLSU) and nuclear (nuWG) gene sequences. *Drusus discolor* and *Allogamus uncatus* were used as outgroup taxa. Bold lines bear nodes with posterior probabilities ≥ 0.95 .

RAMBAUT 2007) to determine the number of generations needed to reach the stationary phase. Consequently, the initial 3000 trees were discarded as burn-in and the remaining trees used to create a 50% majority rule consensus tree.

3. Results

3.1. *Ecclisopteryx* species delimitation

In a B/MCMCMC phylogeny based on concatenated partial sequences of mtCOI, mtLSU and nuWG, with the exception of *E. ivkae* sp.n., monophyly of each putative *Ecclisopteryx* species was highly supported ($pp \geq 0.95$; Fig. 2). *Ecclisopteryx dalecarlica* specimens from different parts of its range (i.e., Norway, Germany, Bulgaria and Romania, Table 1) formed a highly supported clade, as did *E. guttulata* specimens ($pp \geq 0.98$; Fig. 2). All specimens of *E. keroveci* sp.n. including the *Ecclisopteryx* larvae collected from the Jabučica River also formed a highly supported monophyletic clade ($pp = 0.99$; Fig. 2). *Ecclisopteryx keroveci* sp.n. and *E. ivkae* sp.n. formed a highly supported monophyletic clade ($pp = 1$; Fig. 2);

however, *E. ivkae* sp.n. haplotypes occurred in a basal polytomy, thus this species was not recovered as monophyletic (Fig. 2). *Ecclisopteryx dalecarlica* + *E. guttulata* were the sister clade to *E. keroveci* sp.n. + *E. ivkae* sp.n., and this relationship was also highly supported ($p = 0.99$; Fig. 2).

Mitochondrial COI haplotypes (fragment length 440 bp) of *E. keroveci* sp.n. adults and larvae sampled at the mouth of the Jabučica River were either identical or differed at a maximum of 5 nucleotide positions (= 1.1%). Overall, mtCOI haplotypes of *E. keroveci* sp.n. differed across the whole region by at most 8 nucleotide positions (21 specimens); hence, intraspecific uncorrected p -distances ranged from 0–1.8% (Fig. 3). A similar case was observed in *E. ivkae* sp.n., as mtCOI haplotypes from the two sequenced sampling sites (both at the Cetina River) were found to differ by maximally 4 nucleotide positions (7 specimens), with intraspecific uncorrected p -distance ranging from 0–0.9% (Fig. 3). Overall, interspecific uncorrected p -distances of mtCOI haplotypes ranged from 1.6–2.7% between *E. keroveci* sp.n. and *E. ivkae* sp.n., 8.2–10.5% between *E. dalecarlica* and *E. keroveci*, and 7.7–9.5% between *E. dalecarlica* and *E. ivkae* sp.n. (Fig. 3). Uncorrected p -distances of the same mtCOI fragment between the other *Ecclisopteryx* species ranged from 6.1–13.2% (Fig. 3).

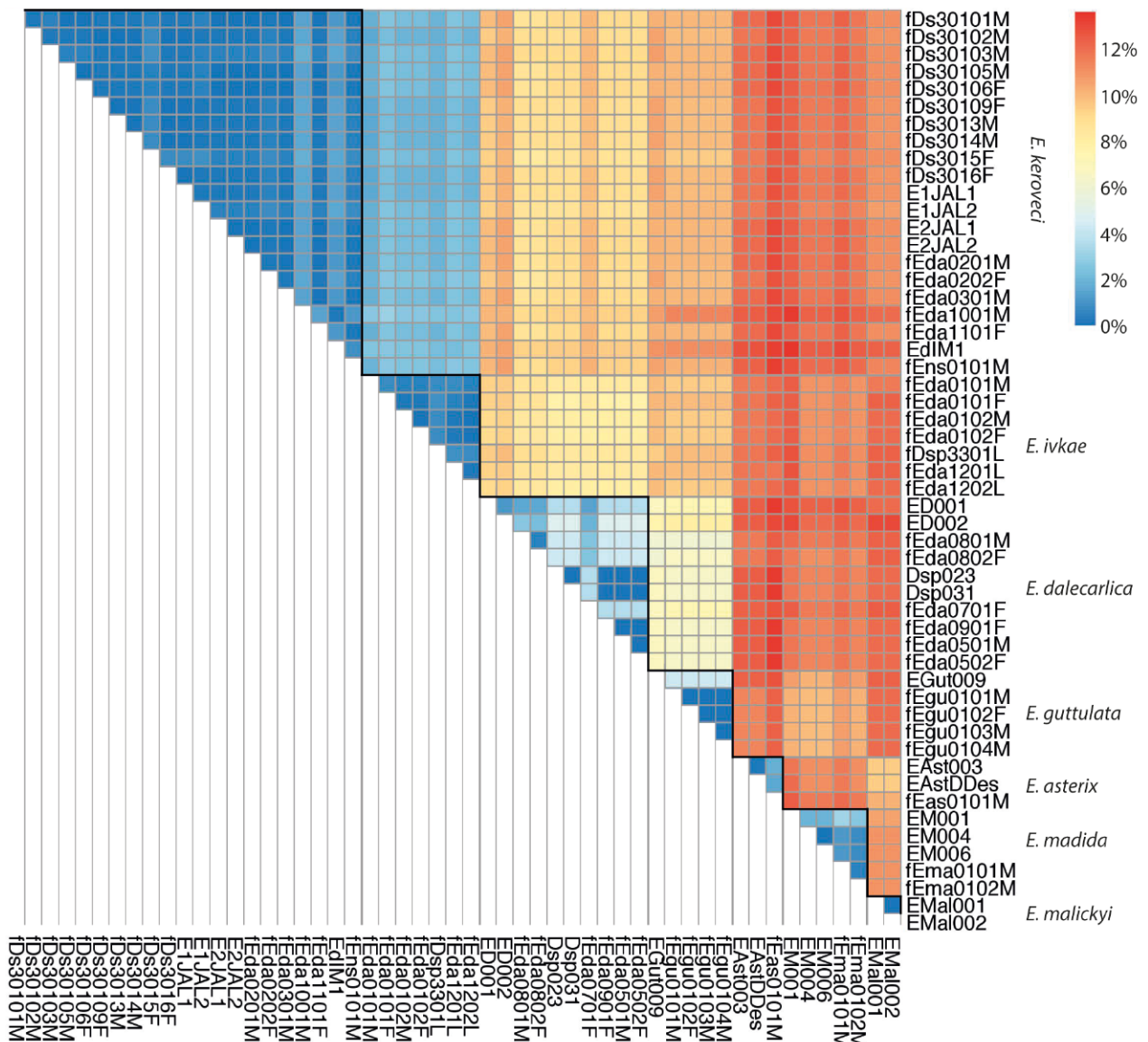


Fig. 3. Colour heat map showing inter- and intraspecific uncorrected *p*-distances of the partial mitochondrial COI gene sequence (440 bp) between seven *Ecclisopteryx* species. Intraspecific *p*-distances are outlined by the black line. For detailed information on haplotypes see Table 1.

3.2. Description of *Ecclisopteryx keroveci* Previšić, Graf & Vitecek sp.n.

Adults. General appearance: light brown; sternites and tergites brown; cephalic, thoracic and abdominal setal areas pale, yellowish; body setation light brown; legs light brown; haustellum and intersegmental teguments pale, whitish. Male maxillary palps 3-segmented. Spur formula (male and female): 1-2-3. Forewing length: male 9.8–12.1 mm (N=20), female 10–12.5 mm (N=20).

Male terminalia (Fig. 4A–E): Tergite VIII brown with lighter areas around alveoli and somewhat darker stripe medially lacking setae; setation concentrated posteriorly, around spinate area, anterior part of tergite VIII with few setae. Spinate area mushroom-shaped in dorsal view and flanked by membranous areas.

Segment IX rhombus-shaped in lateral view, transversely dilated: in ventral view distance from lateral most point of segment IX to straight anteroposterior line originating from lateral most point of inferior appendages is approximately 10% of total width of segment IX on each side.

Superior appendages (cerci sensu SNODGRASS 1935) in lateral view round, simple, without further modifications. Intermediate appendages (paraprocts sensu SNODGRASS 1935) reduced as typical for genus, membranous dorsal and ventral protuberances rounded. Inferior appendages (gonopods sensu SNODGRASS 1935) in lateral view bipartite with well sclerotized dorsal part and less sclerotized, seemingly membranous, ventral part; dorsal part prolonged caudally with distinct dorsally turned tip: with broad and shallow lateral concavity, somewhat bifurcated with 2 rounded tips of unequal length (dorsal

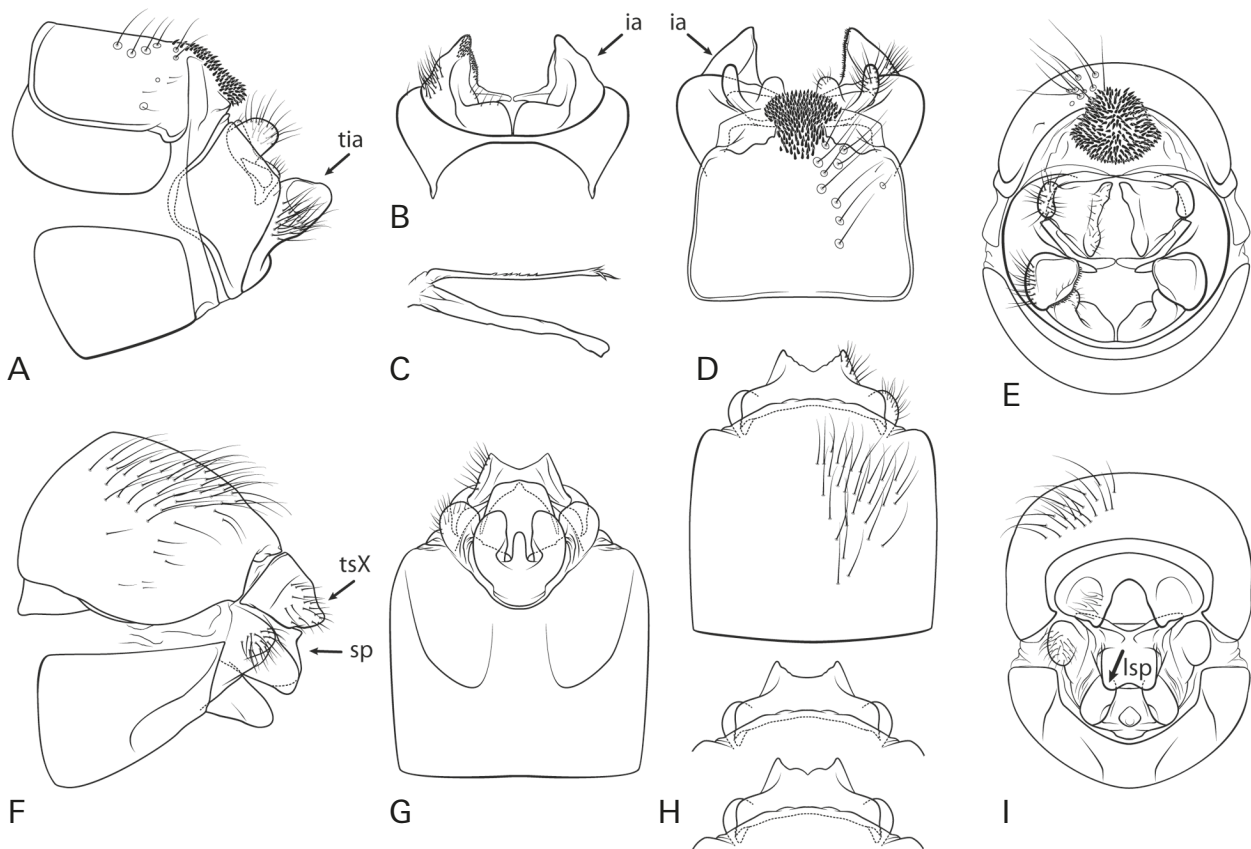


Fig. 4. *Ecclisopteryx keroveci* sp.n.; male genitalia, **A**: lateral view, **B**: ventral view, **C**: aedeagus and parameres, **D**: dorsal view and **E**: caudal view; female genitalia, **F**: lateral view, **G**: ventral view, **H**: dorsal view and variability in female genitalia, dorsal view and **I**: caudal view. — Abbreviations: tia=tip of inferior appendages, ia=inferior appendages, tsX=tip of segment X, sp=supragenital plate, lsp=lobes of supragenital plate.

one shorter than ventral one), in dorsal view triangular and slender with tips separated by small indentation, caudal parts and median margins strongly sclerotized and covered with spines; setation of appendices inferiores concentrated laterally on dorsal part, forming setal brush.

Aedeagus slender with distinct terminal protuberance (in lateral view only) and parameres of equal length. Parameres fused at their bases, with 2 major concentrations of thorn-like spines on dorsal surface; several well developed distal thorn-like spines and medial group of smaller thorn-like spines, the latter with bulbous bases.

Female terminalia (Fig. 4F–I): Lateral lobe of segment IX membranous, triangular in lateral view with dorsal sclerotized setose part, the latter evenly rounded in dorsal and ventral view. Segment X wider proximally than distally, in dorsal view with 2 lateral lobes and median triangular excision of varying shape (Fig. 4H); lateral lobes laterally slightly concave, tips sharp and distinct in dorsal and ventral views, curved somewhat dorsally in lateral view; approximately as long as supragenital plate; ventrally unsclerotized. Supragenital plate in lateral view quadrangular with small dorsal process; in ventral view quadrangular; in caudal view quadrangular with 2 indistinct ventral lobes. Vulvar scale with 3 lobes in ventral view: 2 lateral lobes, roundly oval with converging tips;

1 median, well developed, about half as long as lateral lobes and of greater length than width.

Fifth instar larvae. Body length of larva 10–13.1 mm, head width 1.3–1.5 mm (N=10). Case slightly curved, consisting of mineral particles (Fig. 7A), 10.2–13.7 mm long, slightly attenuating posteriorly (width at anterior opening 2.8–4.5 mm and at posterior opening 1.4–2.9 mm).

Head: Light to chestnut brown with dark muscle attachment spots, with yellowish-white rings around the eyes (Fig. 7B). 18 pairs of primary setae (# 1, 4, 10, 11 white; 13, 16 light brown, rest dark brown) and additional spines on parietalia present (i.e. between eyes and anterior head margin; N=12–20, light brown, 100–300 µm length) (Figs. 7B, 8A). Frontoclypeus bell-shaped; carinae bearing antennae; ventral apotome bell-shaped, yellowish-brown. Mandibles typical for grazers, lacking teeth.

Thorax: Pronotum light to chestnut brown, with dark muscle attachment spots, posterior margin thickened and darkly striped (Fig. 7B,C). Dorsal profile in lateral view lacking distinct ridge, in dorsal and lateral view medially with a delicate step (Figs. 7B, 8B). Two setal rows along anterior border of pronotum: (1) dense fringe of short, curved, fine, yellow setae; (2) widely-spaced, continuous row of long, straight, dark setae meeting at anterior

pronotal midline; in total, 110–130 dark setae of varying lengths (100–300 µm) distributed over each pronotal half. Small, white recumbent setae present on pronotal surface. Prosternal horn present.

Mesonotal sclerites light brown, with dark muscle attachment spots and lateral and posterior margins darkly sclerotised (Fig. 7C). Anterior mesonotal setal group (*sa1*) consisting of 7–13 setae; posterior group, *sa2* (26–41 setae) and lateral group, *sa3* (30–40 setae) connected, not clearly separated.

Metanotum divided into 3 pairs of light brown sclerites. Anteromedian sclerites (*sa1*) ellipsoid, distance between them smaller than their length (Fig. 7C); 19–30 setae per sclerite. Posteromedian sclerites small (*sa2*), with 16–21 setae; lateral sclerites (*sa3*) with 30–40 setae. A row of setae present between posteromedian sclerites (*sa2*); a small setal group of 20–30 setae present between each lateral (*sa3*) and posteromedian sclerite.

Legs light brown to yellowish with numerous setae on coxae, trochanters and femora; tibiae and tarsi bearing less setae. Foreleg coxa, femur and tibia each wider than those of mid- and hind legs. Whole dorsal and ventral margins of all coxae and femora covered with setae. Forefemora each with 3–6 yellow and 2–5 dark setae on ventral-edge, midfemora each with 6–13 dark and hind femora each with 6–10 dark setae on ventral edge. Additional setae present at both anterior and posterior faces of all femora; ventral trochanteral brush present at distal section of foretrochanters. Setae present at ventral margin (proximal and distal sections) of all trochanters. Dorsal setae only at distal third of mid- and hind tibiae.

Abdomen: First abdominal segment with 1 dorsal and 2 lateral fleshy protuberances. Setal areas *sa1*, *sa2* and *sa3* fused, resulting in continuous transverse row of setae anterior to the dorsal protuberance, reaching to the dorsal section of each lateral protuberance. Setal group posterior to dorsal protuberance lacking (Fig. 7C). Lateral protuberances lacking posterior sclerites; in front of each lateral protuberance lies continuous band of anterolateral setae, linking with each dorsal and ventral *sa3* setal group. First abdominal sternum with fused setal areas *sa1*, *sa2* and *sa3*, resulting in a continuous field of setae, with small sclerites at the base of individual setae.

Single filamentous gills present on segments II–VII. Dorsal pre- and post-segmental gills present on segments II–VII; ventral pre- and post-segmental gills present on segments II–VII. Lateral pre-segmental gills present on segments II and V and post-segmental gills on segments II–IV. Lateral fringe extends from anterior border of segment III to anterior border of segment VIII. Number of posterodorsal setae on segment VIII 2–6.

Etymology. The name of *keroveci* was given in honour of our colleague, Prof. Dr. Mladen Kerovec, who has enthusiastically supported our studies of Drusinae in the Balkans for many years.

Type material. *Ecclisopteryx keroveci* sp.n.: Holotype ♂: Bosnia and Hercegovina, mouth of Jabučica River, N 43.29022 E

18.61733, 765 m asl, 04.vii.2012, leg. Previšić A., Ivković M., Mihaljević Z., Miliša M.; deposited in the Biology Centre, Oberösterreichisches Landesmuseum, Linz, Austria. Paratypes: 30 ♂ and 49 ♀, same data; deposited in the first author's collection at the Faculty of Science in Zagreb. 10 5th instar larvae, same location, 14.v.2008 and 02.vi.2009, leg. Previšić A., Graf W.

3.3. Description of *Ecclisopteryx ivkae* Previšić, Graf & Vitecek sp.n.

All morphological characters of adults and 5th-instar larvae identical to *Ecclisopteryx keroveci* sp.n. except:

Adults. General appearance: brown; cephalic, thoracic and abdominal setal areas pale, yellowish to light brown; body setation light brown to brown. Forewing length: male 10.4–12.5 mm (N=7), female 12.4–14.0 mm (N=9).

Male terminalia (Fig. 5A–E): Tergite VIII brown, lacking a darker median stripe; setation evenly distributed over the whole surface of tergite VIII with larger setae posteriorly. Superior appendages (cerci sensu SNODGRASS 1935) in lateral view somewhat elongated. Dorsal part of inferior appendages (gonopods sensu SNODGRASS 1935) in lateral view not turned dorsally, with a deep and broad lateral concavity, faintly bifurcated with 2 tips of more or less equal length, in dorsal view broadly triangular with the tips separated by a distinct indentation. Parameres fused at their bases, with 3 major concentrations of thorn-like spines on dorsal surface: several well developed distal thorn-like spines and medial group of smaller thorn-like spines divided into 2 groups of thorn-like spines.

Female terminalia (Fig. 5F–I): Segment X in dorsal view with 2 lateral lobes and a deep and round median excision leaving 2 distinct median protrusions in lateral, ventral and dorsal view; tips of lateral lobes rounded, not curved dorsally in lateral view, somewhat longer than the supragenital plate. Supragenital plate quadrangular in lateral and ventral view; in caudal view hourglass-shaped. Vulvar scale with 3 lobes in ventral view: 2 lateral lobes, quadrangular with converging tips; 1 median lobe, about half as long as the lateral lobes and triangular in shape.

Fifth instar larva. Body length of larva 8.0–9.5 mm, head width 1.5–1.7 mm (N=8). Case slightly curved, consisting of mineral particles (Fig. 7D), 7.5–10.8 mm long, slightly attenuating (width at anterior opening 2.8–4.8 mm and at posterior opening 1.7–1.8 mm).

Head: Brown to brownish black with granular surface sculpturing. Primary setae # 1, 4, 10, 11 white; the remaining setae dark brown; 1–7 dark brown spines on parietalia (200–375 µm length) present (Figs. 7F, 8C,D).

Thorax: Pronotum brown to brownish black with granular surface sculpturing (Fig. 7E). Dorsal profile in lateral view with small but distinct ridge, in dorsal view medially with a delicate step-like structure (Figs. 7F, 8D). In total, 110–120 dark setae of varying lengths (100–300 µm) distributed over each pronotal half. Mesonotum sclerites chestnut brown, with dark muscle attachment

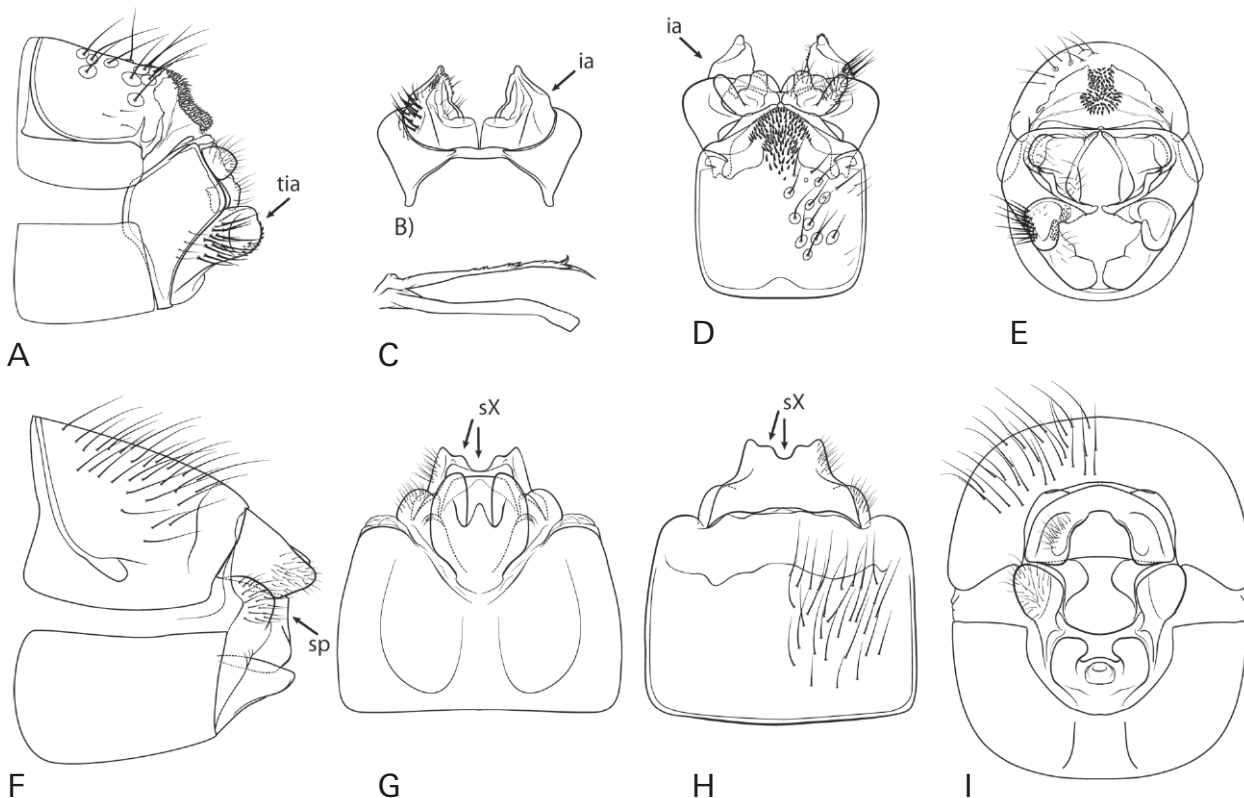


Fig. 5. *Ecclisopteryx ivkae* sp.n.; male genitalia, **A:** lateral view, **B:** ventral view, **C:** aedeagus and parameres, **D:** dorsal view and **E:** caudal view; female genitalia, **F:** lateral view, **G:** ventral view, **H:** dorsal view and **I:** caudal view. — Abbreviations: tia=tip of inferior appendages, ia=inferior appendages, sp=supragenital plate, sX=segment X.

spots and lateral and posterior margins darkly sclerotised (Fig. 7E,F). Anterior mesonotal setal group (*sa1*) consisting of 3–7 setae; posterior group, *sa2* (20–32 setae) and lateral group, *sa3* (28–40 setae) connected, not clearly separated. Anteromedian metanotum sclerites (*sa1*) quadrangular, distance between them smaller than their length (Fig. 7E); 16–28 setae per sclerite. Posteromedian metanotum sclerites small (*sa2*), with 15–19 setae; lateral sclerites (*sa3*) with 20–32 setae. A row of setae present between posteromedian sclerites (*sa2*); a small setal group of 16–22 setae present between each lateral (*sa3*) and posteromedian sclerite.

Legs chestnut brown with numerous setae on coxae, trochanters and femora; tibiae and tarsi with only small number of setae. Forefemora each with 3–4 yellow and 2–5 dark ventral-edge setae, midfemora each with 6–9 dark and hind femora each with 3–7 dark ventral edge setae. Number of posterodorsal setae on segment VIII 4–9.

Etymology. The name of *ivkae* was given in honour of Ivka Previšić, the first author's grandmother.

Type material. *Ecclisopteryx ivkae* sp.n.: Holotype ♂: Cetina River, Glavaš spring N 43.976697 E 16.430150, 386 m asl, 02.vi.2011, leg. Previšić A.; deposited in the Biology Centre, Oberösterreichisches Landesmuseum, Linz, Austria. Paratypes: 4 ♂ and 2 ♀, same data; 1 ♂ and 1 ♀ 31.v.2005, leg. Previšić A.; 1 ♀ 07.vi.2007, leg. Graf W.; 2 ♀ 02.vi.2012, leg. Previšić A.; deposited in the first author's collection at the Faculty of Science in Zagreb. 8 5th instar larvae: same location, 04.x.2013 (N=4, leg. Kučinić M.) and 07.xi.2013 (N=4, leg. Previšić A.).

3.4. Differential diagnosis of *Ecclisopteryx keroveci*, *E. ivkae*, *E. dalecarlica* and other Drusinae species

Adult males. *Ecclisopteryx keroveci* and *E. ivkae* males are morphologically most similar to *E. dalecarlica* males, but differ distinctly in several features. They can be separated using the following key:

- 1 Tips of inferior appendages distinctly indented in lateral view (Fig. 6A; arrow tia), inferior appendages elongate and slender in ventral view (Fig. 6B; arrow ia): ***E. dalecarlica***
- 1' Tips of inferior appendages lacking a distinct indentation in lateral view, inferior appendages more or less triangular and broad in ventral view (Figs. 4A,B & 5A, B; arrows tia & ia): **2, *E. ivkae* & *E. keroveci***
- 2 Posterior edge of tip of inferior appendages ventrally elongated and arched dorsally (Fig. 4A; arrow tia), tips in dorsal view lacking a distinct shoulder (Fig. 4D; arrow ia): ***E. keroveci***
- 2' Posterior edge of tip of inferior appendages more or less straight in lateral view, lacking a clear ventral elongation (Fig. 5A; arrow tia), tips in dorsal view with a distinct shoulder (Fig. 5D; arrow ia): ***E. ivkae***

Adult females. *Ecclisopteryx keroveci* and *E. ivkae* females are morphologically most similar to *E. dalecarlica* females, but differ in several features. Species can be distinguished using the following key:

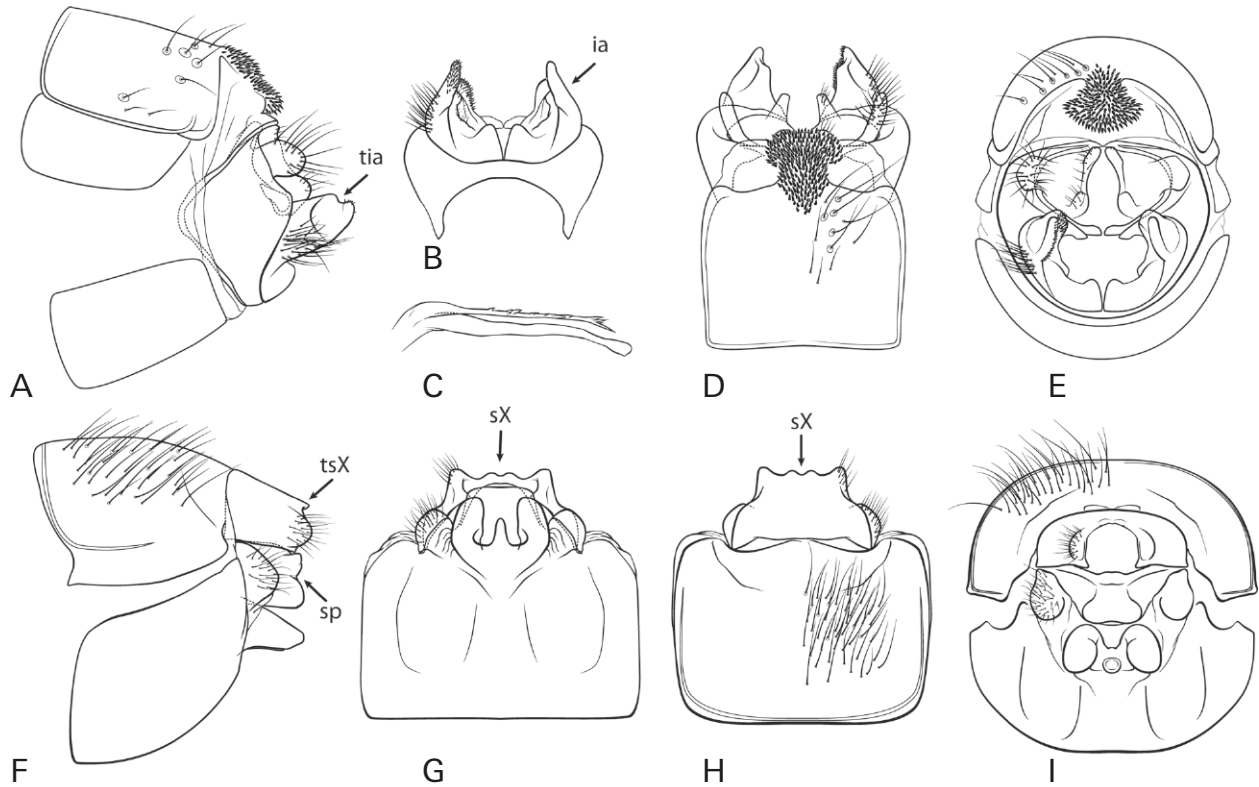


Fig. 6. *Ecclisopteryx dalecarlica* Kolenati, 1848; male genitalia, **A**: lateral view, **B**: ventral view, **C**: aedeagus and parameres, **D**: dorsal view and **E**: caudal view; female genitalia, **F**: lateral view, **G**: ventral view, **H**: dorsal view and **I**: caudal view. — Abbreviations: tia=tip of inferior appendages, ia=inferior appendages, sp=supragenital plate, tsX=tip of segment X, sX=segment X.

- 1** In lateral view, segment X with a distinct protuberance (Fig. 6F; arrow tsX) and supragenital plate with a sharp median incision (Fig. 6F; arrow sp); and in dorsal and ventral view segment X with 2 distinct median lobes, somewhat shorter than outer lobes (Fig. 6G,H; arrows sX): *E. dalecarlica*
- 1'** In lateral view, tips of segment X somewhat curved dorsally (Fig. 4F; arrow tsX) and supragenital plate longer ventrally than dorsally (Fig. 4F; arrow sp), in caudal view with 2 distinct ventral lobes (Fig. 4I; arrow lsp): *E. keroveci*
- 1''** In lateral view, supragenital plate lacking an indentation (Fig. 5F; arrow sp); in dorsal and ventral view, segment X with distinct, round median incision, leaving 2 mediolateral lobes (Fig. 5G,H; arrows sX): ... *E. ivkae*

Larvae. A summary of morphological features for the identification of Limnephilidae and Drusinae larvae was given by WARINGER (1985). Within the framework of the limnephilid key by WARINGER & GRAF (2011), *E. keroveci* is keyed together with *E. dalecarlica* and *Drusus trifidus*, whereas *E. ivkae* is keyed together with *E. guttulata* and *E. madida*.

(A) The fifth instar larva of *E. keroveci* can be separated from the larva of *D. trifidus* and *E. dalecarlica* using the following key:

- 1** Colouration of head capsule and body sclerites blackish brown; additional spines lacking on parietalia;

additional spines on pronotum short and yellow; lateral fringe extending from anterior margin of abdominal segment III to first 1/3 of segment VIII:

- *D. trifidus*
- 1'** Colouration of head capsule and body sclerites yellow or brown (Figs. 7B,C, 8A,E); additional spines present on parietalia (Figs. 7B, 8A,E,F); additional spines on pronotum long and brown (Fig. 7B) or short and blackish brown (Fig. 8F); lateral fringe extending from anterior margin of abdominal segment III to end of segment VIII: **2, *E. dalecarlica* & *E. keroveci***
- 2** Colouration of head capsule and body sclerites yellow (Fig. 8E,F); additional spines on parietalia and pronotum blackish, stout and of roughly the same length (80–100 µm; Fig. 8E,F): *E. dalecarlica*
- 2'** Colouration of head capsule and body sclerites light brown to chestnut brown (Figs. 7B, 8A); the additional spines on parietalia and pronotum light brown, pointed, varying in length (100–300 µm; Figs. 7B, 8A): *E. keroveci*
- (B)** The fifth instar larva of *E. ivkae* can be separated from the larvae of *E. guttulata* and *E. madida* using the following key:
 - 1** Colouration of head capsule and body sclerites brownish-red (Fig. 8G,H); pronotum with a pronounced median notch in anterior view (Fig. 8G): *E. guttulata*

Table 2. Synoptic key for the diagnosis of the larvae of the genus *Ecclisopteryx* and *Drusus trifidus* (Limnephilidae: Drusinae). Distribution of *Ecclisopteryx* species following GRAF et al. 2008, 2011; GRAF & SCHMIDT-KLOIBER 2011, unpublished data; endemism indicated by a cross (†); ¹ also present on British Islands.

Species	Parietalia: presence and number of additional spines per parietale	Lateral fringe extending on abdominal segments:		Mesonotal sclerites, colour:	Pronotum with		Distribution
		beginning at	ending at		sharp ridge (lateral view)	pronounced median notch (anterior view)	
<i>E. keroveci</i> [†]	yes (12–20)	anterior margin of III	end of VIII	light brown to chestnut brown	no (Fig. 8B)	no (Fig. 8A)	Western Balkan region
<i>E. ivkae</i> ¹	yes (1–7)	anterior margin of III	end of VIII	brownish-black	yes (Fig. 8D)	no (Fig. 8C)	Dalmatia
<i>E. dalecarlica</i> ¹	yes (stout, 14–20)	anterior margin of III	end of VIII	yellow	no (Fig. 8F)	no (Fig. 8E)	central & northern Europe
<i>E. guttulata</i>	yes (≥ 16)	anterior margin of III	first third of VIII	brownish-red	yes (Fig. 8H)	yes (Fig. 8G)	central & southern Europe
<i>E. madida</i>	yes (> 20)	anterior margin of III	first third of VIII	black	yes	no (Fig. 8I)	central–eastern Europe
<i>E. asterix</i> [†]	no	last third of III	first third of VIII	brownish-black	no	no	Karawanken/Soboth
<i>E. malickyi</i> [†]	no	last third of III	first third of VIII	brownish-black	no	no	Lessinian Alps
<i>D. trifidus</i>	no	anterior margin of III	first third of VIII	anterior: brown to brownish-black, posterior: beige	no	no	central & western Europe

- 1' Colouration of head capsule and body sclerites blackish brown (Figs. 7D–F, 8C,D); pronotum without a pronounced median notch in anterior view (Fig. 8C,I): **2, *E. ivkae* & *E. madida***
- 2 Number of additional spines on each parietale is 1–7 (Figs. 7F, 8C,D); lateral fringe ending at the end of abdominal segment VIII: ***E. ivkae***
- 2' Number of additional spines on each parietale is > 20 (Fig. 8I); lateral fringe ending at the first 1/3 of abdominal segment VIII: ***E. madida***

The most important morphological features enabling separation of all *Ecclisopteryx* species (and *D. trifidus*) are summarised in Table 2 and Fig. 8. For reliable identification, distribution ranges of these species should also be kept in mind: *D. trifidus* does not occur in the Dinaric Western Balkan ecoregion (ER5; GRAF et al. 2008; GRAF & SCHMIDT-KLOIBER 2011) (Table 2); *Ecclisopteryx asterix* and *E. malickyi* are very restricted in their distribution ranges and are not known from ecoregion ER5 (GRAF et al. 2008, 2011; GRAF & SCHMIDT-KLOIBER 2011). Also, larvae of the two latter species differ from all other *Ecclisopteryx* species by lacking additional spines on the parietalia, and are easily differentiated from larvae of *E. keroveci* and *E. ivkae* by various features summarized in Table 2.

3.5. Ecology, habitat and phenology of *Ecclisopteryx keroveci*

Larval habitat characteristics at the type locality of *E. keroveci* (mouth of the Jabučica River) indicate a preference for rhithral sections of streams. The collection site is

approximately 9 km downstream from the spring, where the stream is about 4 m wide and has a mean current velocity of 0.1 ms⁻¹ in July; water temperature in July 2012 was 12.7°C (10.00 am). However, strong discharge dynamics have been observed. Substrate was mainly composed of larger fractions, i.e., megalithal (30%), macrolithal (35%) and mesolithal (25%), and rocks were substantially covered with algae. Larvae of *E. keroveci* were collected on the surface of algae-covered stones, which, in agreement with larval mandibular morphology, indicates that *E. keroveci* is a grazer (PAULS et al. 2008; WARINGER et al. 2010). Adults of *E. keroveci* were collected in early July 2012; however data on its flight period are still incomplete.

At the mouth of the Jabučica River, *E. keroveci* was sympatric with the trichopterans *Rhyacophila armenica* Guerin, 1834, *Rhyacophila balcanica* Radovanović, 1953, *Rhyacophila moscaryi* Klapálek, 1894, *Rhyacophila nubila* (Zetterstedt, 1840), *Rhyacophila obliterated* McLachlan, 1863, *Rhyacophila trescavicensis* Botosaneanu, 1960, *Rhyacophila tristis* Pictet, 1834, *Glossosoma conformis* Neboiss, 1963, *Glossosoma discophorum* Klapálek, 1902, *Agapetus ochripes* Curtis, 1834, *Synagapetus slavorum* Botosaneanu, 1960, *Dipletrona atra* McLachlan, 1878, *Hydropsyche dinarica* Marinković-Gospodnetić, 1979, *Hydropsyche instabilis* (Curtis, 1834), *Hydropsyche mostarensis* Klapálek, 1898, *Hydropsyche tabacarui* Botosaneanu, 1960, *Polycentropus excisus* Klapálek, 1894, *Polycentropus flavomaculatus* (Pictet, 1834), *Polycentropus ierapetra dirfisi* Malicky, 1974, *Lype reducta* (Hagen, 1868), *Brachycentrus montanus* Klapálek, 1892, *Micrasema minimum* McLachlan, 1876, *Drusus biguttatus* (Pictet, 1834), *Limnephilus hirsutus* (Pictet, 1834), *Potamophylax luctuosus* (Piller & Mitterpa-



Fig. 7. *Ecclisopteryx keroveci* sp.n., larva; **A:** larva in its case, **B:** head and pronotum, left lateral view, **C:** thorax and first abdominal segment, dorsal view. *Ecclisopteryx ivkae* sp.n., larva; **D:** larva in its case, **E:** thorax, dorsal view and **F:** head and pronotum, left lateral view. Scale bar is 1 mm.

cher, 1783), *Lepidostoma basale* (Kolenati, 1848), *Odonotocerus albicorne* (Scopoli, 1763), *Beraemyia schmidi* Botosaneanu, 1960, *Oecismus monedula* (Hagen, 1859), *Sericostoma flavicorne* Schneider, 1845 and the plecopterans *Perla marginata* (Panzer, 1799), *Perla pallida* Guerin, 1838, *Dinocras megacephala* (Klapálek, 1907), *Isoperla tripartita* Illies, 1954 and *Chloroperla rus-*

sevi Braasch, 1969 (MARINKOVIĆ-GOSPODNETIĆ 1970, our data). Hence, the caddisfly community at the mouth of the Jabučica River is quite species rich (e.g. PREVIŠIĆ et al. 2007). The finding of *Polycentropus ierapetra dirfis* is particularly noteworthy, since it was previously recorded only from the Hellenic Western Balkan ecoregion (ER6; GRAF et al. 2008; GRAF & SCHMIDT-KLOIBER 2011; MA-

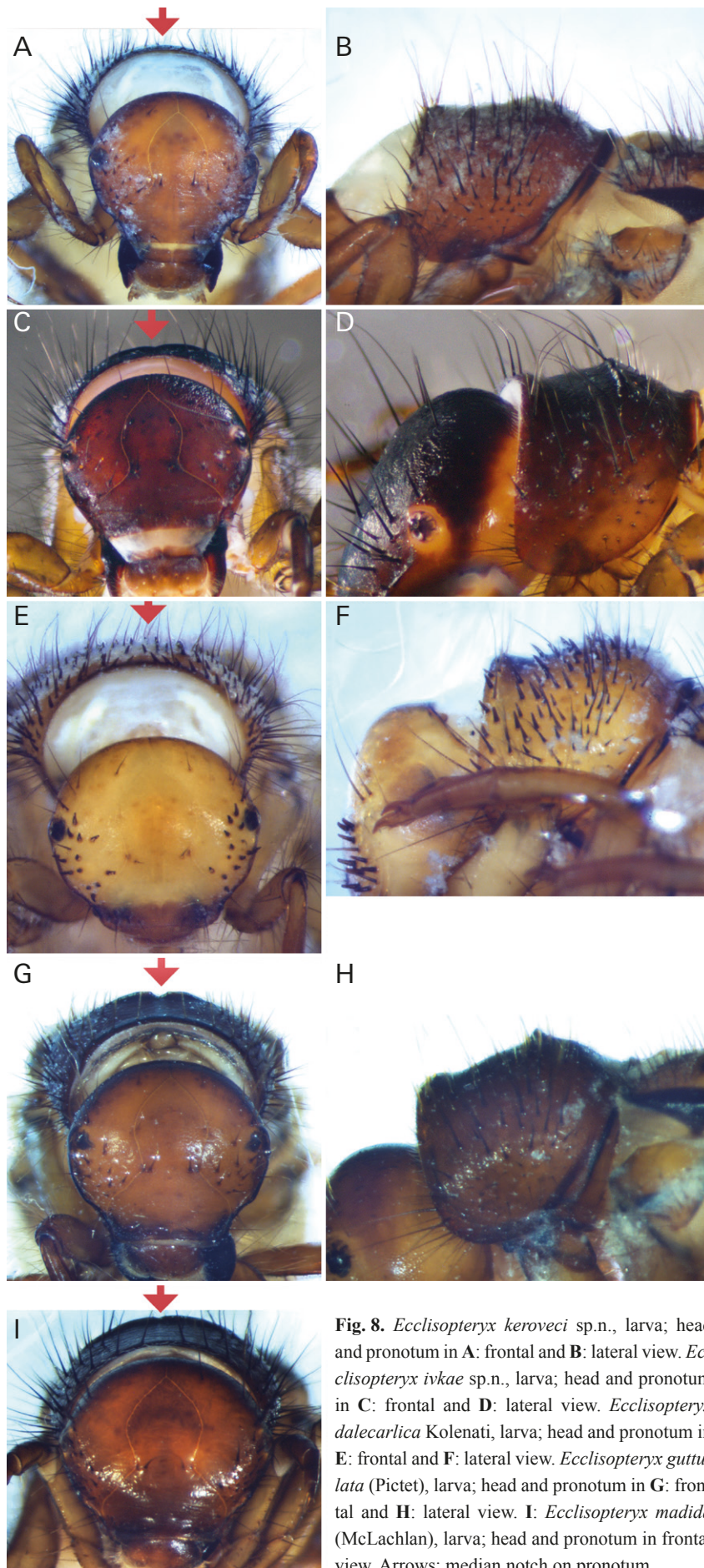


Fig. 8. *Ecclisopteryx keroveci* sp.n., larva; head and pronotum in **A**: frontal and **B**: lateral view. *Ecclisopteryx ivkae* sp.n., larva; head and pronotum in **C**: frontal and **D**: lateral view. *Ecclisopteryx dalecarlica* Kolenati, larva; head and pronotum in **E**: frontal and **F**: lateral view. *Ecclisopteryx guttulata* (Pictet), larva; head and pronotum in **G**: frontal and **H**: lateral view. **I**: *Ecclisopteryx madida* (McLachlan), larva; head and pronotum in frontal view. Arrows: median notch on pronotum.

LICKY 2004). This collection site represents the north-westernmost distribution of the species and considerably extends its range to the Dinaric Western Balkan ecoregion (ER5 sensu ILLIES 1978).

3.6. Ecology, habitat and phenology of *Ecclisopteryx ivkae*

Larval habitat characteristics at the type locality of *E. ivkae* (Cetina River, Glavaš spring) indicate a preference for crenal sections of streams. Larvae were collected approximately 50 m downstream of the spring. Here the stream was about 5 m wide, with a mean current velocity of 0.21 ms⁻¹ and water temperatures ranging from 8.4 to 12.9°C throughout the year (POPIJAČ 2007). Substrate was mainly composed of smaller fractions (microlithal and mesolithal) with some larger stones and submersed vegetation. Larvae of *E. ivkae* were collected on the surface of algae-covered stones, which, in concordance with larval mandibular morphology, indicates that *E. ivkae* is also a grazer (PAULS et al. 2008; WARINGER et al. 2010). Additionally, adults were collected at the site 2.4 km downstream, indicating a possible preference for rhithral sections as well. Adults were generally collected in late May and early June (in 2005, 2007 and 2012).

At Glavaš spring, *E. ivkae* was sympatric with the trichopterans *Rhyacophila balcanica* Radovanović, 1953, *Rhyacophila fasciata* Hagen, 1859, *Glossosoma discophorum* Klapálek, 1902, *Hydropsyche* sp., *Tinodes dives* (Pictet, 1834), *Annitella apfelbecki* Klapálek, 1899, *Chaetopteryx fusca* Brauer, 1857, *Grammotaulius nigropunctatus* (Retzius, 1783), *Limnephilus flavicornis* (Fabricius, 1787), *Limnephilus lunatus* Curtis, 1834, *Limnephilus vittatus* (Fabricius, 1798), *Micropterna nycterobia* McLachlan 1875, *Micropterna testacea* (Gmelin, 1789), *Stenophylax permistus* McLachlan, 1895, *Allogamus uncatatus* (Brauer, 1857), *Halesus digitatus* (Schrank, 1781), *Sericostoma flavicorne* Scheider, 1845 and *Odontocerum albicorne* (Scopoli, 1763) and the plecopterans *Leuctra mortoni* Kempny, 1899, *Nemoura cinerea* (Retzius, 1783), *Protonemura autumnalis* Rauser, 1956, *Protonemura hrabei* Rauser, 1956, *Isoperla illyrica* Tabacaru, 1971, *Isoperla inermis* Kacanski & Zwick, 1970, *Isoperla tripartita* Illies, 1954, and *Brachyptera tristis* (Klapálek, 1901) (POPIJAČ & SIVEC 2010; WARINGER et al. 2009; our data).

4. Discussion

4.1. Species delimitation and larval affiliation

Association of adults and larvae of the two new species collected at the mouth of the Jabučica River and the spring reach of the Cetina River, respectively, is supported by molecular genetic analyses at both intra- and

interspecific levels. Additionally, larvae collected at both localities exhibit a unique combination of morphological characters and are clearly distinct from each other and the other *Ecclisopteryx* species (see 3.4. for details).

Despite the tremendous importance of species level identification of larvae in applied science (e.g., water quality monitoring, conservation biology) (e.g. HERING et al. 2004), ca. 60% of Drusinae larvae remain unknown (WARINGER et al. 2013). In particular, species level identification offers enhanced resolution of trait-environment relationships, particularly in ecologically sensitive taxa. Thus, species-level resolution has the potential to improve the quality of ecological assessments that use caddisflies or other sensitive aquatic insects as quality indicators (e.g. SCHMIDT-KLOIBER & NIJBOER 2004). The morphological characteristics of the larva of *E. keroveci* and *E. ivkae* now allow clear identification of these species and will enable better ecological characterisation of Drusinae in the region. When this information is integrated in national and international databases it will be of great value for further use in ecological investigations.

The B/MCMCMC phylogenetic analysis presented here to discriminate the species is based on the combination of three gene fragments (mtCOI, mtLSU, nuWG) previously demonstrated to successfully resolve phylogenetic relationships among species of Drusinae (PAULS et al. 2008). The phylogeny suggests a highly supported sister clade relationship of (*E. keroveci* + *E. ivkae*) + (*E. dalecarlica* + *E. guttulata*) and monophyly of all putative *Ecclisopteryx* species except *E. ivkae*, which is recovered in a basal polytomy (Fig. 2). Interspecific *p*-distances of mtCOI sequence data between the two new species, *E. keroveci* and *E. ivkae* (1.6–2.7%), are similar to the observed intraspecific variability of the same mtCOI fragment in populations of some Drusinae species (e.g. KUČINIĆ et al. 2008; PAULS et al. 2009; PREVIŠIĆ et al. 2009, 2014). However, in several closely related Drusinae species, interspecific *p*-distances of the same mtCOI fragment are similar or even lower than observed between *E. keroveci* and *E. ivkae* in the current study (e.g., WARINGER et al. 2007; KUČINIĆ et al. 2011). Further, intraspecific *p*-distances of the mtCOI gene fragment in *E. keroveci* show relatively high divergence of easternmost and westernmost populations (e.g., 1.6% between haplotypes from eastern Montenegro (Brodavac) and western Croatia (Velika Belica), 1.8% between haplotypes from eastern Bosnia (Sušjeska NP) and the latter, Fig. 3). Nevertheless, the morphological and molecular data at hand suggests that these specimens all belong to a single species.

In contrast to the highly variable mtCOI gene, differences between closely related species in nuWG and particularly in mtLSU are much lower (PAULS et al. 2008), and thus provide less information. Probably for this reason the B/MCMCMC phylogeny does not fully support the delimitation of the two species, as *E. ivkae* forms a basal polytomy. Including additional (or more variable) loci would improve the present analysis. However, in our case, the striking differences in larval morphology of northern European populations of *E. dalecarlica* initially

led us to think that two or more new species might occur, especially since larval characters in Drusinae are stable and vary little among species (e.g. KUČINIĆ et al. 2011; WARINGER & GRAF 2011; WARINGER et al. 2013). Furthermore, as indicated in our study, morphology of male and female genitalia of *E. keroveci* and *E. ivkae* differs distinctly between these species and *E. dalecarlica* (see 3.4 for details).

Thus, the integration of genetic and morphological differences, justifies the separation of *E. ivkae* and *E. keroveci* as two distinct species, as both taxa exhibit a unique combination of both genetic and morphological character states. Additional and equally comprehensive studies might uncover the existence of other localised allopatric lineages in *Ecclisopteryx* species in the Balkans, regardless of their current taxonomic status.

4.2. Distribution of *E. keroveci* and *E. ivkae*

With the addition of the 2 new *Ecclisopteryx* species, this genus currently comprises 7 species: 3 species relatively widespread in Europe, 1 regional endemic, and 3 micro-endemics (Table 2 & Fig. 1; GRAF et al. 2008, 2011; GRAF & SCHMIDT-KLOIBER 2011). Unlike the 2 *Ecclisopteryx* range restricted endemics confined to different parts of the Alps (*E. malicky* is known exclusively from the south-central Alps, and *E. asterix* from the south-eastern Alps; GRAF et al. 2008, 2011; GRAF & SCHMIDT-KLOIBER 2011), *E. keroveci* and *E. ivkae* show rather unique distribution patterns in the Western Balkans (Fig. 1B). Although both are allopatric and endemic, *E. keroveci* seems to have a wider, disjunct distribution in the region.

Previous records of both *E. dalecarlica* (URBANIĆ 2004; STANIĆ-KOŠTROMAN 2009; PREVIŠIĆ & POPIJAČ 2010; IBRAHIMI 2011; VUČKOVIĆ 2011; VUČKOVIĆ et al. 2011) and *E. guttulata* (RADOVANOVIĆ 1935; MARINKOVIĆ-GOSPODNETIĆ 1970) in the region are misidentifications of these 2 new, formerly unknown cryptic endemics. This conclusion is further supported since specimens from most the localities where *E. dalecarlica* and/or *E. guttulata* were presumed to have been collected were available for the current study; these all proved to be either of the 2 new species. The only historical record we could examine is a single male from central Bosnia, collected at the end of the 19th century, from Klapálek's collection (the National Museum, Prague, Czech Republic). It was originally listed as *E. guttulata*, but it proved to be *E. keroveci*. The findings of RADOVANOVIĆ (1935) and MARINKOVIĆ-GOSPODNETIĆ (1970) could not be checked because the material was not available for study.

4.3. The Western Balkans – a hotspot of freshwater biodiversity under threat

The description of *E. keroveci* and *E. ivkae* increases the number of endemic Western Balkan caddisflies: of

36 Drusinae species distributed in the region, 28 are endemics. Most of these are restricted to very small geographic areas (GRAF et al. 2008; OLÁH 2010, 2011; GRAF & SCHMIDT-KLOIBER 2011; OLÁH & KOVÁCS 2013). In the Dinaric Western Balkan ecoregion (ER5) 64% of Drusinae species are endemic (14 of 22 species); similarly, in the Hellenic Western Balkan ecoregion (ER6) 65% are endemic (13 of 20; GRAF et al. 2008; OLÁH 2010, 2011; GRAF & SCHMIDT-KLOIBER 2011; OLÁH & KOVÁCS 2013).

Hence, the Western Balkans is a diversity centre for highland caddisflies inhabiting isolated “island habitats” such as coldwater springs and streams (e.g. Drusinae, *Chaetopteryx* species; KUČINIĆ et al. 2013). Such habitat preferences coupled with low dispersal abilities, specific life history traits, and historical processes causing further fragmentation and isolation of habitats (e.g., karstification; PREVIŠIĆ et al. 2009, 2014) most likely result in high diversification rates. A remarkable degree of cryptic diversity was recently observed in the Western Balkans not only in groundwater fauna (e.g. ZAKŠEK et al. 2009), but also in widespread and commonly known surface aquatic species, such as the crayfish *Austropotamobius torrentium* (KLOBUČAR et al. 2013) and the amphipod *Gammarus fossarum* (WEISS et al. 2014). Furthermore, a considerable fraction of the existing endemic diversity across various animal groups is considered cryptic in many parts of Europe, e.g., in isolated southern European mountain ranges (ESSL et al. 2013). All this indicates that high degrees of cryptic diversity can be expected in many groups of aquatic organisms in the Balkans, and highlights the need for more comprehensive research of insufficiently investigated freshwater biodiversity and biogeography.

Near natural streams in all Western Balkan countries are increasingly threatened by human activities, especially the increase in construction of small power plants (FREYHOF 2012; SCHWARZ 2012). According to FREYHOF (2012), construction of dams and its inevitable side effects pose the most serious threat to freshwater diversity in the Balkans, the most important “hotspot” of European threatened biodiversity. Such adverse environmental impacts also threaten the existence of yet-to-be-discovered cryptic species.

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