ARTHROPOD SYSTEMATICS & PHYLOGENY

75 (3): 497–521
20.12.2017

SENCKENBERG

High cryptic diversity in aquatic insects: an integrative approach to study the enigmatic *Leuctra inermis* species group (Plecoptera)

Simon Vitecek *[,1,4,#], Gilles Vinçon[2,#], Wolfram Graf[3] & Steffen U. Pauls[4]

[1] Department for Limnology and Bio-Oceanography, Universität Wien, UZA I, Althanstrasse 14, 1090 Vienna, Austria; Simon Vitecek [simon.vitecek@senckenberg.de] — [2] 55 Bd Joseph Vallier, F 38100 Grenoble, France; Gilles Vinçon [gvincon@gmail.com] — [3] Institute of Hydrobiology and Aquatic Ecology Management, University of Natural Resources and Life Sciences, Gregor-Mendel-Straße 33, 1180 Vienna, Austria; Wolfram Graf [wolfram.graf@boku.ac.at] — [4] Senckenberg Research Institute and Natural History Museum, Senckenberganlage 25, 60325 Frankfurt am Main, Germany; Steffen U. Pauls [steffen.pauls@senckenberg.de] — * Corresponding author; # Equally contributing authors

Accepted 05.x.2017.
Published online at www.senckenberg.de/arthropod-systematics on 11.xii.2017.

Editors in charge: Gavin Svenson & Klaus-Dieter Klass

Abstract

Within the genus *Leuctra* (Plecoptera: Leuctridae) the *L. inermis* species group comprises 17–18 species in which males lack the characteristic tergal abdominal ornamentation of many *Leuctra* species, and females have an accessory receptacle in the dorsal portion of the vagina. Taxonomically the group is challenging, and congruence of existing morphological species concepts and phylogenetic relationships of taxa is hitherto not assessed. Here, we estimate phylogenetic relations of morphologically defined species by concatenated maximum likelihood (ML) and Bayesian combined species tree and species delimitation analysis. We aim to clarify the status of 15 European species of the *L. inermis* group and 2 potential new species. To this end, we infer relationships on a 2580 bp, 4 loci (mtCOI, mt12S, nuH3, nu28S) molecular sequence dataset. We further depict abdominal terminalia with their morphological characteristics for 15 *L. inermis* group species, and describe 2 micro-endemic species from the Southern Alps and the Apennine Mountains corroborated by distinct morphological and molecular characteristics. Our analyses support close relationships of the *L. inermis* group species and corroborate the *a priori* morphological definition of all included species. However, relationships among species are largely unresolved, indicative of a putatively recent diversification of the group. Phylogenetic inference suggests sister taxon relationships between morphologically similar species. Morphological variation within single species is linked to geographical location of populations. Our results thus suggest that further study on the differentiation of geographically isolated populations as distinct lineages or species is warranted. Based on the high number of regional endemic lineages and species we highlight the necessity to protect high-altitude aquatic habitats.

Key words

Leuctridae, STACEY, integrative taxonomy, *Leuctra fochettii*, *Leuctra grafi*, new species, aquatic biodiversity.

1. Introduction

Plecoptera are a group of highly niche-specific aquatic insects widely used as indicators of water quality (Rosenberg & Resh 1993; Graf et al. 2002–2011; Moog 2002–2011; Hering et al. 2003, 2006; Birk et al. 2012; DeWalt et al. 2015). The group comprises many cold-stenotopic taxa that contribute to its high diversity and interesting zoogeography (Illies 1965; Hynes 1976; Zwick 2000; Graf et al. 2009, 2017; DeWalt et al. 2015). European Plecoptera exhibit high degrees of endemism, and more than half of the known species (264 of 514) are considered regional or micro-endemics (Graf et al. 2009, 2017). Population fragmentation and ensuing endemism in stone-

flies are often linked to the relatively poor dispersal capacity of both mature and immature stages (FOCHETTI et al. 2011; ELBRECHT et al. 2014). Evolutionary history and extant diversity patterns of Plecoptera are moreover shaped by allopatric diversification driven by historic glaciation, orogenesis, and small-scale population fragmentation (ZWICK 2000; FOCHETTI & TIERNO DE FIGUEROA 2008; FOCHETTI et al. 2009, 2011; WEISS et al. 2011; THEISSINGER et al. 2013). Ecologically, a slight majority of Plecoptera taxa behave as detritivores, while other species (particularly systellognathan ones) are carnivores (HYNES 1976; SILVERI et al. 2008; GRAF et al. 2009, 2017; FENOGLIO et al. 2010; BOTTOVÁ et al. 2013; DEWALT et al. 2015). Larval habitats of Plecoptera comprise lotic and lentic waterbodies, and the majority of species prefer cool to cold temperature conditions (HYNES 1976; GRAF et al. 2009, 2017).

Among European Plecoptera, the family Leuctridae (Plecoptera, Arctoperlaria, Euholognatha) is second only to Nemouridae in number of described species (TIERNO DE FIGUEROA & FOCHETTI 2014; GRAF et al. 2017). In Europe, Leuctridae are represented by the genera *Pachyleuctra* [3 species], *Tyrrhenoleuctra* [5 species], and *Leuctra* [151 species] (TIERNO DE FIGUEROA & FOCHETTI 2014; GRAF et al. 2017). The overwhelming majority of the taxa are mostly small, darkly coloured, and save a few exceptions, are macropterous. Males of the genus *Leuctra* exhibit diagnostic tergal ornamentation patterns on abdominal segments. Differences in the sclerotized tergal processes of the males and form of external genitalia in both sexes are the also most important characters in Leuctridae taxonomy (e.g., RAVIZZA & VINÇON 1998). Differentiation of larval stages in Leuctridae is rarely achieved, and only few species can be reliably identified as larvae (ZWICK 2004; GRAF & SCHMIDT-KLOIBER 2010). Also, adults of some species groups exhibit little morphological differentiation and are difficult to distinguish (RAVIZZA 2002).

A taxonomically particularly challenging species group of European *Leuctra* is the *L. inermis* group. Males exhibit reduced abdominal tergal sclerotization without raised dorsal processes; the female genital tract develops an accessory receptacle on the dorsal side of the vagina, and the external species-specific female genitalic characters seem to oscillate between two major forms (AUBERT 1957; ZWICK 1973). The first comprehensive treatment of the group was conducted by AUBERT (1957), who recognized 10 species in 3 phyletic species groups based on similarities in sclerotization/pigmentation patterns in males and external structure of male and female genitalia. Five additional species included by Aubert due to their similarity to the *L. inermis* group have since been recognised as more closely related to other groups (BERTHÉLEMY 1968; RAVIZZA & VINÇON 1998). Recent descriptions of additional species increase total taxonomic richness of the *L. inermis* group to 17–18 species: *L. alosi* Navas, 1919, *L. ameliae* Vinçon & Ravizza, 1996, *L. apenninicola* Ravizza, 1988, *L. balcanica* Raušer, 1965, *L. flavomaculata* Mosely, 1935, *L. garumna* Vinçon & Ravizza, 1996, *L. handlirschi* Kempny, 1899, *L. inermis* Kempny, 1899, *L. insubrica* Aubert, 1949, *L. kempnyi* Mosely, 1932, *L. metsovonica* Aubert, 1966, *L. pusilla* Krno, 1985, *L. quadrimaculata* Kis, 1962, *L. rauscheri* Aubert, 1957, *L. silana* Aubert, 1953 (likely a synonym of *L. inermis* Kempny; G. Vinçon unpubl. data), *L. simplex* Zhiltzova, 1960, *L. teriolensis* Kempny, 1900, and *L. uncinata* Martynov, 1928.

Based on scarce autecological data, European species of the group can be assumed to differ little in their preferred ecological niche. No ecological data exist for the Caucasian *L. simplex* and *L. uncinata.* Larvae of *L. inermis* group species mostly occur in crenal to rhithral stretches of cold-water streams in montane to alpine habitats, and behave as detritivores (GRAF et al. 2009, 2017). To our current knowledge, adult emergence peaks in spring to summer; we thus assume a univoltine annual life cycle (BO & FENOGLIO 2009; GRAF et al. 2009, 2017). As in other Leuctridae, larvae of the group cannot be reliably identified based on their morphology.

Taxonomically, the *L. inermis* group could contain high micro-endemic species-level or subspecies-level diversity: they share ecological traits (e.g., habitat preferences, altitudinal distribution, phenology) with other groups of aquatic insects comprising many endemic species such as, among others, Rhyacophilidae (Insecta, Trichoptera), Drusinae (Trichoptera, Limnephilidae), *Consorophylax* (Trichoptera, Limnephilidae, Stenophylacini) or Pediciidae (Insecta, Diptera) (PREVIŠIĆ et al. 2014; DENÉS et al. 2015; IBRAHIMI et al. 2015, 2016; GRAF et al. 2015; VITECEK et al. 2015a,b; GRAF & VITECEK 2016). Also, sexual selection via development of distinct drumming call dialects could enhance divergence of populations (BOUMANS & JOHNSEN 2014). Consequently, other allopatric, micro-endemic taxa of the *L. inermis* group can be assumed to exist.

Alternatively, different morphologically defined species of the *L. inermis* group as previously recognized could represent morphological variants of a few widespread species (*cf.* FOCHETTI et al. 2011). Intriguingly, geographical ranges of many species of the *L. inermis* group overlap, and result in syntopic occurrence of certain taxa (BOJKOVÁ & SOLDÁN 2011; PETROVIC et al. 2014; GRAF et al. 2005; GRAF 2010; GRAF & SCHMIDT-KLOIBER 2010; KROČA 2011). Indeed, these reported distribution patterns could be due to the existence of morphological variants within several currently unrecognized widespread species (FOCHETTI et al. 2011).

In this contribution, we test species boundaries of morphologically defined taxa in the enigmatic *L. inermis* group. To this end, we infer phylogenetic relationships between 15 currently accepted and 2 potentially new European species using sequence data from 4 genes (mtCOI, mt12S, nuH3, nu28S). We predict that present grouping of species within the *L. inermis* group is correct. Additionally, we describe 2 new species – *L. grafi* sp.n. and *L. fochettii* sp.n. – and examine their relationship to the currently recognized *L. inermis* group members.

Table 1. PCR primers and PCR cycling conditions.

Fragment	Primers & Primer Concentration		PCR Cycling conditions	Taq Kit
mtCOI5-P	HCO2198-L & LCO1490-L (Nelson et al. 2007)	0.25 µM	5′ 95°C, 5 × (30″ 95°C, 1′ 44°C, 1′ 72°C), 15 × (30″ 95°C, 30″ 48°C, 1′ 72°C), 20 × (30″ 95°C, 30″ 50°C, 1′ + (10″ * n) 72°C)	peqGOLD HotTaq
mtCOI3-P	Jerry & S20 (Pauls et al. 2006)	0.25 µM	5′ 95°C, 35 × (45″ 95°C, 30″ 45°C, 45″ 72°C), 5′ 72°C	peqGOLD HotTaq
mt12S	12SAI & 12SBI (Simon et al. 1994)	0.25 µM	1′ 98°C, 40 × (10″ 95°C, 30″ 52°C, 30″ 72°C), 2′ 72°C	peqGOLD HotTaq
nuH3	H3F & H3R (Ogden & Whiting 2003)	0.5 µM	30″ 98°C, 14 × (10″ 98°C, 30″ 62°C, 20″ 72°C), 22 × (10″ 98°C, 30″ 50°C, 20″ 72°C), 7′ 72°C	Q5 Hot Start High-Fidelity 2X Master Mix
nu28S	Rd1a & Rd4b (Crandall et al. 2000)	0.25 µM	2′ 95°C, 3 × (40″ 95°C, 40″ 55°C, 90″ 72°C), 3 × (40″ 95°C, 40″ 52°C, 90″ 72°C), 32 × (40″ 95°C, 40″ 48°C, 90″ 72°C), 5′ 72°C	Q5 Hot Start High-Fidelity 2X Master Mix

Table 2. Substitution models and partition schemes used in phylogenetic analysis. CP = codon position; UP = unpartitioned.

Fragment	UP	CP 1	CP 2	CP 3
Substitution models used in BI				
mtCOI5-P	—	HKY	TN93 + Γ	TN93 + Γ
mtCOI3-P	—	JC	K2 + I	TN93 + Γ
mt12S	HKY + Γ	—	—	—
nuH3	—	JC	JC	HKY + Γ
nu28S	T92 + Γ + I	—	—	—
Partitioning scheme used in ML analyses				
mtCOI5-P	—	IV	V	VI
mtCOI3-P	—	IV	V	VI
mt12S	I	—	—	—
nuH3	—	II	IV	III
nu28S	II	—	—	—

2. Materials and methods

2.1. Collection and comparative morphology

Adult specimens were collected with sweep nets and beat sheets; collected specimens were stored and kept in 70–96% EtOH until examination in the laboratory. Morphological characteristics of specimens were examined and photographed using a Zeiss StereoLumar V.12 dissecting microscope equipped with an AxioCamErc5s camera and the Zeiss-native image processing software ZEN. Image series through different focus levels were stacked using CombineZP (Hadley 2008; Brecko et al. 2014) to create single extended-depth-of-focus images. Keyence VHX-1000 and VHX-5000 digital microscopes were used to obtain additional images. To preserve as much tissue as possible for potential future studies, the majority of specimens were not cleared. True colour images were converted to grey-scale, and can be retrieved from the Electronic Supplement. Nomenclature of morphological characteristics follows Aubert (1957) and Ravizza (2002) (Fig. 3).

2.2. Specimen repositories

The specimens used to generate the molecular dataset are stored in the collection of the Senckenberg Forschungsinstitut und Naturmuseum Frankfurt [SFNF]. All further specimens referred to in this contribution are permanently deposited in the following collections:

1. Collection Gilles Vinçon [coll. Vinçon] — Postal address: 55 Bd Joseph Vallier, F 38100 Grenoble, France; contact: Gilles Vinçon [gvincon@gmail.com]
2. Collection Wolfram Graf [coll. Graf] — Postal address: Institut für Hydrobiologie und Gewässermanagement, Universität für Bodenkultur Wien, Gregor-Mendel-Straße 33, 1180 Vienna, Austria; contact: Wolfram Graf [wolfram.graf@boku.ac.at]
3. Musee de Zoologie Lausanne [MZL] — Postal address: Palais de Rumine, Place de la Riponne 6, 1014 Lausanne, Switzerland; contact: Anne Freitag [anne.freitag@vd.ch]
4. Senckenberg Forschungsinstitut und Naturmuseum Frankfurt [SFNF] — Postal address: Senckenberganlage 25, 60325 Frankfurt am Main, Germany; contact: Steffen Pauls [steffen.pauls@senckenberg.de]

2.3. Molecular methods

Whole genomic DNA was extracted from the thorax using the DNEasy Blood and tissue Kit (Qiagen) according to the manufacturer's protocol. To maximise the amount of resuspended DNA and increase final DNA concentration, we eluted final DNA from the spin columns with 70 µl sterile ddH_2O, vacuum concentrated the DNA elutions and resuspended the pellets in 18 µl sterile ddH_2O. Standard PCR procedures and primers were used (Table 1). PCR reactions were set up in 10 µl reactions. Diluted, unpurified PCR products were sequenced on an ABI 3730XL capillary sequencer at Senckenberg BiK-F Laboratory Centre using the PCR primers for mtCOI-3P, mt12S, nuH3, and nu28S loci, while standard HCO2198 and LCO1490 primers (Folmer et al. 1994) were used to sequence mtCOI-5P.

Table 3. Summary and collection details of specimens used in phylogenetic analysis. — ***Abbreviations***: Leg., *legit*; Det., *determinavit*. Asterisks indicate outgroup taxa; BOLD ID, unique specimen identifier under which the molecular data can be retrieved from the BOLD database.

Specimen identifier	Taxon	Sampling date	Leg.	Det.	Latitude	Longitude	BOLD ID
Leal0102	*Leuctra alosi*	09.vi.2013	Graf	Vinçon	42.6504	2.0791	SPLEU001-17
Leal0201	*Leuctra alosi*	13.vii.2012	Graf	Vinçon	42.4845	2.4130	SPLEU002-17
Leal0202	*Leuctra alosi*	13.vii.2012	Graf	Vinçon	42.4845	2.4130	SPLEU003-17
Leal0301	*Leuctra alosi*	11.vii.2012	Graf	Vinçon	42.3859	2.0958	SPLEU004-17
Leal0302	*Leuctra alosi*	11.vii.2012	Graf	Vinçon	42.3859	2.0958	SPLEU005-17
Leal0402	*Leuctra alosi*	12.vii.2012	Graf	Vinçon	42.4864	2.4139	SPLEU006-17
Leal0501	*Leuctra alosi*	12.vi.2013	Graf	Vinçon	43.0844	–2.8277	SPLEU007-17
Leal0502	*Leuctra alosi*	12.vi.2013	Graf	Vinçon	43.0844	–2.8277	SPLEU008-17
Leam0101	*Leuctra ameliae*	30.vi.2012	Graf	Vinçon	45.2133	6.9575	SPLEU009-17
Leam0201	*Leuctra ameliae*	02.vii.2012	Graf	Vinçon	44.9484	6.8534	SPLEU010-17
Leam0202	*Leuctra ameliae*	02.vii.2012	Graf	Vinçon	44.9484	6.8534	SPLEU011-17
Lete0201	*Leuctra ameliae*	01.vii.2012	Graf	Vitecek/Vinçon	45.218	6.8884	SPLEU012-17
Leap0201	*Leuctra apenninicola*	19.vii.2009	Vinçon	Vinçon	44.1321	10.7789	SPLEU013-17
Leap0202	*Leuctra apenninicola*	19.vii.2009	Vinçon	Vinçon	44.1321	10.7789	SPLEU014-17
Leap0301	*Leuctra apenninicola*	03.iv.2015	Vinçon	Vinçon	43.7572	12.0661	SPLEU015-17
Leap0302	*Leuctra apenninicola*	03.iv.2015	Vinçon	Vinçon	43.7572	12.0661	SPLEU016-17
Leba0101	*Leuctra balcanica*	04.–08.vi.2012	Neu, Bálint	Vinçon	42.7431	24.6434	SPLEU017-17
Leba0102	*Leuctra balcanica*	04.–08.vi.2012	Neu, Bálint	Vinçon	42.7431	24.6434	SPLEU018-17
Lefl0101	*Leuctra flavomaculata*	08.vii.2012	Graf	Vinçon	43.223	2.6248	SPLEU019-17
Lefo0101	*Leuctra fochettii*	25.vii.2015	Graf	Vinçon	45.7566	11.1575	SPLEU020-17
Lefo0102	*Leuctra fochettii*	25.vii.2015	Graf	Vinçon	45.7566	11.1575	SPLEU021-17
Lefo0201	*Leuctra fochettii*	16.vii.2016	Graf	Vinçon	45.7569	11.1572	SPLEU022-17
Lefo0202	*Leuctra fochettii*	16.vii.2016	Graf	Vinçon	45.7569	11.1572	SPLEU023-17
Lesp0101	*Leuctra fochettii*	08.vi.2008	Vinçon	Vinçon	45.7519	11.1868	SPLEU024-17
Lesp0102	*Leuctra fochettii*	08.vi.2008	Vinçon	Vinçon	45.7519	11.1868	SPLEU025-17
Lega0101	*Leuctra garumna*	25.vii.2012	Graf	Vinçon	42.9644	0.8639	SPLEU026-17
Lega0102	*Leuctra garumna*	25.vii.2012	Graf	Vinçon	42.9644	0.8639	SPLEU027-17
Legr0103	*Leuctra grafi*	28.vi.2016	Vinçon	Vinçon	44.1362	10.1642	SPLEU028-17
Legr0104	*Leuctra grafi*	28.vi.2016	Vinçon	Vinçon	44.1362	10.1642	SPLEU029-17
Legr0201	*Leuctra grafi*	28.vi.2016	Vinçon	Vinçon	44.2954	10.233	SPLEU030-17
Legr0202	*Leuctra grafi*	28.vi.2016	Vinçon	Vinçon	44.2954	10.233	SPLEU031-17
Leha0102	*Leuctra handlirschi*	20.vi.2013	Graf	Vinçon	43.819	6.8768	SPLEU032-17
Leha0201	*Leuctra handlirschi*	16.iv.2015	Graf	Vinçon	46.6022	14.354	SPLEU033-17
Leha0301	*Leuctra handlirschi*	28.vi.2016	Vinçon	Vinçon	44.3011	10.2148	SPLEU034-17
Leha0302	*Leuctra handlirschi*	28.vi.2016	Vinçon	Vinçon	44.3011	10.2148	SPLEU035-17
Lein0201	*Leuctra inermis*	22.v.2012	Graf	Vinçon	47.3742	10.0218	SPLEU036-17
Lein0202	*Leuctra inermis*	22.v.2012	Graf	Vinçon	47.3742	10.0218	SPLEU037-17
Lein0301	*Leuctra inermis*	19.iv.2015	Graf	Vinçon	46.7788	14.0051	SPLEU038-17
Lein0401	*Leuctra inermis*	07.vii.2012	Graf	Vinçon	45.1466	5.6171	SPLEU039-17
Lein0501	*Leuctra inermis*	08.vii.2012	Graf	Vinçon	45.0722	5.3973	SPLEU040-17
Lein0502	*Leuctra inermis*	08.vii.2012	Graf	Vinçon	45.0722	5.3973	SPLEU041-17
Lein060Xi	*Leuctra inermis*	30.vi.2013	Graf	Vinçon	46.7667	14.9167	SPLEU042-17
Lein060Xii	*Leuctra inermis*	30.vi.2013	Graf	Vinçon	46.7667	14.9167	SPLEU043-17
Lein0701	*Leuctra inermis*	14.vi.2013	Graf	Vinçon	42.2816	–2.9608	SPLEU044-17
Lein0702	*Leuctra inermis*	14.vi.2013	Graf	Vinçon	42.2816	–2.9608	SPLEU045-17
Lein0901	*Leuctra inermis*	13.vii.2009	Vinçon	Vinçon	42.1717	14.1000	SPLEU046-17
Lein0902	*Leuctra inermis*	13.vii.2009	Vinçon	Vinçon	42.1717	14.1000	SPLEU047-17
Leis0101	*Leuctra insubrica*	07.vi.2013	Graf	Vinçon	45.5282	7.6758	SPLEU048-17
Leis0102	*Leuctra insubrica*	07.vi.2013	Graf	Vinçon	45.5282	7.6758	SPLEU049-17
Leke0101	*Leuctra kempnyi*	22.vii.2012	Graf	Vinçon	42.9555	–0.8270	SPLEU050-17
Leke0201	*Leuctra kempnyi*	23.vii.2012	Graf	Vinçon	42.957	–0.8264	SPLEU051-17
Leke0202	*Leuctra kempnyi*	23.vii.2012	Graf	Vinçon	42.957	–0.8264	SPLEU052-17
Leke0301	*Leuctra kempnyi*	22.vii.2012	Graf	Vinçon	42.9541	–0.8178	SPLEU053-17
Leke0302	*Leuctra kempnyi*	22.vii.2012	Graf	Vinçon	42.9541	–0.8178	SPLEU054-17
Leke0401	*Leuctra kempnyi*	12.vi.2013	Graf	Vinçon	43.0844	–2.8277	SPLEU055-17
Leke0402	*Leuctra kempnyi*	12.vi.2013	Graf	Vinçon	43.0844	–2.8277	SPLEU056-17
Leme0101	*Leuctra metsovonica*	05.vii.2010	Graf	Vinçon	41.7059	20.6667	SPLEU057-17
Leme0102	*Leuctra metsovonica*	05.vii.2010	Graf	Vinçon	41.7059	20.6667	SPLEU058-17

Table 3 continued.

Leme0201	*Leuctra metsovonica*	05.vii.2010	Graf	Vinçon	41.3991	21.1998	SPLEU059-17
Lepu0X01	*Leuctra pusilla*	unknown	Graf	Vinçon	42.0973	14.0309	SPLEU060-17
Lepu0101	*Leuctra pusilla*	19.vii.2016	Graf	Vinçon	44.1597	7.5425	SPLEU061-17
Lepu0201	*Leuctra pusilla*	28.vi.2016	Vinçon	Vinçon	44.2954	10.2330	SPLEU062-17
Lepu0202	*Leuctra pusilla*	28.vi.2016	Vinçon	Vinçon	44.2954	10.2330	SPLEU063-17
Lequ0101	*Leuctra quadrimaculata*	05.vii.2010	Previšić	Vinçon	41.3991	21.1998	SPLEU064-17
Lera040Xi	*Leuctra rauscheri*	19.iv.2014	Graf	Vinçon	46.4692	14.3358	SPLEU065-17
Lera040Xii	*Leuctra rauscheri*	19.iv.2014	Neu	Vinçon	46.4692	14.3358	SPLEU066-17
Leha0X01	*Leuctra rauscheri*	12.vii.2009	Graf	Vinçon	46.3993	11.0320	SPLEU067-17
Lera0201	*Leuctra rauscheri*	02.–10.vii.2006	Bálint	Vinçon	46.3096	10.4925	SPLEU068-17
Lera0202	*Leuctra rauscheri*	02.–10.vii.2006	Bálint	Vinçon	46.3096	10.4925	SPLEU069-17
Lera0501	*Leuctra rauscheri*	11.vii.2012	Graf	Vinçon	42.3859	2.0958	SPLEU070-17
Lera0701	*Leuctra rauscheri*	30.vi.2012	Graf	Vinçon	45.2103	6.9699	SPLEU071-17
Lete0301	*Leuctra teriolensis*	30.vi.2012	Graf	Vinçon	45.2103	6.9699	SPLEU072-17
Lehi0101	*Leuctra hippopus**	14.vi.2013	Graf	Vinçon	43.2816	2.9608	SPLEU073-17
Lerv0101	*Leuctra ravizzai**	01.vii.2012	Graf	Vinçon	45.218	6.8884	SPLEU074-17
Tyta0101	*Tyrrhenoleuctra tangerina**	13.iv.2013	Vinçon	Vinçon	34.0872	–4.1812	SPLEU075-17
Tyta0102	*Tyrrhenoleuctra tangerina**	13.iv.2013	Vinçon	Vinçon	34.0872	–4.1812	SPLEU076-17

Sequences were edited in Geneious R6 (Kearse et al. 2012) and aligned using MAFFT v7 (Katoh & Standley 2013) as implemented in Geneious R6. For phylogenetic analysis, mt12S and nu28S fragments were not partitioned; partial sequence data of protein-coding genes (mtCOI, nuH3) was partitioned by codon position. Nucleotide substitution models for each partition were selected according to the Bayesian Information Criterion in the model test module of Mega v6.06 (Tamura et al. 2013) and PartitionFinder v. 1.1.3 (Lanfear 2011) (Table 2).

Phylogenetic relationships in the *L. inermis* group were inferred on a 20 species (including 15 ingroup species, 2 new putative new species, and 3 outgroup species [*Tyrrhenoleuctra tangerina*, *L. hippopus*, *L. ravizzai*]; 75 terminals), 4 loci (mtCOI, mt12S, nuH3, nu28S), 2580 bp molecular dataset (Table 3, Electronic Supplement Table S1, Electronic Supplement Dataset S1) using Bayesian Inference (BI) and Maximum Likelihood (ML) methods.

A species tree was estimated using STACEY v1.2.2 (Jones 2015, 2017) as implemented in BEAST2 (Bouckaert et al. 2014) using the corresponding nucleotide substitution models (Table 2). Single specimen identity was used as species trait. We estimated relationships between specimens and minimal clusters (Jones 2015, 2017) assuming a birth-death speciation tree prior under equal ploidy settings (Vitecek et al. 2017). Further, we assumed a collapse height of 1×10^{-4} while estimating collapse weight on a uniform prior around [0,1]; relative death rates were assumed to follow a uniform prior on [–0.5, 0.5]; population scale factor and birth-death collapse growth rates were assumed to follow a log-normal distribution around [4 ± 2.75]. Combined species tree and species delimitation estimation was run 3 × independently for 7×10^9 generations (sampling every 5,000th generation) to assure topological convergence between runs. Log files were examined in Tracer v1.6 (Rambaut et al. 2014) to assess whether runs had reached a stationary phase. Support for tree topologies estimated via STACEY was assessed by constructing a maximum clade credibility tree running TreeAnnotator v1.8.3 (Drummond et al. 2012) after discarding the first 75% of the sample as burn-in.

Additionally, a ML tree based on a concatenated dataset was estimated via RAxML (Stamatakis 2014) 3 × independently to assure topological convergence. Prior to concatenation, we inferred single-locus trees via RAxML using the corresponding partition schemes to assess potential incongruence between loci (Vitecek et al. 2015c). We found no supported conflicts between single-locus tree topologies (Electronic Supplement 1 Figures S1–S5). ML analyses of the concatenated dataset were then performed using the partition scheme identified by PartitionFinder (Table 2) with the default GTR + Γ nucleotide substitution model. Bootstrap support for ML topologies was inferred using the fast bootstrap algorithm with 1000 replicates.

Molecular species delimitation based on STACEY tree samples was performed via speciesDA.jar as outlined in Jones (2015), using a collapse height of 0.0003. ML trees were analysed with web-based bPTP (Zhang et al. 2013; http://species.h-its.org/) using default settings to infer species number estimates.

3. Results

3.1. Properties of the molecular dataset and phylogenetic analyses

Final alignments of mtCOI (1199 bp), mt12S (360 bp), nuH3 (301 bp), and nu28S (720 bp) comprised 36.61%, 16.38%, 21.93%, and 14.31% variable sites, respectively; 33.11%, 15.55%, 15.61%, and 6.66% of sites were parsimony-informative.

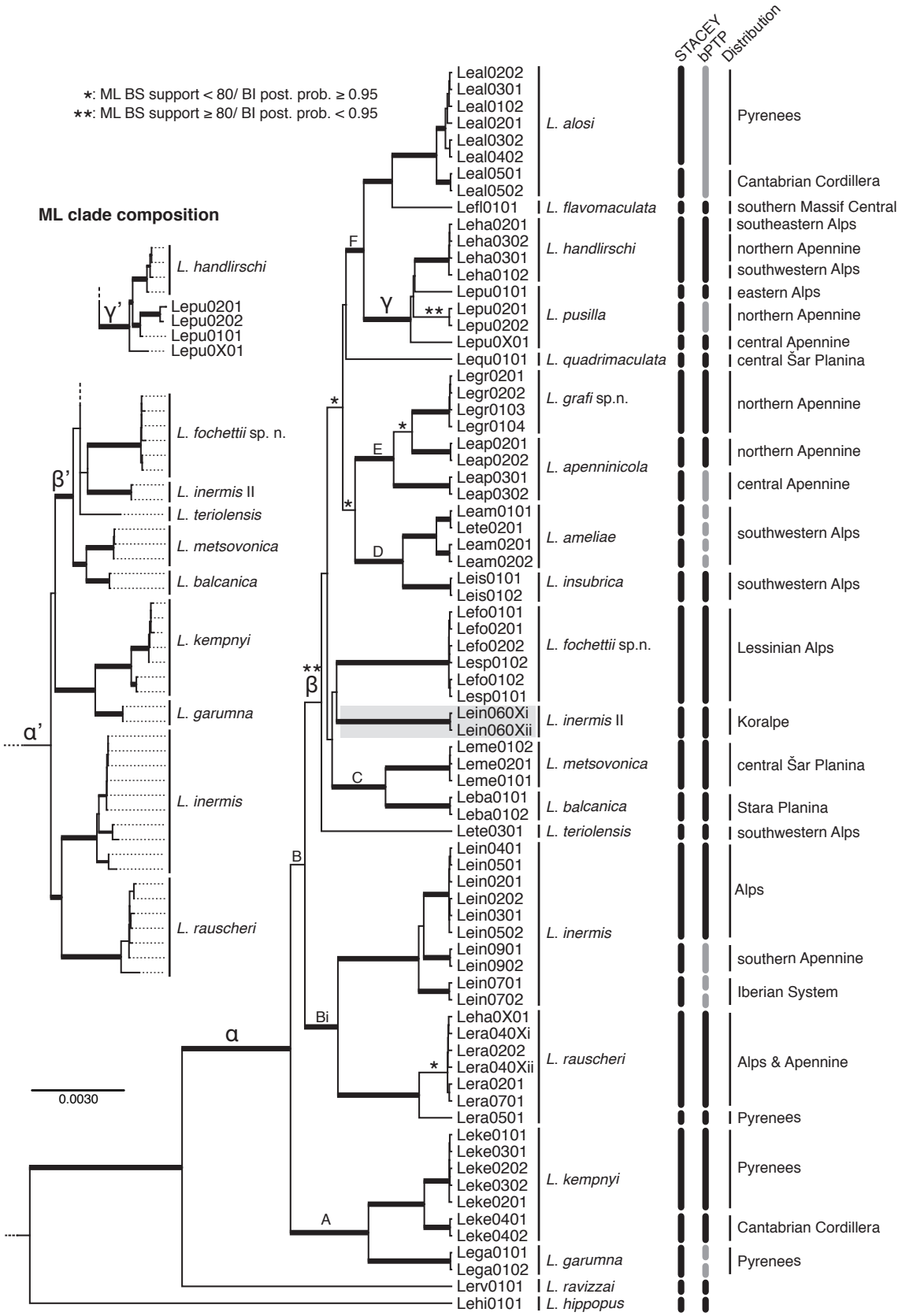


Fig. 1. Results of phylogenetic inference and molecular species delimitation using STACEY and bPTP algorithms. Maximum clade credibility B/MCMC STACEY species or minimal cluster tree using unique specimen identifiers as species traits, based on 2580 bp sequence data from partial sequences of 4 loci (mtCOI, mt12S, nuH3, nu28S). Bold branches indicate bootstrap support ≥ 80 and posterior probabilities ≥ 0.95. Alternative position of clades or specimens inferred through ML analysis at nodes α, β and γ are presented as pruned clades α', β' and γ'. Majuscules at branches indicate clades referred to in the text. Highly supported delimited molecular taxonomic units ('species') recovered by both analytical approaches are demarcated by solid black blocks, grey blocks indicate entities that were delineated but are not highly supported in bPTP analyses. Additionally, origin of delimited entities is provided. See Table 3 for specimen details; scale bar represents substitution rate.

All analyses suggest a close relatedness of the *Leuctra inermis* group taxa (Fig. 1, clades α, α') and recover the morphologically defined species (Figs. 3–20) as distinct clades. Topologies of final BI maximum clade credibility trees were convergent among independent runs. Likewise, optimal ML trees inferred on concatenated sequence data recovered the same topologies in independent runs (Fig. 1).

Both ML and BI analyses procure similar topologies and recover all species as highly supported clades and corroborate the new species *L. fochettii* sp.n. and *L. grafi* sp.n. However, relationships within the *L. inermis* group are largely unresolved in all analyses. Further, ML and BI suggest different placement of certain taxa: **(1)** BI recovers *L. kempnyi* + *L. garumna* as highly supported sister to an unsupported clade comprising all other *L. inermis* group species whereas ML analysis suggests *L. rauscheri* + *L. inermis* as highly supported sister to an unsupported clade comprising all other *L. inermis* group species (Fig. 1, clades α, α'); **(2)** BI places *L. teriolensis* as sister to a larger clade comprising *L. alosi*, *L. ameliae*, *L. apenninicola*, *L. balcanica*, *L. flavomaculata*, *L. fochettii* sp.n., *L. grafi* sp.n., *L. handlirschi*, *L. metsovonica*, *L. inermis* II, *L. insubrica* and *L. pusilla*; ML inference places *L. teriolensis* as sister to a larger clade comprising *L. alosi*, *L. ameliae*, *L. apenninicola*, *L. flavomaculata*, *L. fochettii* sp.n., *L. grafi* sp.n., *L. handlirschi*, *L. inermis* II, *L. insubrica* and *L. pusilla* – however, neither scenario is supported (Fig. 1, clades β, β'); **(3)** BI suggests different placement of *L. pusilla* specimens relative to another than ML analysis, but this is not supported – ML inference suggests a highly supported clade containing *L. pusilla* [central Apennine] + (*L. pusilla* [eastern Alps + northern Apennine] + *L. handlirschi*) (Fig. 1, clades γ, γ').

Within the *L. inermis* group, several sister species/clade relationships are highly supported: in both analyses, *L. kempnyi* + *L. garumna* is recovered (Fig. 1, clade A). A highly supported clade comprising the majority of *L. inermis* specimens (*L. inermis*) is recovered as sister to *L. rauscheri* (Fig. 1, clade Bi). Both analyses show highly supported sister taxon relationships between *L. metsovonica* and *L. balcanica* (Fig. 1, clade C), between *L. ameliae* and *L. insubrica* (Fig. 1, clade D), between (*L. appenninicola* I + *L. appenninicola* II) and *L. grafi* sp.n. (Fig. 1, clade E) and between ((*L. handlirschi* + *L. pusilla* [relationships of single specimens not resolved in BI]) and (*L. alosi* + *L. flavomaculata*)) (Fig. 1, clade F). BI further suggests a highly supported relationship between clades D, E, *L. quadrimaculata* and clade F (Fig. 1). Both analyses recover a highly supported clade of two specimens of *L. inermis* as sister of *L. fochettii* sp.n., but this relation is not supported (*L. inermis* II [Lein0601, Lein0602], highlighted grey in Fig. 1).

3.2. Molecular variation of intraspecific lineages

Within recognized species both BI and ML phylogenetic inferences as well as molecular species delimitation methods recover distinct geographic population structure (Figs. 1, 2). Molecular species delimitation via STACEY infers a total of 30 molecular taxonomic units ('species'); bPTP suggests 23 highly supported and 10 moderately supported molecular taxonomic units ('species') (Fig. 1). Both methods highly support *L. fochettii* sp.n. and *L. grafi* sp.n. (Fig. 1).

Specimens of *L. alosi* populations from the Iberian Cantabrian Cordillera (population code Leal05) are distinct from those collected in the Pyrenees (Leal01–Leal04) (Figs. 1, 2B). In *L. ameliae* two distinct clades are recovered as distinct taxa, corresponding to 2 populations ((Leam01+Lete02), Leam02) (Figs. 1, 2C). In *L. apenninicola* specimens from isolated mountain ranges in the Apennine peninsula are recovered as distinct lineages (Figs. 1, 2C). In *L. inermis*, specimens from extra-Alpine populations (Lein07, Lein09) are distinct from Alpine *L. inermis* populations (Lein02–Lein05) ((Alpine *L. inermis* + Iberian *L. inermis*) + Apennine *L. inermis*); additionally, *L. inermis* II (Lein06) is highly supported by molecular species delimitation (Figs. 1, 2A). Specimens of *L. kempnyi* from Pyrenees (Leke01–Leke03) and the Iberian Cantabrian Cordillera (Leke04) are recovered as distinct (Figs. 1, 2L). In *L. pusilla* a specimen from the Eastern Alps (Lepu01), a specimen from the central Apennine (Lepu0X) and 2 specimens from the northern Apennine (Lepu02) were found to differ from another in phylogenetic and species delimitation analyses (Figs. 1, 2N). Similar to *L. inermis*, Pyrenean (Lera05) and Alpine (Lera02, Lera04, Lera07) specimens of *L. rauscheri* are molecularly differentiated (Figs. 1, 2P).

3.3. Variation of morphological characters

The distinction of regional intraspecific lineages is also apparent in genital morphology. Variation of male and female primary and secondary genitalia structures within species corresponds well to phylogenetic structure of single-species clades, and population structure as recovered by molecular species delimitation.

4. Discussion

4.1. Phylogenetic resolution and taxonomic conclusions

While clades corresponding to phenotypically defined species were highly supported in phylogenetic analyses, relationships between species and clades were largely unresolved. This is likely due to the low differentiation between clades in 3 of the 4 partially sequenced loci used in phylogenetic inference (ranging from roughly 6–16% parsimony-informative sites). Moreover, the low variability of these loci necessitates rather cautious interpretation of molecular species delimitation within the *L. inermis*

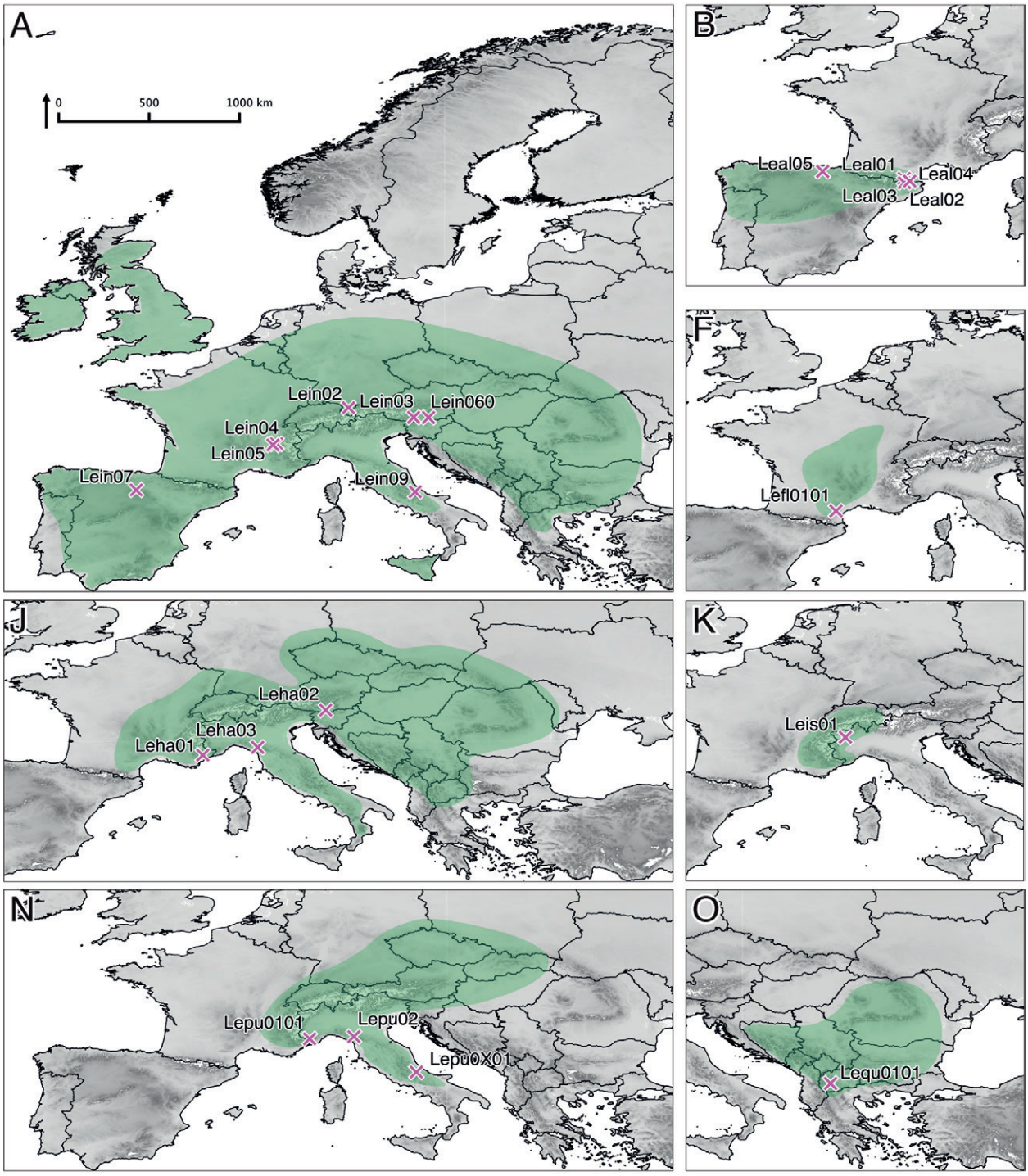
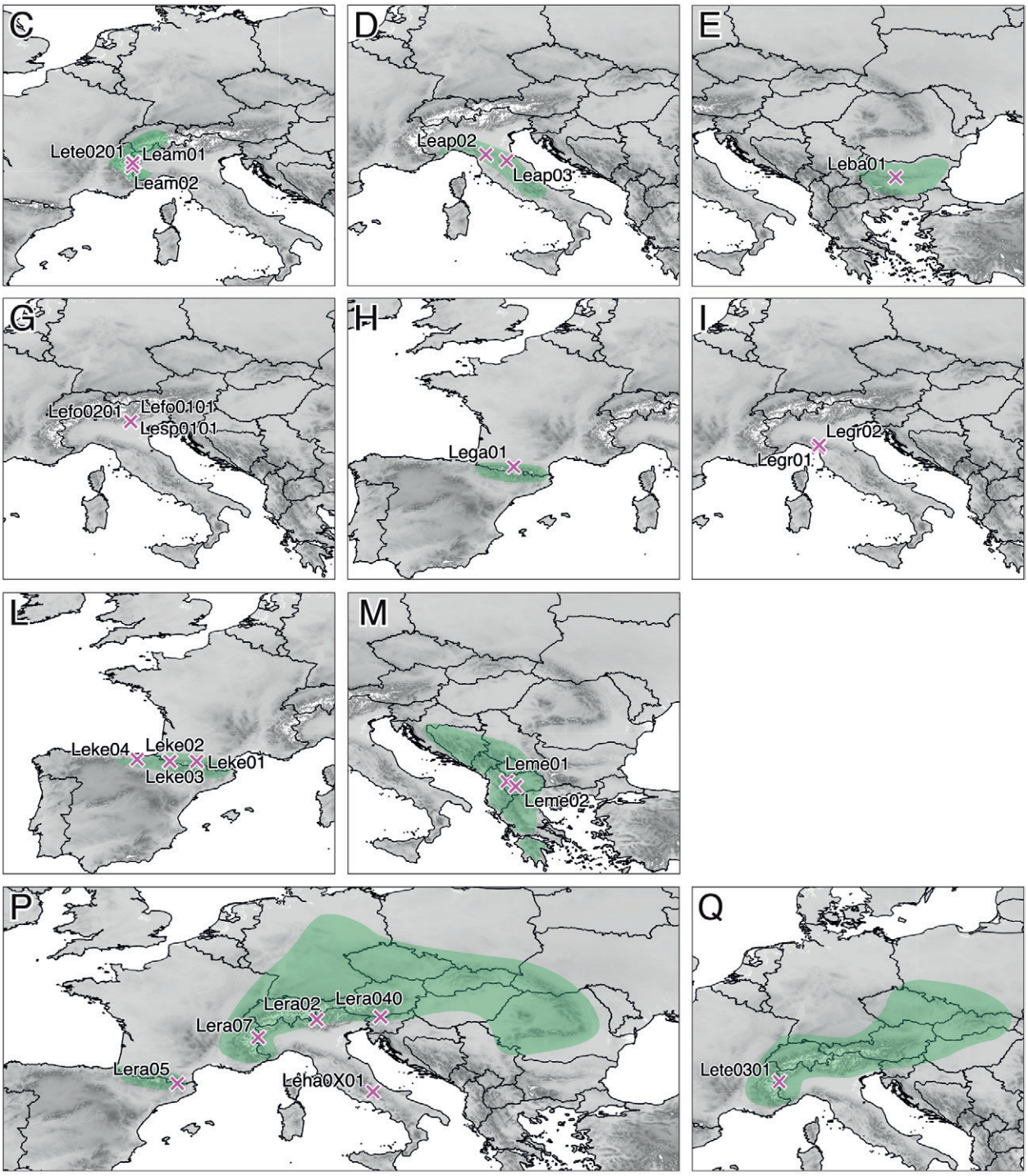


Fig. 2. Distribution of *Leuctra inermis* group species: **A**: *L. inermis*; **B**: *L. alosi*; **C**: *L. ameliae*; **D**: *L. apenninicola*; **E**: *L. balcanica*; **F**: *L. flavomaculata*; **G**: *L. fochettii* sp.n.; **H**: *L. garumna*; **I**: *L. grafi* sp.n.; **J**: *L. handlirschi*; **K**: *L. insubrica*; **L**: *L. kempnyi*; **M**: *L. metsovonica*;

species group, as the patterns observed could be due to an over-representation of the most variable sequence data.

Nevertheless, the morphological assessment of large series and the present molecular dataset supports description and delineation of the morphologically highly distinct *L. fochettii* sp.n. and *L. grafi* sp.n. in all analyses. However, we refrain from drawing additional taxonomic conclusions at this time. As the group is taxonomically challenging any systematic decision should consider taxonomic stability and applicability. While we observed morphologically and phylogenetically distinct entities within single species we cannot fully assess morphological or molecular apomorphies based on the current sampling for these lineages. For example we require more material of the population *L. inermis* II (currently represented by 5 specimens) to fully assess morphological variation.

Further studies based on a larger number of specimens should therefore be conducted for some species. A larger, integrative revisionary treatment of the group based on a more extensive geographic sampling is worthwhile.



N: *L. pusilla*; **O**: *L. quadrimaculata*; **P**: *L. rauscheri*; **Q**: *L. teriolensis*. Green areas indicate reported ranges of species, collection sites of specimens analysed in this study are identified by magenta crosses and the corresponding population code (see Table 3 for specimen details).

4.2. Taxonomic units in the *L. inermis* group

Traditionally recognized species of the *L. inermis* species group (Figs. 4–20) were consistently recovered as distinct clades in phylogenetic inference, corroborating molecular differentiation (Gattoliat et al. 2016). All analyses support *L. grafi* sp.n. and *L. fochettii* sp.n. as distinct entities. However, relationships between species (as minimal clusters or highly supported terminal ML clades) are not fully resolved.

Interestingly, ranges of some morphologically and molecularly well-defined widespread species overlap in the Alps, the Pyrenees and the Carpathians (Fig. 2): Alpine species like *L. rauscheri*, *L. teriolensis*, *L. inermis*, and *L. handlirschi* occur syntopically, a pattern also found in the Pyrenees in *L. alosi*, *L. kempnyi* and *L. inermis*. This indicates that effective mechanisms of reproductive isolation act to stabilize morphologically and ecologically close species. Male drumming calls and corresponding female preferences could represent a highly

effective pre-copulatory isolating mechanism. Indeed, species-specific drumming calls of males have been reported for several Plecoptera species, and can be used to identify species (RUPPRECHT 1968; ZIEGLER & STEWART 1977; MURÁNYI et al. 2014; BOUMANS & JOHNSEN 2015). However, drumming signals have not been extensively studied in the *L. inermis*-group, and no published recordings are known. Also, hybridization between *Leuctra* species occurs among some species (BOUMANS & TIERNO DE FIGUEROA 2016). This suggests that reproductive isolation is not solely based on pre-mating isolation, but that other pre- or postzygotic isolating mechanisms could also be in place.

4.3. Greater diversity in the *L. inermis* group?

Phylogenetic inference, molecular species delimitation and morphological variation of species indicate an interesting mix of sympatric and allopatric diversification processes leading to the present-day distribution patterns in the *L. inermis* group. In particular, morphological and molecular data congruently characterized species in the present study. Such integrative approaches combining morphological and molecular methods enhance taxonomic resolution and can be used to characterize cryptic aquatic diversity (PAULS et al. 2010; PREVIŠIĆ et al. 2014a). Recently, several new aquatic insect species were discovered based on similar integrative taxonomic approaches (PAULS et al. 2010; PREVIŠIĆ et al. 2014a; GRAF et al. 2015; IBRAHIMI et al. 2015, 2016; VITECEK et al. 2015a,b).

High degrees of endemism in the *L. inermis* group (only 4 species occur in more than 2 ecoregions *sensu* ILLIES 1978) could be a result of repetitive disruptive events that separated distinct populations. These could be induced by historic climate change or orogenesis and in turn enhance allopatric speciation. Indeed, similar effects of climatic oscillations and orogenetic processes were demonstrated in the Drusinae subfamily (Insecta: Trichoptera) (PAULS et al. 2006; PREVIŠIĆ et al. 2014b). Diversification and speciation in geographically isolated populations is likely mediated by ecological constraints imposed by habitat preferences, dispersal capability and phenology. Leuctridae are mostly cold-stenotopic, and their optimal temperature niche is predominately confined to montane areas of Europe (RAVIZZA & VINÇON 1998; RAVIZZA 2002; GRAF et al. 2009, 2017). Consequently, species of the group do not colonize highly connected habitats such as low-land streams, reducing population inter-breeding potential (BILTON et al. 2001; FINN et al. 2006; HUGHES 2007).

Nevertheless, there is clear evidence that some cold-stenotopic Leuctridae disperse over large distances to colonize distant islands (DOBRIN & GIBERSON 2005; DEWALT & SOUTH 2015). This astounding feat is likely achieved through surface skimming, a locomotion mode most commonly observed in Plecopterans (MARDEN et al. 2000). However, while dispersal capabilities of *Leuctra* species are remarkable for such tiny insects, maximal overland dispersal distances are estimated to be less than 600 m for the majority of the population (BRIERS et al. 2004; MACNEALE et al. 2005). Moreover, in *L. inermis* only minor proportions of individuals (0.1 – 0.2%) have been found to travel as far as 1000 m from their native stream overland (BRIERS et al. 2004). This limited overland dispersal potential is likely due to higher surface roughness of mountaineous landscapes that increases dispersal costs. Moreover, whether individuals dispersing overland contribute to gene flow between populations is unclear.

While wind potentially increases dispersal distances, strong wind also has been demonstrated to reduce Plecoptera flight propensity (BRIERS et al. 2003). Low air temperatures as frequently prevailing in high altitude have also been demonstrated to reduce flight activity in several species of aquatic insects, including stoneflies (WARINGER 1991; KUUSELA & HUUSKO 1996; BRIERS et al. 2003; FINN et al. 2006). Integrating this information, we assume that these taxa do not readily traverse between potential habitats.

In *Leuctra* numerous species co-occur, resulting in local fauna comprising both endemics and widely distributed species (POPIJAČ & SIVEC 2009; VINÇON & PARDO 2009; BOJKOVÁ et al. 2010; BOUMANS & BRITTAIN 2012; BOJKOVÁ & SOLDÁN 2013; BOUMANS & TIERNO DE FIGUEROA 2016). Thus, geographic isolation of morphologically similar species is likely not the only cause for reproductive/genetic isolation in Leuctridae. Diversification may additionally be controlled by localized evolution of specific female preferences for male drumming call patterns, resulting in exclusive regional drumming patterns (RUPPRECHT 1972; ZIEGLER & STEWART 1977; STEWART & SANDBERG 2004; LÓPEZ-RODRÍGUEZ & TIERNO DE FIGUEROA 2009; TIERNO DE FIGUEROA et al. 2011a,b; BOUMANS & JOHNSEN 2015). Thus, assessment of drumming call variation within and between species could help to elucidate taxonomic boundaries in the *L. inermis* group.

Species diversity in the *L. inermis* group is likely higher than currently recognized. The discovery of *L. grafi* sp.n. and *L. fochettii* sp.n. support the notion that numerous additional microendemic species are likely to exist. Both new species were collected in relatively isolated mountain systems, suggesting fragmentation among highland habitats as an important driver of allopatric speciation. Interestingly, the observed diversity patterns of micro-endemic species support the separateness and ecological significance of the Dinodal (a biome formed by permanently running, fast and turbulent streams on steep slopes and hard substrates that likely facilitated persistance of certain taxa in central European refugia during the glaciations) for generation and maintenance of European biodiversity (MALICKY 1983, 2000, 2006; MALICKY et al. 1983; PAULS et al. 2006; GRAF et al. 2011; WEISS et al. 2011; TRIZZINO et al. 2014). Further, comparative morphology also supports the hypothetical existence of conspecific, geographically isolated populations. Morphologically, molecularly and geographically differentiated populations of *L. alosi*, *L. inermis*, *L. ap-*

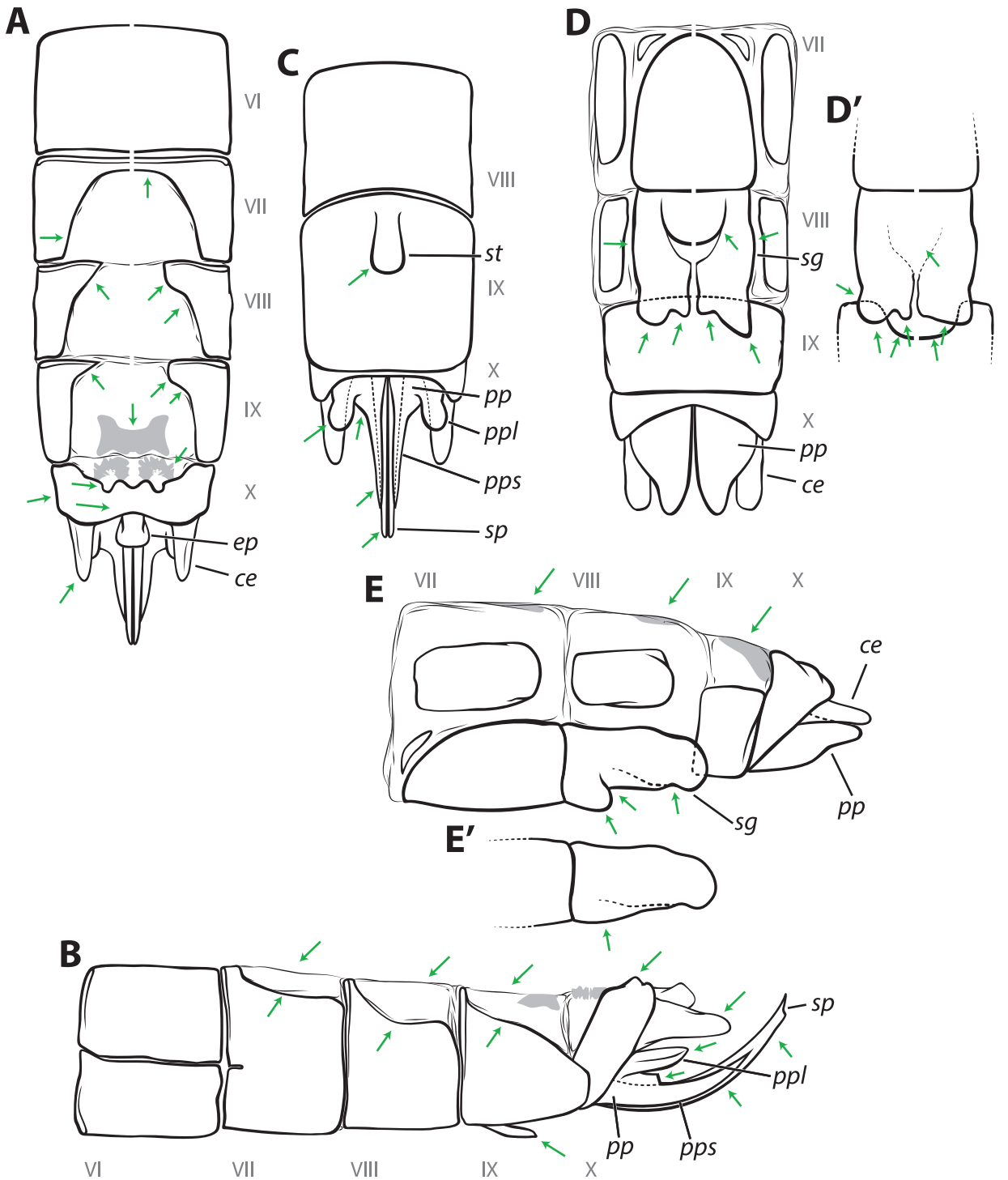


Fig. 3. Guide to the morphological characteristics of *Leuctra inermis* group species, including a synopsis of common character states. **A**: generic *L. inermis* group male, dorsal view (left and right halves depict different character states); **B**: generic *L. inermis* group male, lateral view; **C**: generic *L. inermis* group male, ventral view; **D**: generic *L. inermis* group female with anteromedian protuberance on subgenital plate, ventral view (left and right halves depict different character states); **D'**: generic *L. inermis* group female without anteromedian protuberance on subgenital plate, ventral view (left and right halves depict different character states); **E**: generic *L. inermis* group female with anteromedian protuberance on subgenital plate, lateral view; **E'**: generic *L. inermis* group female without anteromedian protuberance on subgenital plate, lateral view. — ***Abbreviations***: *ce*, cerci; *ep*, epiproct; *pp*, paraproct; *ppl*, lobes of paraproct; *pps*, stylus of paraproct; *sg*, subgenital plate; *sp*, specilla; grey areas indicate sclerotized spots surrounded by unsclerotized cuticle; roman numerals (in grey) identify abdominal segments. Green arrows indicate diagnostic characters like sclerotization patterns of male abdominal tergites or shape of parts of the subgenital plate. — Illustration neither to scale nor proportionally correct nor taxonomically exhaustive. Del. Vitecek.

enninicola and *L. kempnyi* may represent distinct species – however, considering the ranges of some species (*L. inermis*, *L. handlirschi*, *L. rauscheri*, *L. teriolensis*), the currently available dataset does not support conclusive taxonomic decisions. Clarification of the systematic status of these morphological variants should ideally be

achieved based on an integrative examination of specimens from the whole distribution range that includes most or all known variants (PREVIŠIĆ et al. 2014a).

5. Diagnosis and description of the new species

5.1. *Leuctra fochettii* sp.n. Vinçon & Graf

Fig. 9

Material. ***Holotype:*** ♂: Italy, Venetia, Venetian Prealps, Lessini Mountains, vicinity of Campogrosso Pass, northern slope, 1300–1350 m a.s.l., 45.751912°N 11.186812°E, leg. Vinçon 08.vi.2008, deposited in the MZL. — ***Paratypes:*** 1 ♂ (voucher ID for 1 ♂ Lesp0101; currently in coll. Vitecek, will be transferred to SFNF), 1 ♀ (voucher ID for 1 ♀ Lesp0102; currently in coll. Vitecek, will be transferred to SFNF), 8 ♂♂, 12 ♀♀: same locality and date (in coll. Vinçon). — ***Additional material:*** 40 ♂♂, 43 ♀♀: same locality and date (in coll. Vinçon). 2 ♂♂, 1 ♀: same locality, leg. Vinçon 11.ix.2008 (in coll. Vinçon). 15 ♂♂, 17 ♀♀: below Campogrosso Pass, southern slope, spring and brook, 1200–1300 m a.s.l., leg. Vinçon 08.vi.2008 (in coll. Vinçon). 32 ♂♂, 38 ♀♀: same location and date, 1100–1200 m a.s.l. (in coll. Vinçon). 7 ♂♂, 1 ♀: Italy, Lessini Mountains, below Fugazze Pass, northern slope, above Camposilvano, spring, 1150–1170 m, leg Vinçon 08.vi.2008 (in coll. Vinçon). 6 ♂♂, 12 ♀♀: Italy, Lessini Mountains, below Fugazze Pass, southern slope, 900–1000 m a.s.l., leg. Vinçon 29.vi.2009 (in coll. Vinçon). 1 ♂, 4 ♀♀: Italy, Lessini Mountains, below Fugazze Pass, southern slope, above San Antonio, spring, 800 m a.s.l., leg. Vinçon 29.vi.2009 (in coll. Vinçon). 28 ♂♂, 17 ♀♀: same locality, leg. Vinçon 13.v.2010 (in coll. Vinçon). 22 ♂♂, 28 ♀♀: Italy, Venetia, Venetian Prealps, south Trento, above Folgaria, above San Sebastiano, brook and spring, 1280 m a.s.l., leg. Vinçon 01.v.2009 (in coll. Vinçon). 2 ♂♂, 1 ♀: same locality, leg. Vinçon 02.v.2009 (in coll. Vinçon). 11 ♂♂ (voucher ID for 1 ♂ Lefo0201), 10 ♀♀ (voucher ID for 1 ♀ Lefo0202) (currently in coll. Vitecek, will be transferred to coll. SFNF): Italy, Venetia, Venetian Prealps, East of Camposilvano, 45.7569°N 11.15721667°E, leg. Graf 16.vii.2016 (in coll. Graf). 1 ♂ (voucher ID for 1 ♂ Lefo0101; currently to coll. Vitecek, will be transferred to coll. Graf), 2 ♀♀ (voucher ID for 1 ♀ Lefo0102; currently in coll. Vitecek, will be transferred to coll. Graf): Italy, Venetia, Venetian Prealps, East of Camposilvano, 45.75661111°N 11.15752778°E, leg. Graf 25.vii.2016 (in coll. Graf).

Diagnosis. A medium-sized, macropterous *Leuctra* species of the *L. inermis* group. ***Males*** of *L. fochettii* sp.n. are most similar to males of *L. rauscheri*, but exhibit (1) abdominal segment IX ventral vesicle suboval, small; (2) abdominal segment X in dorsal view medially narrow with 2 distinct mediolateral incisions of the anterior margin; (3) abdominal segment X in dorsal view medially narrow with a shallow posterior incision; (4) styli broad with subtriangular cross-section; (5) styli in lateral view proximally wider than distally, with a distinct step separating proximal and distal portions. Males of *L. rauscheri* (Fig. 19) have a suboval, large ventral vesicle of abdominal segment IX; a medially narrow abdominal segment X with a medial incision of the anterior margin; a distinct posterior incision of abdominal segment X; styli slender; styli in lateral view evenly curved and evenly tapering. ***Females*** are most similar to females of *L. rauscheri* but exhibit (1) a distinct elongate ventral protuberance of the subgenital plate; (2) posterior lobes of the subgenital plate laterally longer than medially; (3) posterior lobes of subgenital plate with median lobules, projecting posteriorly. Females of *L. rauscheri* (Fig. 19) have a rounded, short ventral protuberance of the subgenital plate; posterior lobes of the subgenital plate medially and laterally of similar length; posterior lobes of subgenital plate without median lobules.

Description. ***Habitus*** as typical for the group and of little diagnostic value: General colour brown to dark brown. Head dark brown with black granulation on occiput. Antennal scapus light brown, flagellum dark brown; distal portion of each antennomere with ring of setae shorter than antennomere width. Pronotum brown to dark brown with darker pattern. Legs brown. Body, wings and legs covered with short setae. Body size in males 5.8–6.5 mm, forewing length 6.8–7.2 mm (n=14); in females 7.6–8 mm, forewing length 8.5–9 mm (n=13).

Male abdominal tergal ornamentation and genitalia: Abdominal terga I–VI fully sclerotized. Abdominal tergum VII medially membranous, anterior portion pigmented, with discontinuous anterior margin. Abdominal tergum VIII with discontinuous anterior margin, medially membranous; medial posterolateral projections ("tips") of anterior margin not projecting into membranous portion; tips distinctly sclerotized, broad, pointed, with basal anterior subtriangular protrusions. Abdominal tergum IX with discontinuous anterior margin, medially membranous; medial projections of anterior margin with basal anterior subtriangular protrusions forming subrectangular tips of lateral sclerotized tergal portions; membranous medial portion medioposteriorly with subrectangular sclerotized spot with subrectangular anteromedial incision. Ventral vesicle of abdominal segment IX suboval; basal pedicle as wide as ventral vesicle. Abdominal segment X in dorsal view medially narrow with a regular anterior margin and 2 distinct mediolateral incisions of the anterior margin; in dorsal view with shallow posterior incision; in dorsal and lateral view posterior margin pilose, elevated. Cerci normal, covered with long setae. Epiproct with short basal process, in dorsal view rounded. Paraproct in ventral and lateral views with suboval lateral lobes; in ventral and caudal view medial styli broad, stout; in lateral view styli proximally wider than distally with a distinct step separating proximal and distal portions, distal portion evenly curved, pointed; styli projecting posterodorsally; styli once their width shorter than the specilla. Specilla short, evenly curved, projecting posterodorsally, with pointed tip.

Female genitalia: Abdominal venter VII covered by wide subtrapezoidal sclerite. Abdominal sternum VIII (subgenital plate) in ventral and lateral views with distinct elongate, rounded anteromedian protuberance; in lateral view distal portion of anteromedian protuberance projecting somewhat posteriorly; in ventral view, lateral margins of subgenital plate slightly sinuous; posterior

lobes of subgenital plate with median lobules, projecting posteriorly; posterior lobes of subgenital plate medially elevated; lateral tips of posterior lobes of subgenital plate subtriangular, laterally longer than medially. Cerci slightly longer than paraproct in lateral and ventral view. ***Larva and eggs***: Unknown.

Biological remarks. To our current knowledge, *L. fochettii* is a cold-stenothermic micro-endemic species restricted to the Lessinian Alps in the Venetian Prealps, where it occurs mainly in high altitude biotopes (1000–1380 m) or in cold springs at lesser altitudes (800 m). The emergence period extends over summer, and adults are on the wing from late spring to early autumn (V–IX). As a result of intensive studies performed all over the Italian Alps, the Lessinian Mountains were identified as hot spot of aquatic biodiversity (Malicky 2006; Trizzino et al. 2014): This mountainous region close to the Po Plain is well separated from the rest of the Alps westwards by the wide glacial valley of the Adige River followed by Garda Lake and northwards and eastwards by another wide glacial valley (the Valsugana Valley and Brenta River Valley). In this region several micro-endemic species occur, especially in the stoneflies with 5 species that are restricted to these refuge mountains: *Isoperla* sp.n., *Protonemura bipartita* Consiglio, 1962, *L. dylani* Graf, 2007, *L. juliettae* Vinçon & Graf, 2011 and *L. grafi* Vinçon & Vitecek, sp.n.; additionally, the caddisfly *Ecclisopteryx malickyi* Morretti, 1991 is reported as an endemic from the same area.

Etymology. Named for Prof. Romolo Fochetti.

5.2. *Leuctra grafi* sp.n. Vinçon & Vitecek

Fig. 11

Material. ***Holotype***: ♂: Italy, Toscana, Apuan Alps, North-Eastern Carrara, Vinca, 44.132778°N 10.179167°E, leg. Graf 17.vii.2007, deposited in the MZL. — ***Paratypes***: 3 ♂♂ (voucher ID for 1 ♂ Legr0101), 3 ♀♀ (voucher ID for 1 ♀ Legr0101) (currently in coll. Vitecek, will be transferred to SFNF); 6 ♂♂, 15 ♀♀: same locality and date (in coll. Vinçon). — ***Additional material***: 10 ♂♂ (voucher ID for 1 ♂ Legr0103; currently in coll. Vitecek, will be transferred to SFNF), 12 ♀♀ (voucher ID for 1 ♀ Legr0104; currently in coll. Vitecek, will be transferred to SFNF): Italy, Northern Apennines, Toscana, Apuan Alps, N.E. Carrara, Vinca, 44.136205°N 10.164215°E, leg. Vinçon 28.vi.2016 (in coll. Vinçon). 3 ♂♂ (voucher ID for 1 ♂ Legr0201; currently in coll. Vitecek, will be transferred to SFNF), 3 ♀♀ (voucher ID for 1 ♀ Legr0202; currently in coll. Vitecek, will be transferred to SFNF): Italy, Northern Apennines, Toscana, above Cerreto pass, southern slope of the pass, glacial circus, 1500 m a.s.l., 44.295403°N 10.233044°E, leg. Vinçon 28.vi.2016 (in coll. Vinçon). 1 ♂: Italy, Northern Apennines, Toscana, Apuane Alps, between Carrara and Vinca, prati di Campocecina, 1300 m a.s.l., leg. Campari & Ravizza 22.vi.1978 (in coll. Vinçon).

Diagnosis. A medium-sized, macropterous *Leuctra* species of the *L. inermis* group. ***Males*** of *L. grafi* sp.n. are most similar to males of *L. apenninicola* and *L. insubrica* [males of these species are characterized by two acute processes extending posterior and median from the anterior margin of tergite VIII]. Males of *L. grafi* sp.n. exhibit (1) abdominal tergum VII with wide, strongly sclerotized anterior margin; (2) discontinuous anterior margin of abdominal tergum VIII with broad, rounded, heavily sclerotized medioposteriad tips lacking anterior subtriangular protrusions; (3) membranous portion of abdominal tergum IX with a sclerotized tergal spot composed of 2 subtriangular patches, fused at their posterior margins; (4) ventral vesicle of abdominal segment IX subrectangular; (5) abdominal segment X in dorsal view medially narrow with 2 distinct incisions on the anterior margin; (6) posterior margin of abdominal segment X in dorsal and lateral view with distinct pilose dorsal protuberance, elevated and projecting dorsal and posterior in lateral view; (7) globular lateral lobes of styli; (8) styli twice their width shorter than the specilla. Males of *L. apenninicola* (Fig. 6) have discontinuous anterior margins of abdominal tergum VIII with thin, rounded medioposteriad tips with anterior subtriangular protrusions; a differently shaped sclerotized spot on membranous tergum of abdominal segment IX; a suboval, anteriorly narrower ventral vesicle of abdominal segment IX; an only slightly elevated pilose dorsal protuberance on posterior margin of abdominal segment X. Males of *L. insubrica* (Fig. 14) have abdominal tergum VII with thin strongly sclerotized anterior margin; discontinuous anterior margins of abdominal tergum VIII with thin, pointed medioposteriad tips with anterior subtriangular protrusions; a differently shaped sclerotized spot on membranous tergum of abdominal segment IX; a suboval, posteriorly slightly narrower ventral vesicle of abdominal segment IX; 2 slightly elevated, rounded pilose dorsal protuberances on posterior margin of abdominal segment X; slender lateral lobes of styli; styli only once their width shorter than the specilla.

Females of the new species are most similar to females of *L. apenninicola*, *L. insubrica*, and *L. inermis* but exhibit (1) an evenly rounded subgenital plate in lateral and ventral view; (2) lateral margins of subgenital plate sinuous; (3) posterior lobes of subgenital plate medially elevated; (4) posterior lobes of subgenital plate without median lobules; (5) tips of posterior lobes of subgenital plate subrectangular, medially and laterally of similar length, slightly projecting laterally. Females of *L. apenninicola* have a distinct rounded anteromedian protuberance of the subgenital plate; tips of posterior lobes of subgenital plate suboval, projecting laterad, laterally longer than medially. Females of *L. insubrica* have posterior lobes of subgenital plate with median lobules; tips of posterior lobes of subgenital plate suboval, projecting laterally. Females of *L. inermis* have straight or posteriorly converging lateral margins of subgenital plate; medially mostly flat posterior lobes of subgenital plate; posterior lobes of subgenital plate with median lobules projecting medially; tips of posterior lobes of subgenital plate rounded, projecting posteriorly.

Description. ***Habitus***: General colour brown to dark brown. Head dark brown with black granulation on

occiput. Antennal scapus light brown, flagellum dark brown; distal portion of each antennal segment with ring of setae shorter than antennal segment width. Pronotum brown to dark brown with darker pattern. Legs brown. Body, wings and legs covered in short setae. Body size in males 4.5–4.9 mm, forewing length 4.8–5 mm (n=23); in females 5.0–6.1 mm, forewing length 5.8–6.3 mm (n=33).

Male abdominal tergal ornamentation and genitalia: Abdominal terga I–VI fully sclerotized. Abdominal tergum VII medially membranous with wide, strongly sclerotized anterior margin. Abdominal tergum VIII with discontinuous anterior margin, medially membranous; medioposteriad projections ("tips") of anterior margin heavily sclerotized, broad, rounded, without basal anterior subtriangular protrusions. Abdominal tergum IX with discontinuous anterior margin, medially membranous; medioanterior projections of anterior margin weakly sclerotized; membranous portion mediposteriorly with sclerotized spot composed of 2 subtriangular patches, fused at their posterior margins. Ventral vesicle of abdominal segment IX subrectangular; basal pedicle as wide as ventral vesicle. Abdominal segment X in dorsal view medially narrow with an irregular anterior margin and 2 distinct incisions of the anterior margin; in dorsal and lateral view with a distinct, elongate suboval, pilose protuberance; in lateral view pilose dorsal protuberance elevated and projecting dorsally and posteriorly. Cerci normal, covered in long setae. Epiproct with short basal process, in dorsal view rounded. Paraproct in ventral and lateral views with globular lateral lobes; medially with evenly curved, pointed styli projecting posterodorsally; styli twice their width shorter than the specilla. Specilla evenly curved, projecting posterodorsally, with pointed tip.

Female genitalia: Abdominal venter VII covered by wide subtrapezoid sclerite. Abdominal sternum VIII (subgenital plate) in ventral and lateral views evenly rounded, without anteromedian protuberance; in ventral view, lateral margins of subgenital plate sinuous; posterior lobes of subgenital plate without median lobules, medially elevated; tips of posterior lobes of subgenital plate subrectangular, medially and laterally of similar length; tips of posterior lobes of subgenital plate projecting slightly laterally. Cerci longer than paraproct in lateral and ventral view.

Larva and eggs: Unknown.

Biological remarks. To our current knowledge, *L. grafi* is a micro-endemic species occurring only in the Apuan Alps in the Northern Apennines and in a restricted part of the Northern Apennines close to Cerreto Pass of the Apuan Alps. It is a crenophilous, cold-stenotopic taxon inhabiting small brooks and springs of montane areas. Flight period is late June to July.

Etymology. Named for Wolfram Graf, trichopterologist, plecopterologist, and collector of the type specimens.

6. Acknowledgements

This research received support from the SYNTHESYS Project (http://www.synthesys.info/) which is financed by European Community Research Infrastructure Action under the FP7 "Capacities" Program, SYNTHESYS grant (DE-TAF-5890) to SV and SUP. Collection trips of WG were conducted in the framework of the FWF-projects P18073-B03 and P23687-B17 (PI: Johann Waringer, Department for Limnology and Bio-Oceanography, University of Vienna). We thank Jean-Luc Gattoliat and Thomas Kaltenbach for sharing PCR protocols and primer sequences. F. Dossi supplied highly appreciated photographs of *L. teriolensis*. Markward Herbert Fischer is thanked for his excellent layouting support. We are most grateful to two anonymous reviewers, Gavin Svenson and Klaus-Dieter Klass whose comments substantially improved this manuscript.

7. References

AUBERT J. 1957. Les *Leuctra* du groupe de *inermis* Kempny et quelques espèces inermes isolées (Plécoptères Leuctridae). – Mitteilungen der Schweizerischen Entomologischen Gesellschaft **4**: 285–312.

BERTHÉLÉMY C. 1968. Contribution à la connaissance des Leuctridae [Plecoptera]. – Annales de Limnologie – International Journal of Limnology **4**: 175–198.

BILTON D.T., FREELAND J.R., OKAMURA B. 2001. Dispersal in Freshwater Invertebrates. – Annual Review of Ecology and Systematics **32**: 159–181.

BIRK S., BONNE W., BORJA A., BRUCET S., COURRAT A., POIKANE S., SOLIMINI A., VAN DE BUND W., ZAMPOUKAS N., HERING D. 2012. Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. – Ecological Indicators **18**: 31–41.

BO T., FENOGLIO S., LÓPEZ-RODRÍGUEZ M.J., TIERNO DE FIGUEROA J.M. 2009. Phenology of adult stoneflies (Plecoptera) of the Curone Stream (Northern Apennines, Italy). – Journal of Freshwater Ecology **24**: 279–283.

BOJKOVÁ J., SOLDÁN T. 2013. Stoneflies (Plecoptera) of the Czech Republic: species checklist, distribution and protection status. – Acta Entomologica Musei Nationalis Pragae **53**: 443–484.

BOJKOVÁ J., SOLDAN T., ZAHRÁDKOVÁ S., CHVOJKA P., TRYZNA M. 2010. Ephemeroptera and Plecoptera of the Bohemian Switzerland National Park, Czech Republic: species diversity and taxocenoses of sandstone watercourses. – Lauterbornia **70**: 91–110.

BOTTOVÁ K., DERKA T., BERACKO P., TIERNO DE FIGUEROA J.M. 2013. Life cycle, feeding and secondary production of Plecoptera community in a constant temperature stream in Central Europe. – Limnologica-Ecology and Management of Inland Waters **43**: 27–33.

BOUCKAERT R., HELED J., KÜHNERT D., VAUGHAN T., WU C.-H., XIE D., SUCHARD M.A., RAMBAUT A., DRUMMOND A.J. 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. – PLoS Computational Biology **10**: e1003537–6.

BOUMANS L., JOHNSEN A. 2014. Species-specific communication bars interspecific mating between syntopic species of *Zwicknia* stoneflies (Plecoptera: Capniidae). – Biological Journal of the Linnean Society **113**: 969–980.

BOUMANS L., MURÁNYI D. 2014. Two new species of *Zwicknia* Murányi, with molecular data on the phylogenetic position of the genus (Plecoptera, Capniidae). – Zootaxa **3808**: 1–91.

BOUMANS L., BRITTAIN J.E. 2012. Faunistics of stoneflies (Plecoptera) in Finnmark, northern Norway, including DNA barcoding of Nemouridae. – Norwegian Journal of Entomology **59**: 196–215.

Boumans L., Tierno de Figueroa J.M. 2016. Introgression and species demarcation in western European *Leuctra fusca* (Linnaeus, 1758) and *L. digitata* Kempny, 1899 (Plecoptera: Leuctridae). – Aquatic Insects **37**: 115–126.

Brecko J., Mathys A., Dekoninck W., Leponce M., VandenSpiegel D., Semal P. 2014. Focus stacking: Comparing commercial top-end set-ups with a semi-automatic low budget approach. A possible solution for mass digitization of type specimens. – ZooKeys **464**: 1–23.

Briers R.A., Cariss H.M., Gee J.H.R. 2003. Flight activity of adult stoneflies in relation to weather. – Ecological Entomology **28**: 31–40.

Briers R.A., Gee J., Cariss H.M., Geoghegan R. 2004. Inter-population dispersal by adult stoneflies detected by stable isotope enrichment. – Freshwater Biology **49**: 425–431.

Crandall K.A., Harris D.J., Fetzner J.W. 2000. The monophyletic origin of freshwater crayfish estimated from nuclear and mitochondrial DNA sequences. – Proceedings of the Royal Society of London B: Biological Sciences **267**: 1679–1686.

De Walt R.E., Kondratieff B.C., Sandberg J.B. 2015. Order Plecoptera. Pp. 933–949 in: Thorp J.H., Rogers D.C. (eds), Thorp and Covich's Freshwater Invertebrates, 4th Edition. – Academic Press, London, Oxford, Boston, New York, San Diego.

De Walt R.E., South E.J. 2015. Ephemeroptera, Plecoptera, and Trichoptera on Isle Royale National Park, USA, compared to mainland species pool and size distribution. – ZooKeys **532**: 137–158.

Dénes A.-L., Kolcsár L.-P., Török E., Keresztes L. 2016. Phylogeography of the micro-endemic *Pedicia staryi* group (Insecta: Diptera): evidence of relict biodiversity in the Carpathians. – Biological Journal of the Linnean Society **119**: 719–731.

Dobring M., Giberson D.J. 2003. Life history and production of mayflies, stoneflies and caddisflies (Ephemeroptera, Plecoptera, and Trichoptera) in a spring-fed stream in Prince Edward Island, Canada: evidence for population asynchrony in spring habitats? – Canadian Journal of Zoology **81**: 1083–1095.

Drummond A.J., Suchard M.A., Xie D., Rambaut A. 2012. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. – Molecular Biology and Evolution **29**: 1969–1973.

Elbrecht V., Feld C.K., Gies M., Hering D., Sondermann M., Tollrian R., Leese F. 2014. Genetic diversity and dispersal potential of the stonefly *Dinocras cephalotes* in a central European low mountain range. – Freshwater Science **33**: 181–192.

Fenoglio S., Bo T., López-Rodríguez M.J., Tierno de Figueroa J.M. 2010. Life cycle and nymphal feeding of *Besdolus ravizzarum* (Plecoptera: Perlodidae), a threatened stonefly. – Insect Science **17**: 149–153.

Finn D.S., Theobald D.M., Black W.C. IV, Poff N.L. 2006. Spatial population genetic structure and limited dispersal in a Rocky Mountain alpine stream insect. – Molecular Ecology **15**: 3553–3566.

Fochetti R., Tierno de Figueroa J.M. 2008. Global diversity of stoneflies (Plecoptera; Insecta) in freshwater. – Hydrobiologia **595**: 365–377.

Fochetti R., Gaetani B., Fenoglio S., Bo T., Lopez-Rodriguez M.J., Tierno De Figueroa J.M. 2011. Systematics and biogeography of the genus *Besdolus* Ricker, 1952 (Plecoptera, Perlodidae): molecules do not match morphology. – Zootaxa **3067**: 49–58.

Fochetti R., Sezzi E., Tierno de Figueroa J.M., Modica M.V., Oliverio M. 2009. Molecular systematics and biogeography of the western Mediterranean stonefly genus *Tyrrhenoleuctra* (Insecta, Plecoptera). – Journal of Zoological Systematics & Evolutionary Research **47**: 328–336.

Folmer O., Black M., Hoeh W., Lutz R., Vrijesnhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. – Molecular Marine Biology and Biotechnology **3**: 294–299.

Gattolliat J.-L., Vinçon G., Wyler S., Pawlowski J., Sartori M. 2016. Toward a comprehensive COI DNA barcode library for Swiss stoneflies (Insecta: Plecoptera) with special emphasis on the genus *Leuctra*. – Zoosymposia **11**: 135–155.

Graf W. 2010. Aktualisierte Check-Liste der Steinfliegen (Insecta: Plecoptera) Österreichs. – Lauterbornia **71**: 175–183.

Graf W., Grasser G., Weinzierl A. 2002. Plecoptera. Part III – Metazaoa. 17 pp. in: Moog O. (ed.), Fauna Aquatica Austriaca, Edition 2002. – Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft, Vienna.

Graf W., Lorenz A.W., Tierno de Figueroa J.M., Lücke S., López-Rodríguez M.J., Davies C. 2009. Distribution and Ecological Preferences of European Freshwater Organisms. Volume 2 – Plecoptera. Edited by Schmidt-Kloiber A., Hering D. – Pensoft Publishers (Sofia-Moscow). 262 pp.

Graf W., Kučinič M., Previšič A., Pauls S.U., Waringer J. 2011. The larva of *Ecclisopteryx malickyi* Moretti, 1991 (Trichoptera: Limnephilidae: Drusinae) with comments on the genus. – Zoosymposia **5**: 136–143.

Graf W., Lorenz A.W., Tierno de Figueroa J.M., Lücke S., López-Rodríguez M.J., Murphy J., Schmidt-Kloiber A. 2017. Dataset "Plecoptera". – www.freshwaterecology.info – the taxa and autecology database for freshwater organisms, version 7.0 (accessed on 10.01.2017).

Graf W., Schmidt-Kloiber A. 2010. Taxonomie und Verbreitung von Steinfliegen – Plecoptera in Österreich. Unterlagen zu „Taxonomie und Ökologie aquatischer wirbelloser Organismen – TEIL VII". – Eigenverlag.

Graf W., Vitecek S. 2016. A new species of Limnephilidae (Insecta: Trichoptera) from the Western Alps (Insecta: Trichoptera). – Zootaxa **4085**: 431–437.

Graf W., Vitecek S., Previšić A., Malicky H. 2015. New species of Limnephilidae (Insecta: Trichoptera) from Europe: Alps and Pyrenees as harbours of unknown biodiversity. – Zootaxa **3911**: 381–395.

Hadley A. 2008. CombineZP. – Available at: http://www.hadleyweb.pwp.blueyonder.co.uk/CZP/News.htm (accessed 12.05.2012)

Hering D., Buffagni A., Moog O., Sandin L., Sommerhäuser M., Stubauer I., Feld C.K., Johnson R., Pinto P., Skoulikidis N., Verdonschot P., Zahrádková S. 2003. The development of a system to assess the ecological quality of streams based on macroinvertebrates – design of the sampling programme within the AQEM project. – International Review of Hydrobiology **88**: 345–361.

Hering D., Feld C.K., Moog O., Ofenböck T. 2006. Cook book for the development of a Multimetric Index for biological condition of aquatic ecosystems: Experiences from the European AQEM and STAR projects and related initiatives. – Hydrobiologia **566**: 311–324.

Hughes J.M. 2007. Constraints on recovery: using molecular methods to study connectivity of aquatic biota in rivers and streams. – Freshwater Biology **52**: 616–631.

Hynes H. 1976. Biology of Plecoptera. – Annual Review of Entomology **21**: 135–153.

Ibrahimi H., Kučinić M., Vitecek S., Waringer J., Graf W., Previšić A., Bálint M., Keresztes L., Pauls S.U. 2015. New records for the Kosovo caddisfly fauna with the description of a new species, *Drusus dardanicus* sp. nov. (Trichoptera: Limnephilidae). – Zootaxa **4032**: 551–568.

Ibrahimi H., Vitecek S., Previšić A., Kučinić M., Waringer J., Graf W., Bálint M., Keresztes L., Pauls S.U. 2016. *Drusus sharrensis* sp. n. (Trichoptera, Limnephilidae), a new species from Sharr National Park in Kosovo, with molecular and ecological notes. – ZooKeys **559**: 107–124.

Illies J. 1965. Phylogeny and Zoogeography of the Plecoptera. – Annual Review of Entomology **10**: 117–140.

Illies J. 1978. Limnofauna Europaea. A Checklist of the Animals Inhabiting European Inland Waters, with an Account of their Distribution and Ecology. 2nd Edition. – Gustav Fischer Verlag, Stuttgart. 552 pp.

JONES G.R. 2015. STACEY: species delimitation and phylogeny estimation under the multispecies coalescent. – bioRxiv: 010199.

Jones G.R. 2017. Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. – Journal of Mathematical Biology **74**: 447–467.

KATOH K., STANDLEY D.M. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. – Molecular Biology and Evolution **30**: 772–780.

KEARSE M., MOIR R., WILSON A., STONES-HAVAS S., CHEUNG M., STURROCK S., BUXTON S., COOPER A., MARKOWITZ S., DURAN C., THIERER T., ASHTON B., MEINTJES P., DRUMMOND A.J. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. – Bioinformatics **28**: 1647–1649.

KNIGHTON J., DAPKEY T., CRUZ J. 2014. Random walk modeling of adult *Leuctra ferruginea* (stonefly) dispersal. – Ecological Informatics **19**: 1–9.

KOVÁCS T., VINÇON G., MURÁNYI D., SIVEC I. 2012. A new *Perlodes* species and its subspecies from the Balkan Peninsula (Plecoptera: Perlodidae). – Illiesia **80**: 182–192.

KROČA J. 2011. *Leuctra quadrimaculata* Kis, 1963 (Plecoptera; Leuctridae) in the Moravskoslezské Beskydy Mts. and Javorníky Mts. (Czech Republic). – Casopis Slezského Zemského Muzea (A) **60**: 1–6.

KUUSELA K., HUUSKO A. 1996. Post-emergence migration of stoneflies (Plecoptera) into the nearby forest. – Ecological Entomology **21**: 171–177.

LANFEAR R., CALCOTT B., HO S.Y.W., GUINDON S. 2012. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. – Molecular Biology and Evolution **29**: 1695–1701.

LÓPEZ-RODRÍGUEZ M.J., TIERNO DE FIGUEROA J.M. 2009. First record of the male mating call in the genus *Tyrrhenoleuctra consiglio* (Plecoptera: Leuctridae). – Entomological News **120**: 224–226.

MACNEALE K.H., PECKARSKY B.L., LIKENS G.E. 2005. Stable isotopes identify dispersal patterns of stonefly populations living along stream corridors. – Freshwater Biology **50**: 1117–1130.

MALICKY H. 1983. Chorological patterns and biome types of European Trichoptera and other fresh-water insects. – Archiv für Hydrobiologie **96**: 223–244.

MALICKY H. 2000. Arealdynamik und Biomgrundtypen am Beispiel der Köcherfliegen (Trichoptera). – Entomologica Basiliensia **22**: 235–259.

MALICKY H. 2006. Mitteleuropäische (extra-mediterrane) Arealkerne des Dinodal am Beispiel von Köcherfliegen (Trichoptera). – Beiträge zur Entomologie **56**: 347–359.

MALICKY H., ANT H., ASPÖCK H., DE JONG R., THALER K. 1983. Argumente zur Existenz und Chorologie mitteleuropäischer (extramediterran-europäischer) Faunen-Elemente. – Entomologia Generalis et Applicata **9**: 101–119.

MARDEN J.H., O'DONNEL B.C., THOMAS M.A., BYE J.Y. 2000. Surface-skimming stoneflies and mayflies: the taxonomic and mechanical diversity of two-dimensional aerodynamic locomotion. – Physiological and Biochemical Zoology **73**: 751–764.

MOOG O. 2002–2011. Fauna Aquatica Austriaca, Lieferung 2011. – Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft, Wien.

MURÁNYI D., GAMBOA M., ORCI K.M. 2014. *Zwicknia* gen. n., a new genus for the *Capnia bifrons* species group, with descriptions of three new species based on morphology, drumming signals and molecular genetics, and a synopsis of the West Palaearctic and Nearctic genera of Capniidae (Plecoptera). – Zootaxa **3812**: 1–82.

NELSON L.A., WALLMAN J.F., DOWTON M. 2007. Using COI barcodes to identify forensically and medically important blowflies. – Medical and Veterinary Entomology **21**: 44–52.

OGDEN T.H., WHITING M.F. 2003. The problem with 'the Paleoptera problem': sense and sensitivity. – Cladistics **19**: 432–442.

PARDO I., ZWICK P. 1993. Contribution to the knowledge of Mediterranean *Leuctra* (Plecoptera: Leuctridae). – Journal of the Swiss Entomological Society **66**: 417–234.

PAULS S.U., LUMBSCH T., HAASE P. 2006. Phylogeography of the montane caddisfly *Drusus discolor*: evidence for multiple refugia and periglacial survival. – Molecular Ecology **15**: 2153–2169.

PAULS S.U., BLAHNIK R.J., ZHOU X., WARDWELL T., HOLZENTHAL R.W. 2010. DNA barcode data confirm new species and reveal cryptic diversity in Chilean *Smicridea* (*Smicridea*) (Trichoptera: Hydropsychidae). – Journal of the North American Benthological Society **29**: 1058–1074.

PETROVIC A., SIMIĆ V., MILOSEVIC D., PAUNOVIC M., SIVEC I. 2014. Diversity and distributional patterns of stoneflies (Insecta: Plecoptera) in the aquatic ecosystems of Serbia (Central Balkan Peninsula). – Acta Zoologica Bulgarica **66**: 517–526.

POPIJAČ A., SIVEC I. 2009. Diversity and distribution of stoneflies in the area of Plitvice Lakes National Park and along the Mediterranean river Cetina (Croatia). – Aquatic Insects **31**: 731–742.

PREVIŠIĆ A., GRAF W., VITECEK S., KUČINIĆ M., BÁLINT M., KERESZTES L., PAULS S.U., WARINGER J. 2014a. Cryptic diversity of caddisflies in the Balkans: the curious case of *Ecclisopteryx* species (Trichoptera: Limnephilidae). – Arthropod Systematics & Phylogeny **72**: 309–329.

PREVIŠIĆ A., SCHNITZLER J., KUČINIĆ M., GRAF W., IBRAHIMI H., KEROVEC M., PAULS S.U. 2014b. Microscale vicariance and diversification of Western Balkan caddisflies linked to karstification. – Freshwater Science **33**: 250–262.

RAMBAUT A., SUCHARD M.A., XIE D., DRUMMOND A.J. 2014. Tracer v1.6. – Available from http://beast.bio.ed.ac.uk/Tracer.

RAVIZZA C. 2002. Atlas of the Italian Leuctridae (Insecta, Plecoptera) with an appendix including Central European species. – Lauterbornia **44**: 1–42.

RAVIZZA C., VINÇON G. 1998. Les Leuctridés (Plecoptera, Leuctridae) des Alpes. – Journal of the Swiss Entomological Society **71**: 285–342.

ROSENBERG D.M., RESH V.H. 1993. Introduction to freshwater biomonitoring and benthic macroinvertebrates. Pp. 1–9 in: ROSENBERG D.M., RESH V.H. (eds), Freshwater Biomonitoring and Benthic Macroinvertebrates. – Chapman and Hall, New York.

RUPPRECHT R. 1968. Das Trommeln der Plecopteren. – Zeitschrift für vergleichende Physiologie **59**: 38–71.

RUPPRECHT R. 1972. Dialektbildung bei den Trommelsignalen von *Diura* (Plecoptera). – Oikos **23**: 410–412.

SILVERI L., TIERNO DE FIGUEROA J.M., MAIOLINI B. 2008. Feeding habits of Perlodidae (Plecoptera) in the hyporheic habitats of Alpine streams (Trentino-NE Italy). – Entomologica Fennica **19**: 176–183.

SIMON C., FRATI F., BECKENBACH A., CRESPI B., LIU H., FLOOK P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. – Annals of the Entomolgical Society of America **87**: 651–701.

STAMATAKIS A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. – Bioinformatics **30**: 1312–1313.

STEWART K.W., SANDBERG J.B. 2004. Description of the nymph and drumming calls of *Megaleuctra complicata* Claassen (Plecoptera: Leuctridae); evolution of drumming in Leuctridae. – Aquatic Insects **26**: 123–129.

TAMURA K., STECHER G., PETERSON D., FILIPSKI A., KUMAR S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. – Molecular Biology and Evolution **30**: 2725–2729.

TIERNO DE FIGUEROA J.M., FOCHETTI R. 2014. A second new species of *Tyrrhenoleuctra* discovered by means of molecular data: *Tyrrhenoleuctra lusohispanica* n. sp. (Plecoptera: Leuctridae). – Zootaxa **3764**: 587–593.

TIERNO DE FIGUEROA J.M., GAETANI B., LUZON-ORTEGA J.M., LÓPEZ-RODRÍGUEZ M.J., FOCHETTI R. 2011a. On the identity of *Isoperla*

curtata (Plecoptera: Perlodidae): behavioural and molecular approaches show the existence of two separate species. – Zootaxa **3000**: 49–58.

Tierno de Figueroa J.M., Luzon-Ortega J.M., López-Rodríguez M.J. 2011b. Intraspecific and interspecific variation in drumming signals in the genus *Capnioneura* (Plecoptera: Capniidae). – Aquatic Insects **33**: 335–341.

Theissinger K., Bálint M., Feldheim K.A., Haase P., Johannesen J., Laube I., Pauls S.U. 2013. Glacial survival and post-glacial recolonization of an arctic–alpine freshwater insect (*Arcynopteryx dichroa*, Plecoptera, Perlodidae) in Europe. – Journal of Biogeography **40**: 236–248.

Trizzino M., Bisi F., Maiorano L., Martinoli A., Petitta M., Preatoni D.G., Audisio P. 2014. Mapping biodiversity hotspots and conservation priorities for the Euro-Mediterranean headwater ecosystems, as inferred from diversity and distribution of a water beetle lineage. – Biodiversity and Conservation **24**: 149–170.

Vinçon G., Pardo I. 2009. The stoneflies (Plecoptera, Insecta) of the northwestern Iberian Peninsula. – Annales de Limnologie **40**: 43–62.

Vitecek S., Previšić A., Kučinić M., Bálint M., Keresztes L., Waringer J., Pauls S., Malicky H., Graf W. 2015a. Description of a new species of *Wormaldia* from Sardinia and a new *Drusus* species from the Western Balkans (Trichoptera, Philopotamidae, Limnephilidae). – ZooKeys **496**: 85–103.

Vitecek S., Kučinić M., Oláh J., Previšić A., Bálint M., Keresztes L., Waringer J., Pauls S.U., Graf W. 2015b. Description of two new filtering carnivore *Drusus* species (Limnephilidae, Drusinae) from the Western Balkans. – ZooKeys **513**: 79–104.

Vitecek S., Graf W., Previšić A., Kučinić M., Oláh J., Bálint M., Keresztes L., Pauls S.U., Waringer J. 2015c. A hairy case: The evolution of filtering carnivorous Drusinae (Limnephilidae, Trichoptera). – Molecular Phylogenetics and Evolution **93**: 249–260.

Vitecek S., Kučinić M., Previšić A., Živić I., Stojanović K., Keresztes L., Bálint M., Hoppeler F., Waringer J., Graf W., Pauls S.U. 2017. Integrative taxonomy by molecular species delimitation: mulit-locus data corroborate a new species of Balkan Drusinae micro-endemics. – BMC Evolutionary Biology **17**: 129.

Waringer J. 1991. Phenology and the influence of meteorological parameters on the catching success of light-trapping for Trichoptera. – Freshwater Biology **25**: 307–319.

Weiss S., Stradner D., Graf W. 2011. Molecular systematics, evolution and zoogeography of the stonefly genus *Siphonoperla* (Insecta: Plecoptera, Chloroperlidae). – Journal of Zoological Systematics and Evolutionary Research **50**: 19–29.

Whiting M.F. 2002. Mecoptera is paraphyletic: multiple genes and phylogeny of Mecoptera and Siphonaptera. – Zoologica Scripta **31**: 93–104.

Zhang J., Kapli P., Pavlidis P., Stamatakis A. 2013. A general species delimitation method with applications to phylogenetic placements. – Bioinformatics **29**: 2869–2876.

Ziegler D.D., Stewart K.W. 1977. Drumming behavior of eleven Nearctic stonefly (Plecoptera) species. – Annals of the Entomological Society of America **70**: 495–505.

Zwick P. 1973. Insecta Plecoptera. Phylogenetisches System und Katalog. – Das Tierreich, Berlin, **94**: 1–465.

Zwick P. 2000. Phylogenetic system and zoogeography of the Plecoptera. – Annual Review of Entomology **45**: 709–746.

Zwick P. 2004. Key to the West Palaearctic genera of stoneflies (Plecoptera) in the larval stage. – Limnologica **34**: 315–348.

Author contributions

WG initiated this study. Specimens were collected mostly by GV and WG. Specimens were identified to species level by GV; comparative morphological analyses to delineate putative new species were performed by GV and SV; photographs were taken by SV, raw photographs were edited by SV and GV. Molecular data were generated by SV; compilation of final molecular data sets and phylogenetic analyses were performed by SV and SUP. The manuscript was drafted by SV, species descriptions were drafted by GV, WG and SV; all authors commented, edited, and approved the final manuscript.

Electronic Supplement Files

at www.senckenberg.de/arthropod-systematics

File 1: vitecek&al-leuctrainermis-asp2017-electronicsupplement-1.pdf – **Table S1**: Extent of the molecular dataset. **Figure S1**: ML tree based on partial mtCOI-5P sequence data. **Figure S2**: ML tree based on partial mtCOI-3P sequence data. **Figure S3**: ML tree based on partial mt12S sequence data. **Figure S4**: ML tree based on partial nuH3 sequence data. **Figure S5**: ML tree based on partial nu28S sequence data. **Dataset S1**: Raw alignments as used in species tree analysis through STACEY.

File 2: vitecek&al-leuctrainermis-asp2017-electronicsupplement-2.pdf – **Figure S6**: Full colour images of *L. inermis* group species.

Zoobank registrations

at http://zoobank.org

Present article: http://zoobank.org/urn:lsid:zoobank.org:pub:95E5F1B2-FABC-4764-9AD9-ACAA1837EA58

***Leuctra fochettii* Vinçon & Graf, 2017**: http://zoobank.org/urn:lsid:zoobank.org:act:A887F62C-ACD7-4880-AA7C-3B4CC2727FBF

***Leuctra grafi* Vinçon & Vitecek, 2017**: http://zoobank.org/urn:lsid:zoobank.org:act:5B738C9E-CE37-4ABB-8999-9F2814BEE76A

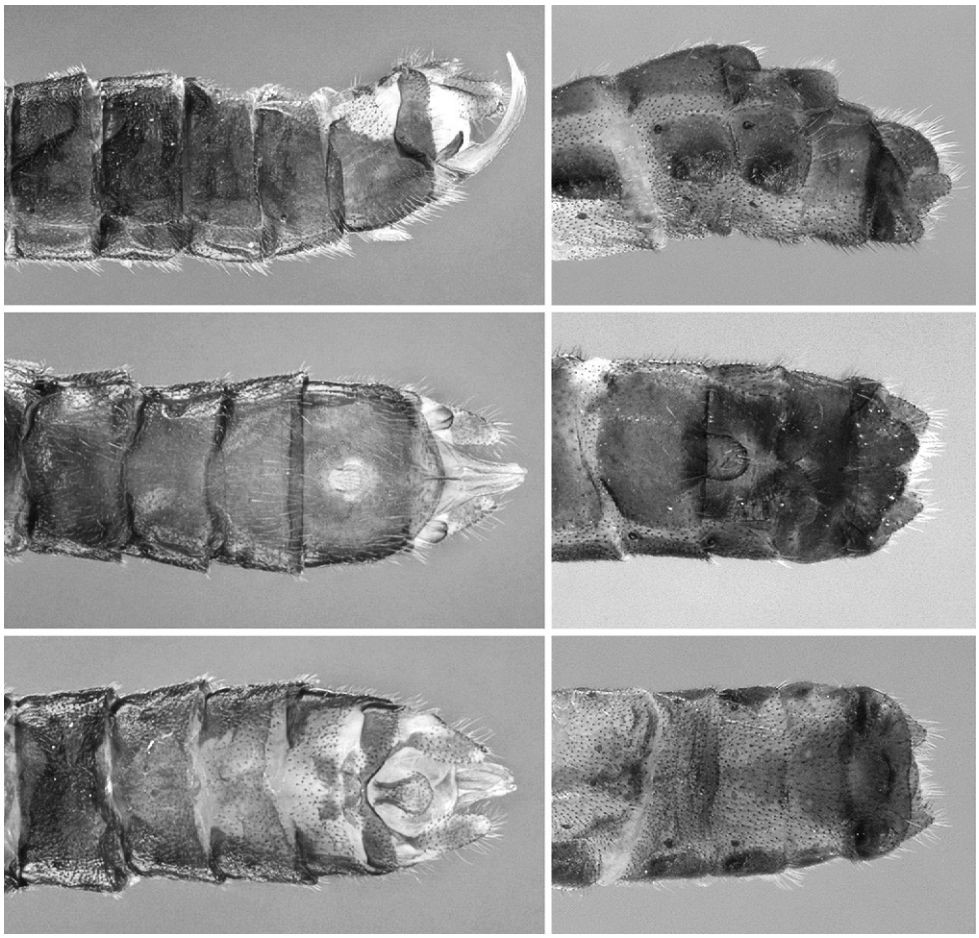


Fig. 5. Terminalia of *Leuctra inermis* group species: *L. ameliae*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

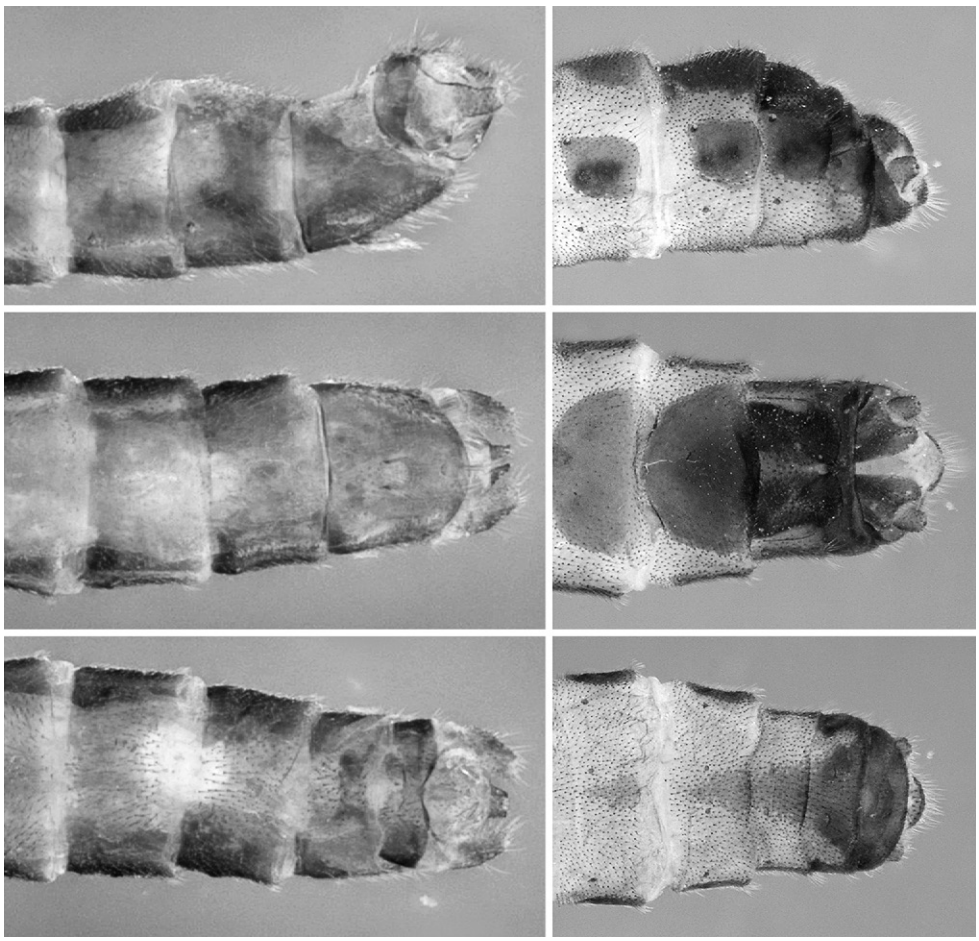


Fig. 4. Terminalia of *Leuctra inermis* group species: *L. alosi*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

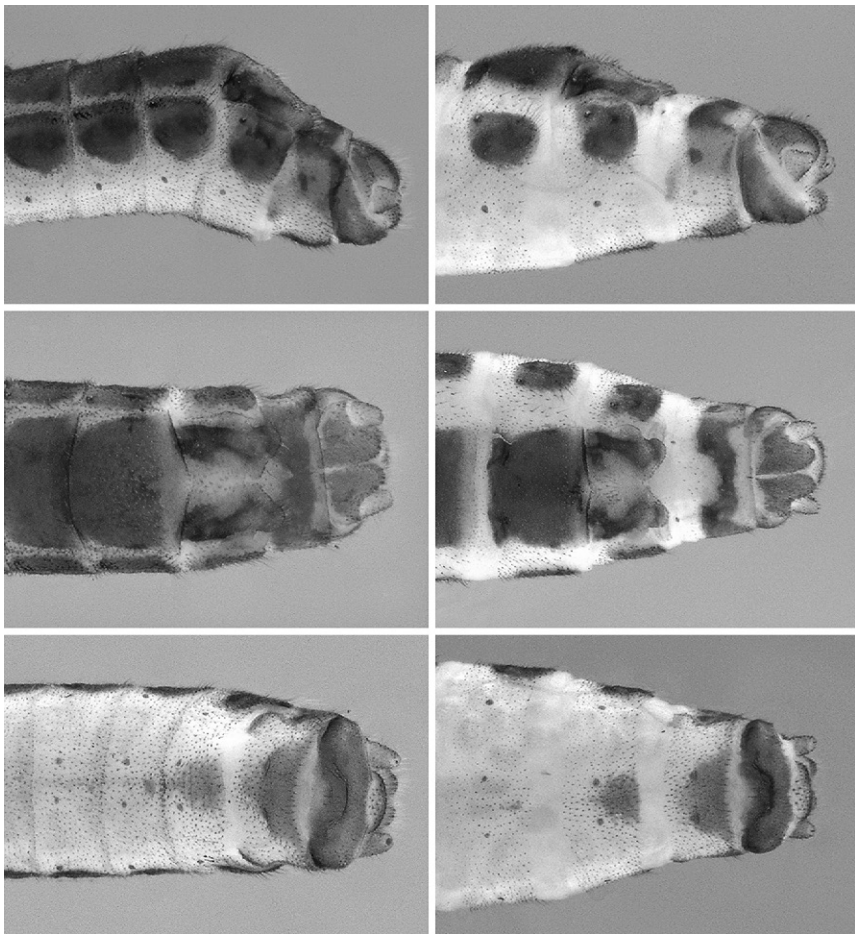


Fig. 7. Terminalia of *Leuctra inermis* group species: *L. balcanica*. Females. From left to right: dorsal view, ventral view, lateral view.

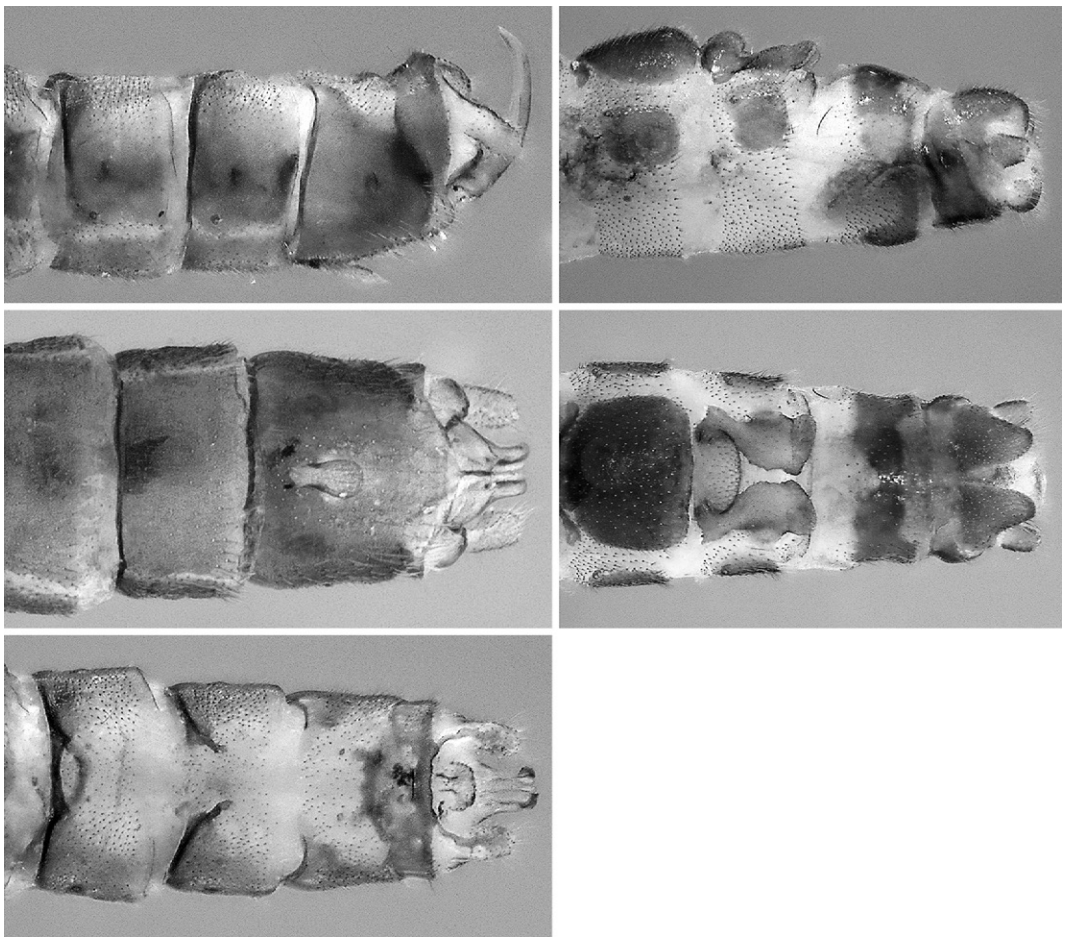


Fig. 6. Terminalia of *Leuctra inermis* group species: *L. apenninicola*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

Fig. 9. Terminalia of *Leuctra inermis* group species: *L. fochetiti* sp.n.. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view. Arrow indicates tip of styli.

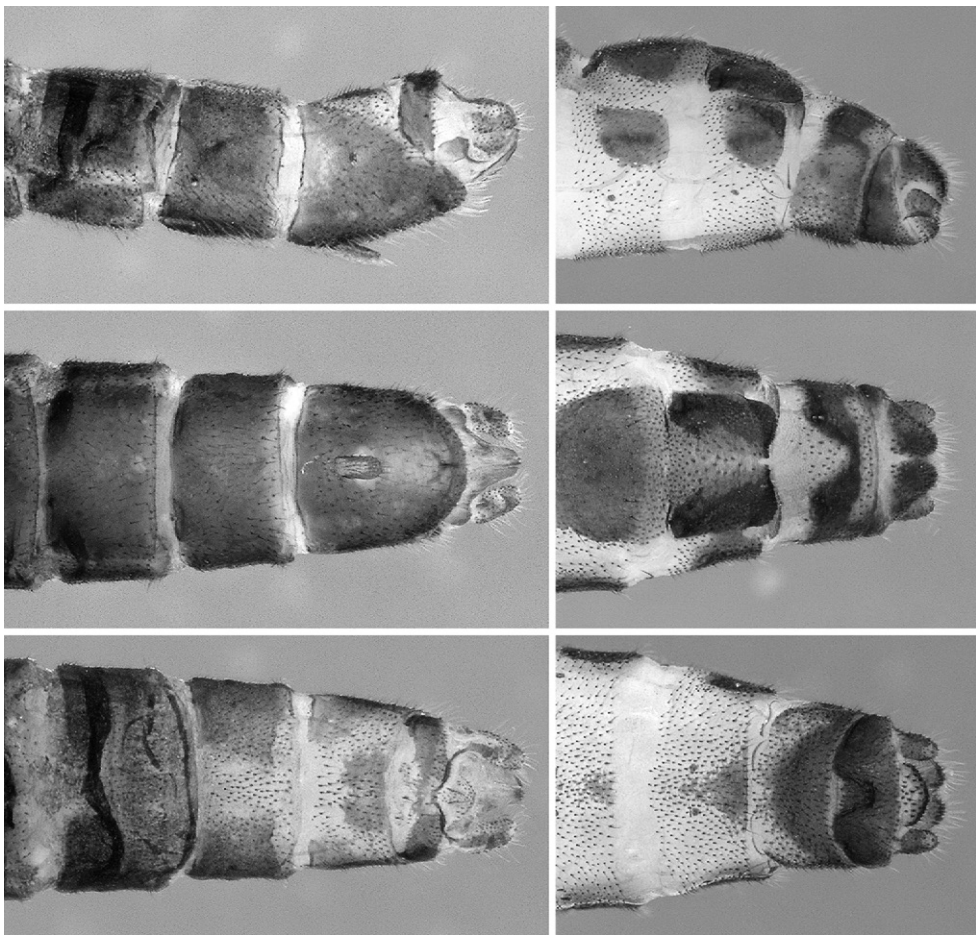


Fig. 8. Terminalia of *Leuctra inermis* group species: *L. flavomaculata*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

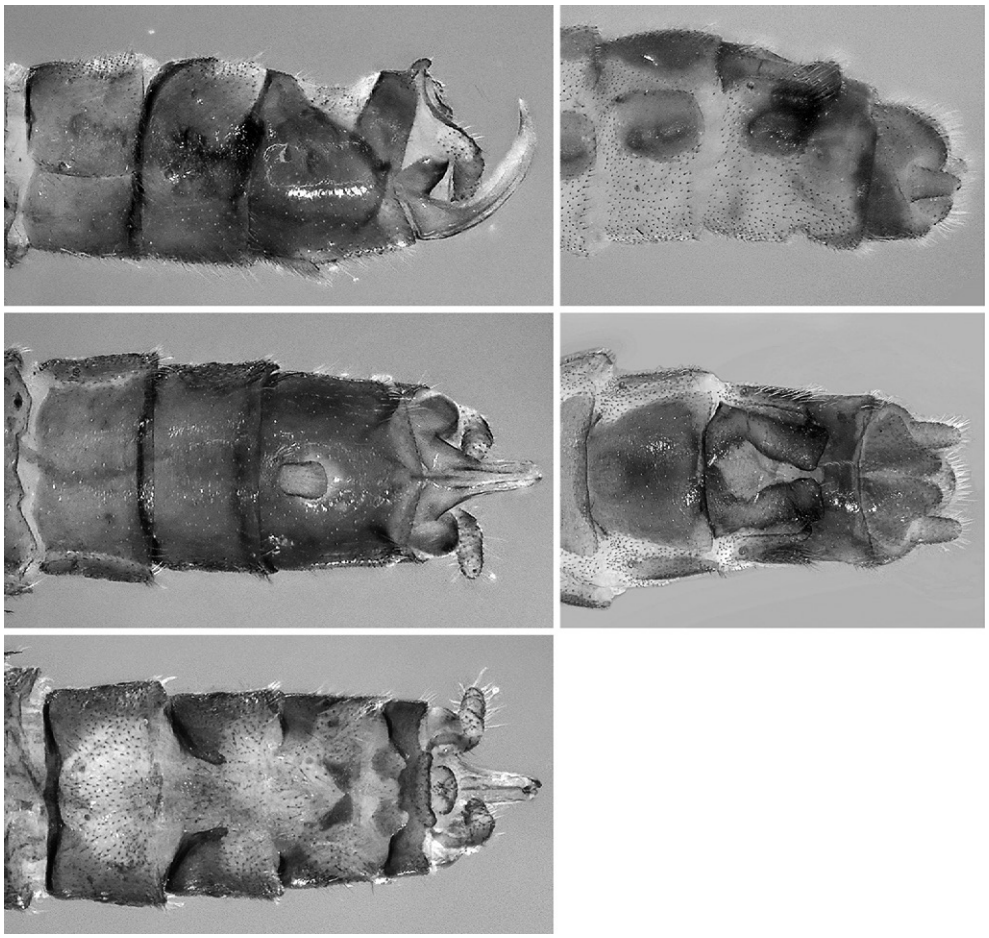


Fig. 11. Terminalia of *Leuctra inermis* group species: *L. grafi* sp.n.. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view. Female dorsal view not shown.

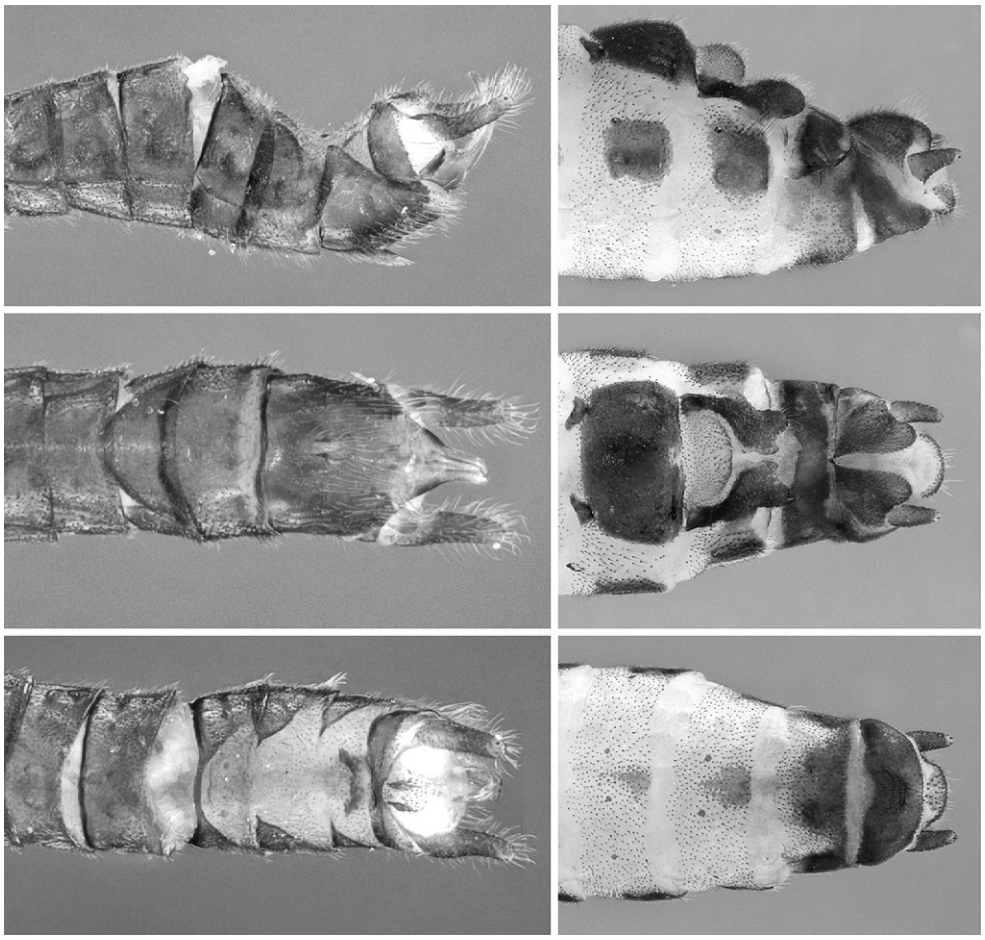


Fig. 10. Terminalia of *Leuctra inermis* group species: *L. garumna*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

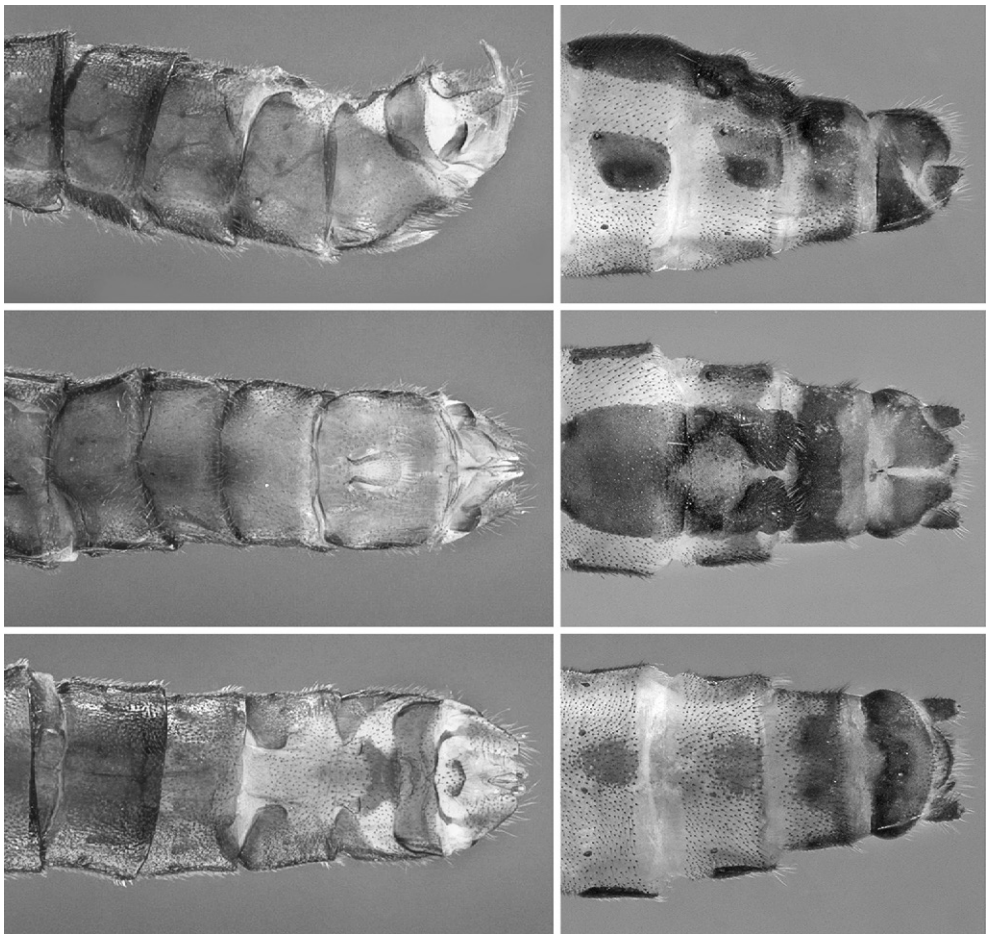


Fig. 13. Terminalia of *Leuctra inermis* group species: *L. inermis*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

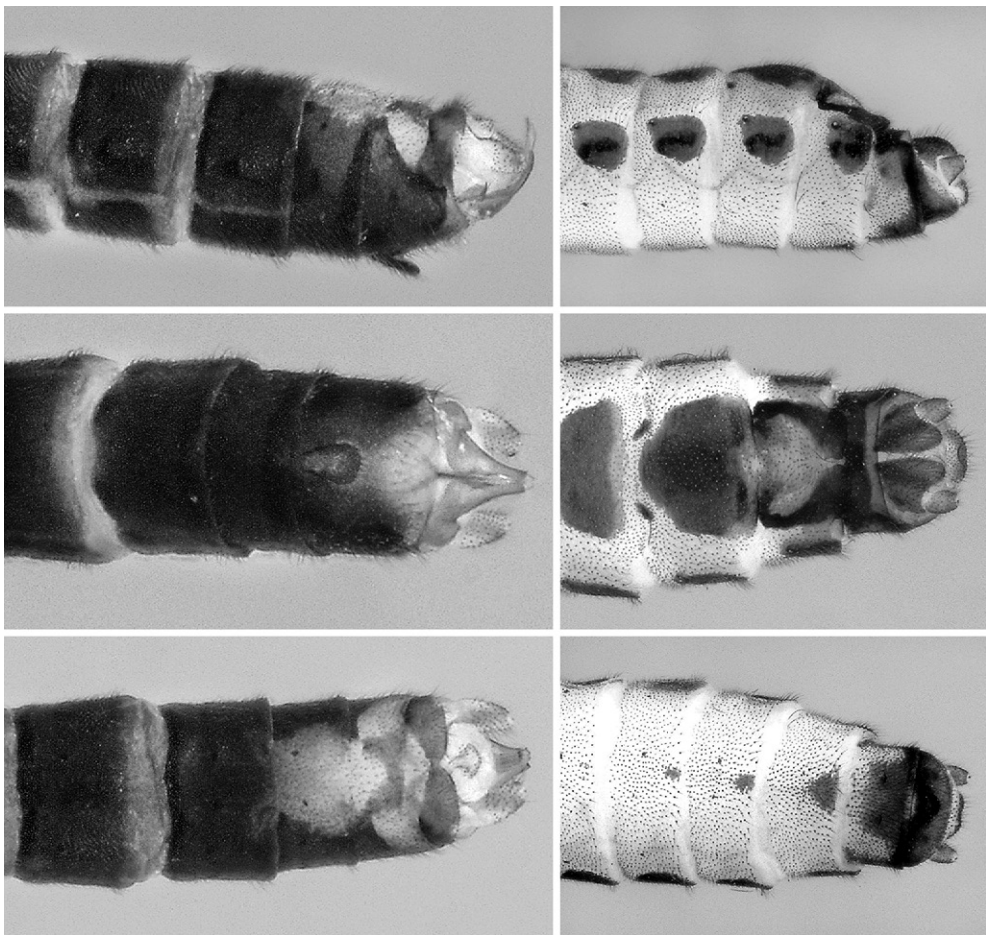


Fig. 12. Terminalia of *Leuctra inermis* group species: *L. handlirschi*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

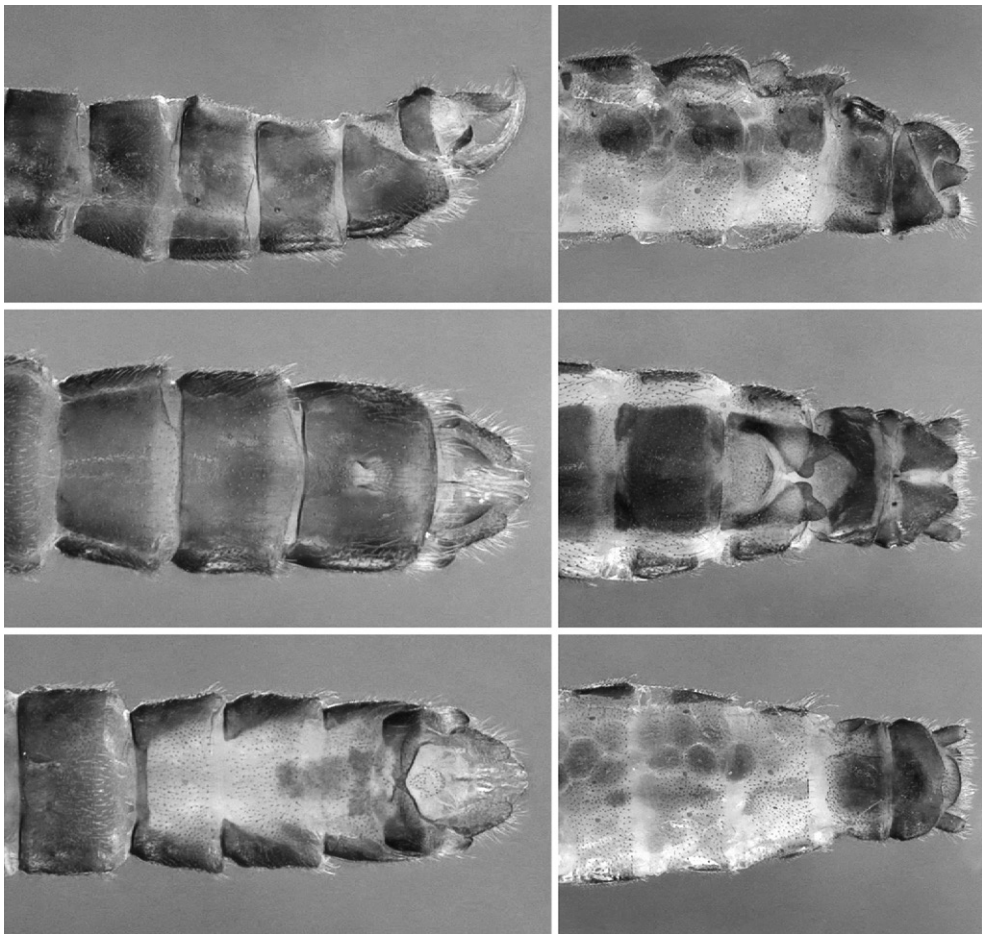


Fig. 15. Terminalia of *Leuctra inermis* group species: *L. kempnyi*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

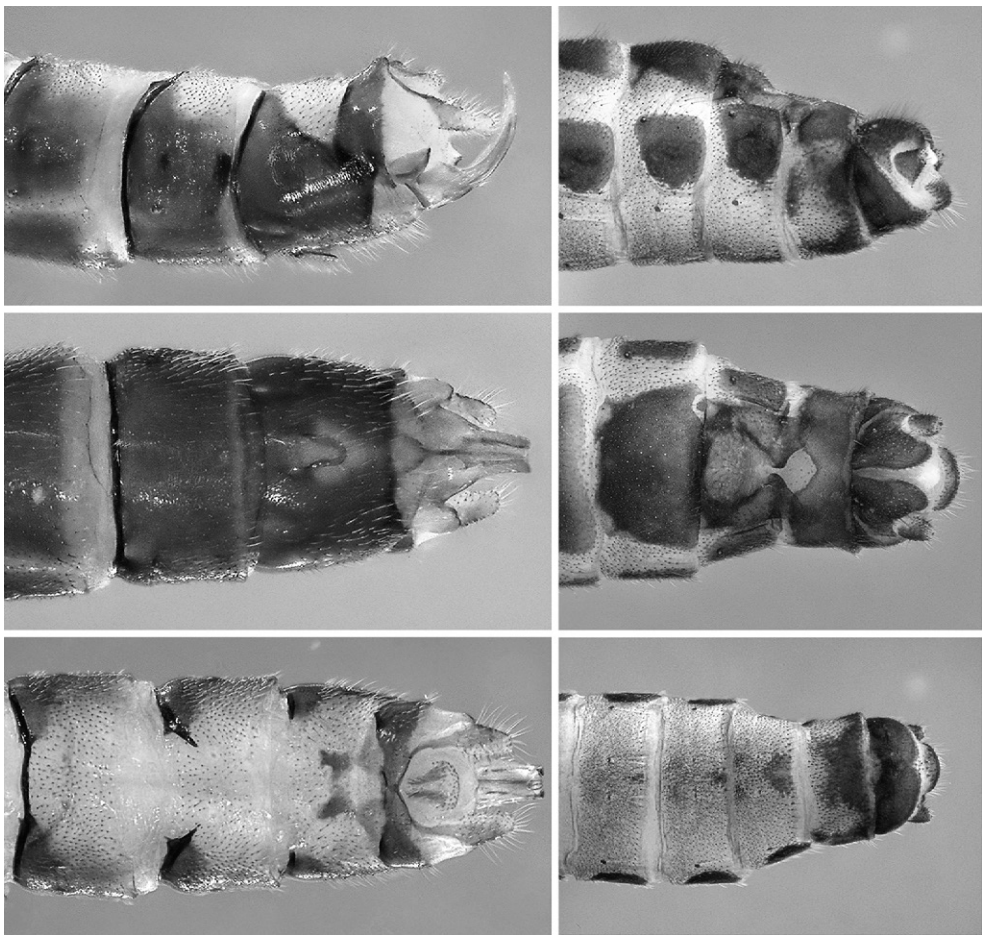


Fig. 14. Terminalia of *Leuctra inermis* group species: *L. insubrica*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

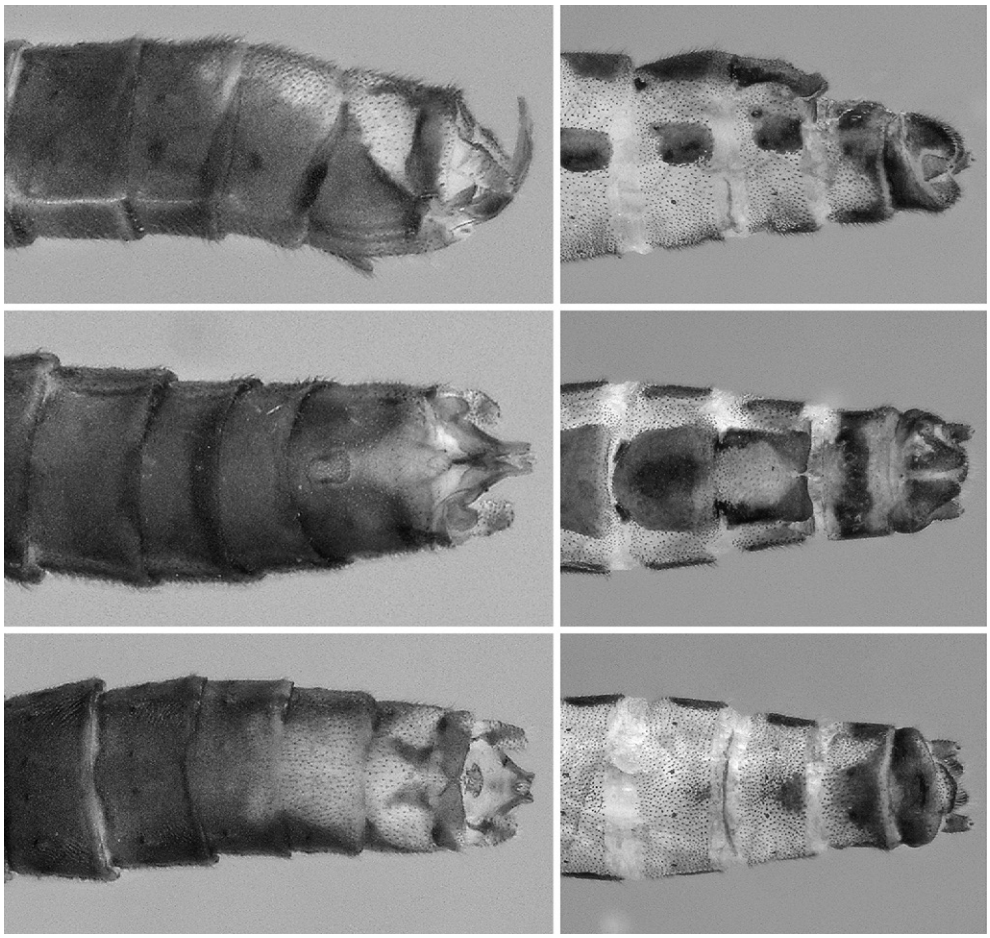


Fig. 17. Terminalia of *Leuctra inermis* group species: *L. pusilla*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

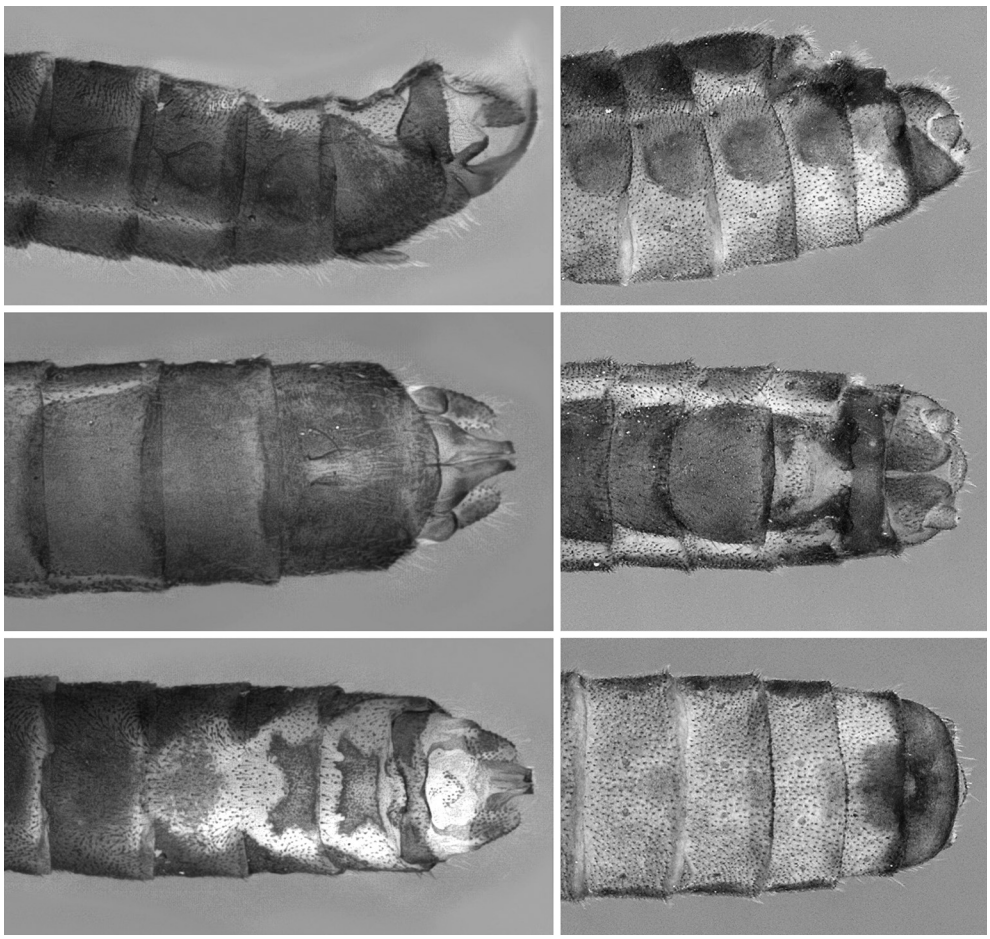


Fig. 16. Terminalia of *Leuctra inermis* group species: *L. metsovonica*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view.

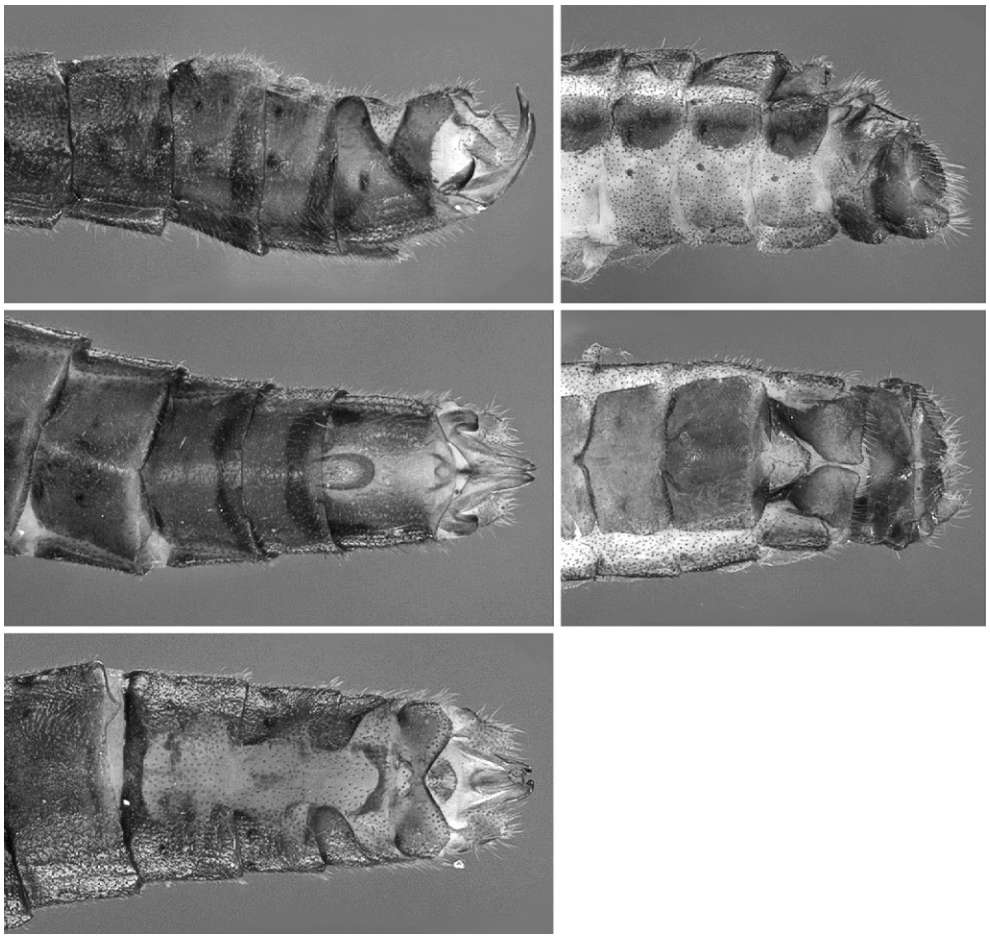


Fig. 19. Terminalia of *Leuctra inermis* group species: *L. rauscheri*. Upper row, male; lower row, female. From left to right: dorsal view, ventral view, lateral view (female dorsal view not shown).

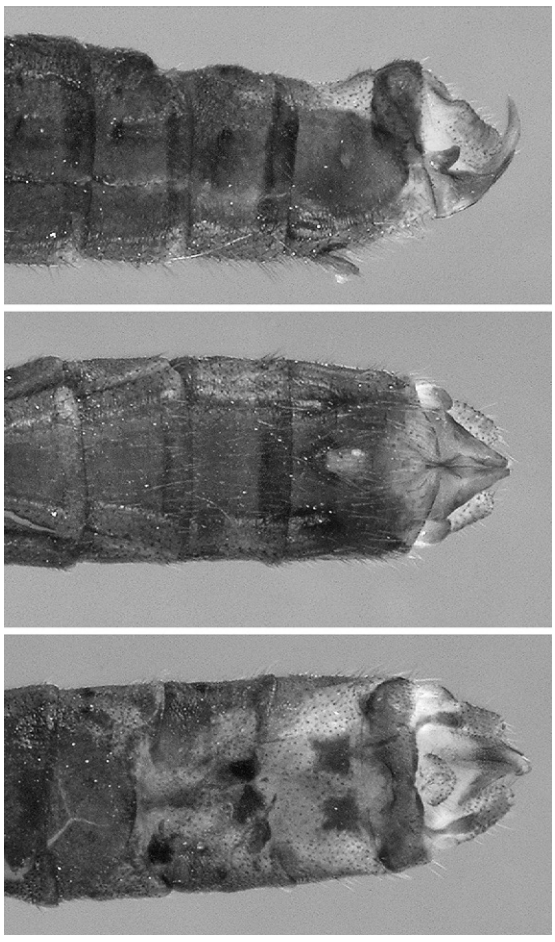


Fig. 18. Terminalia of *Leuctra inermis* group species: *L. quadrimaculata* male. From left to right: dorsal view, ventral view, lateral view.

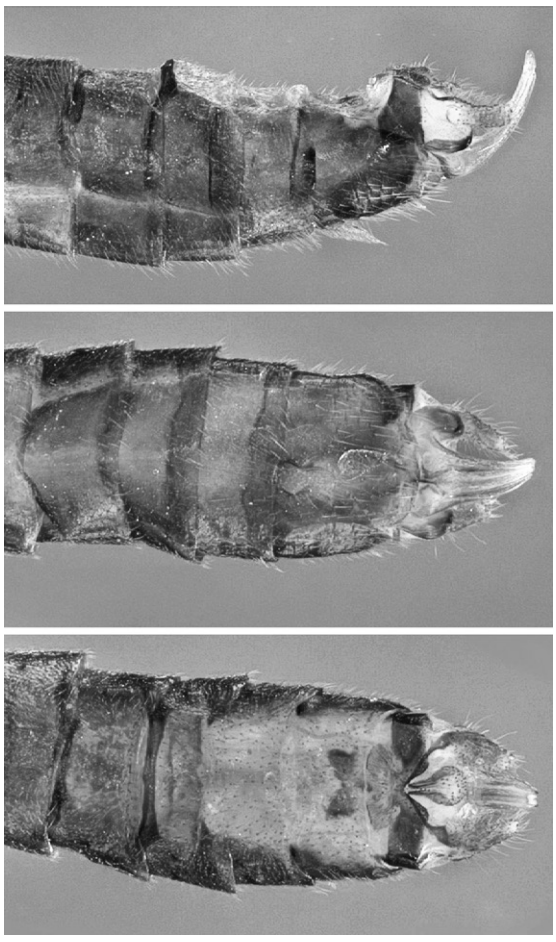


Fig. 20. Terminalia of *Leuctra inermis* group species: *L. teriolensis* male. From left to right: dorsal view, ventral view, lateral view.