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The *Rhyacophila fasciata* Group in Europe: *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (n. sp.) from Greece, Kosovo, Republic of North Macedonia and Serbia (Trichoptera: Rhyacophilidae)

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Abstract

The morphology of all postembryonic stages (larva, pupa, male, and female) of *Rhyacophila macedonica* Karaouzas, Valladolid, & Ibrahimi **n. sp.** from Greece, Republic of North Macedonia, Kosovo and Serbia was examined. Morphological data were supplemented by a molecular analysis of the mitochondrial cytochrome C oxidase subunit I (COI) and compared with samples of the nominate species *Rhyacophila fasciata* Hagen 1859, as well as with other species and subspecies in this group: *Rhyacophila septentrionis* McLachlan 1865, *Rhyacophila denticulata* McLachlan 1879, *Rhyacophila sociata* Navás 1916, *Rhyacophila kykladica* Malicky & Sipahiler 1993, *Rhyacophila fasciata delici* Kucinic & Valladolid 2020, and *Rhyacophila fasciata viteceki* Valladolid & Kucinic 2020. Our results revealed morphological differences between the nominate species and *R. macedonica*, as well as genetic differences among the taxa in the *Rhyacophila fasciata* Group, so we propose *R. macedonica* as a new species of the Group. Based on the new molecular data, we also elevate *R. f. delici* to the status of a distinct species, *R. delici* (*status promotus*).

Key words: Morphology, larva, pupa, imago, COI, ecology, distribution

Περίληψη

Εξετάστηκε η μορφολογία των μεταεμβρυονικών σταδίων (προνύμφη, πλαγγόνα, ενήλικο αρσενικό και θηλυκό) του είδους *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**) από την Ελλάδα, τη Δημοκρατία της Βόρειας Μακεδονίας, το Κόσοβο και τη Σερβία. Τα μορφολογικά δεδομένα συμπληρώθηκαν με μοριακή ανάλυση μιτοχονδριακής υπομονάδας I οξειδάσης του κυτοχρώματος C (COI), τα οποία συγκρίθηκαν με δείγματα του είδους αναφοράς *Rhyacophila fasciata* Hagen 1859, καθώς και με άλλα είδη και υποείδη αυτής της ομάδας: *Rhyacophila septentrionis* McLachlan

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1865, *Rhyacophila denticulata* McLachlan 1879, *Rhyacophila sociata* Navás 1916, *Rhyacophila kykladica* Malicky & Sipahiler 1993, *Rhyacophila fasciata delici* Kucinic & Valladolid 2020 και *Rhyacophila fasciata viteceki* Valladolid & Kucinic 2020. Τα αποτελέσματα μας αποκάλυψαν μορφολογικές και γενετικές διαφορές μεταξύ όλων των ταξινομικών ομάδων της ομάδας *Rhyacophila fasciata*, και ως εκ τούτου προτείνουμε το *R. macedonica* ως ένα νέο είδος. Βάση νέων μοριακών δεδομένων, αλλάζουμε το ταξινομικό καθεστώς του υποείδους *R. f. delici* σε διακριτό είδος *R. delici* (*status promotus*).

Λέξεις κλειδιά: Μορφολογία, προνύμφη, πλαγγόνα, ενήλικο, COI, οικολογία, κατανομή

Resumen

Se ha estudiado la morfología de todas las etapas postembrionarias (larva, pupa, macho y hembra) de *Rhyacophila macedonica* Karaouzas, Valladolid, & Ibrahimi (**n. sp.**) de Grecia, República de Macedonia del Norte, Kosovo y Serbia. Los datos morfológicos se complementaron con un análisis molecular del gen mitocondrial Citocromo Oxidasa subunidad I (COI), que se comparó con muestras de la especie nominal *Rhyacophila fasciata* Hagen 1859, así como con otras muestras de especies y subespecies de este grupo: *Rhyacophila septentrionis* McLachlan, 1865, *Rhyacophila denticulata* McLachlan 1879, *Rhyacophila sociata* Navás 1916, *Rhyacophila kykladica* Malicky & Sipahiler 1993, *Rhyacophila fasciata delici* Kucinic & Valladolid 2020 y *Rhyacophila fasciata viteceki* Valladolid & Kucinic 2020. Nuestros resultados revelaron diferencias morfológicas entre la especie nominal y *R. macedonica,* así como diferencias genéticas entre todos los taxones del Grupo *Rhyacophila fasciata*, por lo que proponemos a *R. macedonica* como una nueva especie del Grupo. Basándonos en nuevos datos moleculares, elevamos el estatus de la subespecie *R. f. delici* a especie, *R. delici* (*status promotus*).

Palabras clave: Morfología, larva, pupa, adulto, COI, ecología, distribución

Introduction

In the introduction of his monograph on genus *Rhyacophila* Pictet 1834 and family Rhyacophilidae Stephens 1836, Schmid (1970) concluded that *Rhyacophila* is one of the most primitive and important genera of caddisflies, because it is present in almost all lotic environments in the Holarctic and Oriental Regions, and because it included 465 species at that time, a number that has increased to 792 valid species (and 30 valid subspecies) to date (GBIF 2020; Morse 2021). Genus *Rhyacophila* shows an evolutionary stage essential for inferring the phylogeny of Trichoptera, mainly due to the male genitalia, where high variability in details is mixed with a high stability in general architecture. Furthermore, in their geographic distribution, many of the species are restricted to small areas, sometimes to only a single mountain, providing the opportunity for phylogenetic and zoogeographical inferences (Schmid 1970).

Pictet (1834) described *Rhyacophila vulgaris* from brooks in the Leman Lake basin and noticed a great variability in the specimens, predicting the future description of new species. Hagen (1859), studying Stephens', Curtis', and Pictet's private collections, classified these specimens in five different species: *R. vulgaris* Pictet 1834, *R. dorsalis* (Curtis 1834), *R. paupera* Hagen 1859, *R. ferruginea* Scopoli 1763, and *R. fasciata* Hagen 1859. The characters that defined *R. fasciata* were similar to those of *R. ferruginea*: dorsal lobe circular, covering the preanal appendages almost totally, but the specimens of *R. fasciata* are bigger in size and wings show three dark transverse bands.

In his study of the British Trichoptera, McLachlan (1865) considered the shape of inferior appendages in *R. ferruginea* similar to that of *R. fasciata* and to that of Scottish specimens of *R. septentrionis*, from which *R. ferruginea* differed only in size. In 1868, McLachlan considered three species, *R. fasciata* (specimens from Carinthia), *R. septentrionis*, and *R. ferruginea*, although he doubted the validity of the last one. Finally, McLachlan (1879), studying several specimens of *R. fasciata* from different countries of Europe, concluded that those from Central Europe could be *R. septentrionis* while those from the Pyrenees could be a new species [this species was described by Navás in 1916 as *R. sociata* (Valladolid *et al.* 2018).] In a recent publication, Valladolid *et al.* (2021) proposed *R. ferruginea* Scopoli 1763 as a *nomen dubium*, due to the difficulty of identifying the original species described, and the resurrection of *Rhyacophila septentrionis* McLachlan 1865.

In 1993 Malicky & Sipahiler proposed the creation of the "*R. fasciata* Complex," including several subspecies, some of them degraded from species: *Rhyacophila fasciata fasciata* Hagen 1859, widely distributed all over Europe, *R. fasciata denticulata* McLachlan 1879 in northern Spain and the French Pyrenees, and *R. fasciata aliena* Martynov

1916 in the Caucasus and eastern Turkey. In addition, this taxa set was complemented by *R. fasciata mysica* Malicky & Sipahiler 1993 in western Turkey, *R. fasciata kykladica* Malicky & Sipahiler 1993 in Greece, and *R. fasciata libanica* Malicky & Sipahiler 1993 in Lebanon (see also Malicky 2004 for the distributions of these subspecies). Recently some of these subspecies were promoted to species (*R. denticulata,* by Valladolid *et al.* 2018; *R. kykladica*, by Valladolid *et al.* 2019) and new species, previously considered as *R. fasciata,* were added to the *Rhyacophila fasciata* Group, such as *R. sociata* Navás 1916 (stat. res.) (Valladolid *et al.* 2018), *R. fasciata delici* Kučinić & Valladolid 2020 (in Valladolid *et al.* 2020) and *R. fasciata viteceki* Valladolid & Kučinić 2020 (in Valladolid *et al.* 2020) and *R. septentrionis* McLachlan 1865 (stat. res.) (Valladolid *et al.* 2021).

Valladolid *et al.* (2021), in their paper on *R. fasciata*, investigated the presence of this species in several European countries, where some of them, such as Greece, the Republic of North Macedonia, Kosovo and Serbia needed more research to confirm the presence of this species. *Rhyacophila fasciata* was cited by Malicky & Sipahiler (1993) and Malicky (2005) from Greece. This species has been recorded mostly from the northcentral and northwestern parts of the country (Malicky 2005; Karaouzas, unpublished data). Its northeastern distribution extends from the Rhodope Mountains in Thrace to west and central Macedonia, including Chalkidiki, with its southward limits to Mount Ossa in Thessaly. The westernmost locality of *R. fasciata* is located in the Louros River in Epirus (Malicky 2005; Karaouzas, unpublished data).

In the Republic of North Macedonia, *Rhyacophila fasciata* was noted in the northeastern and northwestern parts of the country: Kumanski (1997) registered this species in the foothills of Osogovo Mt., in the Bregalnica River at Istibanja, while Slavevska-Stamenković and Hinić (unpublished data) showed that this species is distributed all over the mountain, when they found the species in the upper part of the Kamenicka Reka, Kriva Reka, and Trnovska Reka rivers. On the other hand, the species was recorded in the Pena River below Tetovo (Kovachev *et al.* 1999) in the northwestern part of the country.

In Kosovo this species was cited by Ibrahimi & Sejdiu (2018) and Ibrahimi *et al.* (2012a, 2012b, 2014, 2016, 2018, 2019a, 2019b). The species was found at all three watersheds in Kosovo, namely the Black Sea Basin, the Adriatic Basin, and the Aegean Basin, distributed in different rivers, streams, and streamlets of different altitudes.

In Serbia this species was cited by Djikanović *et al.* (2008), Filipović (1954, 1969), Ibrahimi *et al.* (2017), Marinković-Gospodnetić (1975), Marković *et al.* (1997, 1998, 1999), Paunović *et al.* (2006), Petković *et al.* (2015), Stojanović (2017), Vulić *et al.* (2014), Živić (2005) and Živić *et al.* (2003, 2004, 2005). One of the places where the species was found is the Jastrebac Mountains, located between Niš, Kruševac, and Prokuplje municipalities in Serbia, with the highest peak of Velik Djulica (1492 m a.s.l.). This mountain range is one of the most forested areas in the Balkan Peninsula, covered extensively by deciduous and coniferous forests, and known for its numerous springs, streams, and rivers.

In this paper we present the results of the study of specimens previously identified as *Rhyacophila fasciata* from these three countries. Clear morphological and genetic differences were found in these specimens from Kosovo, the Republic of North Macedonia, and Greece, so we propose a new species for the *Rhyacophila fasciata* Group: *R. macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**). We also discuss the morphological variability amongst males and females of *Rhyacophila fasciata* from Kosovo.

Material and methods

Specimens studied for this project are deposited in: the Karaouzas Private Collection, Athens, Greece (KPC); the Bajgorë Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (BTC); the Bjeshkët e Nemuna Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (BNTC); the Gjilan Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (GJTC); the Gollak Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo, (GOTC); the Karadak Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (KTC); the Ibrahimi Private Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (IPC); the Serbia Trichoptera Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina, Kosovo (STC); the Macedonian National Collection of Invertebrates, Department for Invertebrates at the Institute of Biology

(Faculty of Natural Sciences and Mathematics), Skopje, Republic of North Macedonia (MNCI); and the Collection of Entomology, Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN).

Material examined

Adults. **Greece.** *R. fasciata* (=*R. macedonica*): 1 ♂, Serres, Elaionas, 41.142N, 23.566E, 556 m a.s.l., 20/ix/2018, leg. I. Karaouzas (KPC).

Kosovo. *R. fasciata*: 1 ♂, Mjak, 42.259N, 21.343E, 620 m a.s.l., 17/v/2017, leg. A. Bilalli & M. Musliu (KTC). 1 ♂, Shushtë, 42.281N, 21.359E, 570 m a.s.l., 17/vii/2017, leg. A. Bilalli, H. Ibrahimi & M. Musliu (KTC); 4 ♂♂, 15/xi/2019, leg. H. Ibrahimi [MNCN_Ent 269410, MNCN_Ent 269411, MNCN_Ent 269412, MNCN_Ent 269413 (MNCN)]. 1 ♂ + 3 ♀♀, Samakovë, 42.246N, 21.348E, 630 m a.s.l., 21/v/2017, leg. H. Ibrahimi [1 ♀ MNCN_Ent 296086 (MNCN); 1 ♂ + 2 ♀♀ (KTC)]. 1 ♂, Lugu i Kopilaqës, 42.246N, 21.431E, 1175 m a.s.l., 3/xi/2019, leg. H. Ibrahimi [MNCN_Ent 269414 (MNCN)]. 1 ♂, Dubovë, 42.720N, 20.356E, 500 m a.s.l., 20/v/2020, leg. H. Ibrahimi (BNTC). 3 ♂♂, Kamenicë municipality, 42.658N, 21.733E, 690 m a.s.l., 13/v/2020, leg. H. Ibrahimi (GOTC); 3 ♂♂ + 2 ♀♀, 14/x/2020, leg. H. Ibrahimi (GOTC). 2 ♂♂, Kamenicë municipality, 42.624N, 21.687E, 615 m a.s.l., 19/ix/2020, leg. H. Ibrahimi (GOTC).

R. fasciata (=*R. macedonica*): 1 ♂, Mazhiq, 42.940N, 20.933E, 853 m a.s.l., 18/vi/2009, leg. H. Ibrahimi (IPC). 2 ♂♂, Murgull, 43.074N, 21.066E, 882 m a.s.l., 20/vii/2009, leg. H. Ibrahimi (IPC). 2 ♂♂, Prishtinë, 42.699N, 21.237E, 728 m a.s.l., 15/viii/2009, leg. H. Ibrahimi (IPC). 1 ♂, Mjak, 42.259N, 21.343E, 620 m a.s.l., 17/v/2017, leg. A. Bilalli & M. Musliu (KTC). 2 ♂♂, Selac, 42.979N, 20.959E, 973 m a.s.l., 17/v/2017, leg. A. Bilalli & H. Ibrahimi (BTC). 1 ♂ + 2 ♀♀, Samakovë, 42.246N, 21.348E, 630 m a.s.l., 21/v/2017, leg. H. Ibrahimi [1♀ MNCN_Ent 296085 (MNCN); 1 ♂ + 1 ♀ (KTC)]. 3 ♂♂, Lugu i Kopilaqës, 42.246N, 21.431E, 1175 m a.s.l., 3/xi/2019, leg. H. Ibrahimi [1 ♂ MNCN_Ent 296083 (MNCN); 2 ♂♂ (KTC)]. 1 ♂, Dëbëlldeh, 42.255N, 21.400E, 980 m a.s.l., 05/xi/2019, leg. H. Ibrahimi (KTC). 1 ♂ + 1 ♀, Malishevë, Gjilan, 42.450N, 21.504E, 480 m a.s.l., 16/vii/2020, leg. H. Ibrahimi (GJTC). 4 ♂♂, Kamenicë, 42.589N, 21.657E, 490 m a.s.l., 19/ix/2020, leg. A. Bilalli & H. Ibrahimi (GOTC). 2 ♂♂, Kamenicë municipality, 42.624N, 21.687E, 615 m a.s.l., 19/ix/2020, leg. H. Ibrahimi (GOTC). 2 ♂, Shushtë, 42.281N, 21.359E, 570 m a.s.l., 17/vii/2017, leg. A. Bilalli, H. Ibrahimi & M. Musliu [1 ♂ MNCN_Ent 296084 (MNCN); 1 ♂ (KTC)]. 1 ♂, Dubovë, 42.720N, 20.356E, 500 m, 20/v/2020, leg. H. Ibrahimi (BNTC).

Republic of North Macedonia. *R. fasciata*: 4 ♂♂, river Trnovska, Osogovo Mountain, 42.258N, 22.361E, 820 m a.s.l., 12/viii/2018, leg. J. Hinić & V. Slavevska Stamenković [MNCI_RfT10J01, MNCI_RfT10J02, MNCI_RfT10J03, MNCI_RfT10J04 (MNCI)]. 3 ♂♂, river Kriva before Kriva Palanka, Osogovo Mountain, 42.231N, 22.363E, 710 m a.s.l., 12/viii/2018, leg. J. Hinić & V. Slavevska Stamenković [MNCI_RfT8J01, MNCI_RfT8J02, MNCI_RfT8J03 (MNCI)].

R. fasciata (=*R. macedonica*): 1 ♂, Karadak Mt., Banjaska Reka river, above Banjane village, 42.130N, 21.389E, 640 m a.s.l., 11/viii/2018, leg. J. Hinić & V. Slavevska Stamenković [NMK_SCG005 (MNCI)].

Serbia. *R. fasciata*: 1 ♂, river Kamenica, 44.048N, 20.045E, 540 m a.s.l., 17/xi/2019, leg. D. Stojanov [MNCN_Ent 269451 (MNCN)]. 3 ♂♂ + 2 ♀♀, Jastrebac, 43.416N, 21.377E, 630 m a.s.l., 25/ix/2020, leg. A. Bilalli & M. Musliu (STC).

R. fasciata (=*R. macedonica*): 1 ♂ + 1 ♀, Jastrebac, 43.416N, 21.377E, 630 m a.s.l., 25/ix/2020, leg. A. Bilalli & M. Musliu (STC).

Pupae. **Greece.** *R. fasciata* (=*R. macedonica*): 2 ♂♂P + 1 ♀P, Serres, Agios Ioannis, 41.092N, 23.560E, 60 m a.s.l., 01/v/2013, leg. I. Karaouzas [1 ♂P MNCN_Ent 296080 (MNCN); 1 ♂P + 1 ♀P (KPC)]; 1 ♂P + 1 immature pupa, 21/ix/2018, leg. I. Karaouzas (KPC). 5 ♂♂P + 2 ♀♀P + 2 IP, Serres, Elaionas, 41.142N, 23.566E, 556 m a.s.l., 20/ix/2018, leg. I. Karaouzas (KPC). 2 ♂♂P + 4 ♀♀P + 2 IP, Drama, 41.139N, 24.133E, 82 m a.s.l., 21/ix/2018, leg. I. Karaouzas [1 ♀P MNCN_Ent 296082 (MNCN); 2 ♂♂P + 4 ♀♀P + 2 IP (KPC)].

Larvae. **Greece.** *R. fasciata* (=*R. macedonica*): 1 L, Serres, Agios Ioannis, 41.092N, 23.560E, 60 m a.s.l., 01/v/2013, leg. I. Karaouzas [MNCN_Ent 296081 (MNCN)]; 1 L, 21/ix/2018, leg. I. Karaouzas (KPC). 1 L, Serres, Elaionas, 41.142N, 23.566E, 556 m a.s.l., 20/ix/2018, leg. I. Karaouzas (KPC). 3 L, Drama, 41.139N, 24.133E, 82 m a.s.l., 21/ix/2018, leg. I. Karaouzas (KPC).

Methods

Morphology. Morphological characters of adults, pupae, and larvae were investigated under a binocular microscope. Paired hook plates of pupae and selected parts of male genitalia (parameres) were photographed using a compound microscope where multiple stacked images were integrated with focal stacking software (Auto-Montage Essentials, Syncroscopy©).

The descriptions of *R. fasciata* by the following authors were consulted: Eidel (1974), Hagen (1859), Higler (2005), Klapálek (1893), Lepneva (1964), Malicky (2004), Malicky & Sipahiler (1993), McLachlan (1865, 1868, 1879), Nielsen (1942), Novák (1963), Pitsch (1993b), Rinne & Wiberg-Larsen (2017), Schmid (1970), Sedlak (1985), Tobias & Tobias (2010), and Waringer & Graf (1997, 2011), all summarized by Valladolid *et al.* (2021).

Terminology for larval structures, including setae and sensorial pits (Fig. 1), follows the publications by Williams & Wiggins (1981) and Friedrich *et al.* (2015); for adult genitalia Schmid (1970); and for nomenclature of wing venation Ulmer (1909) and Holzenthal *et al.* (2007) (Figs 8b, 8c).

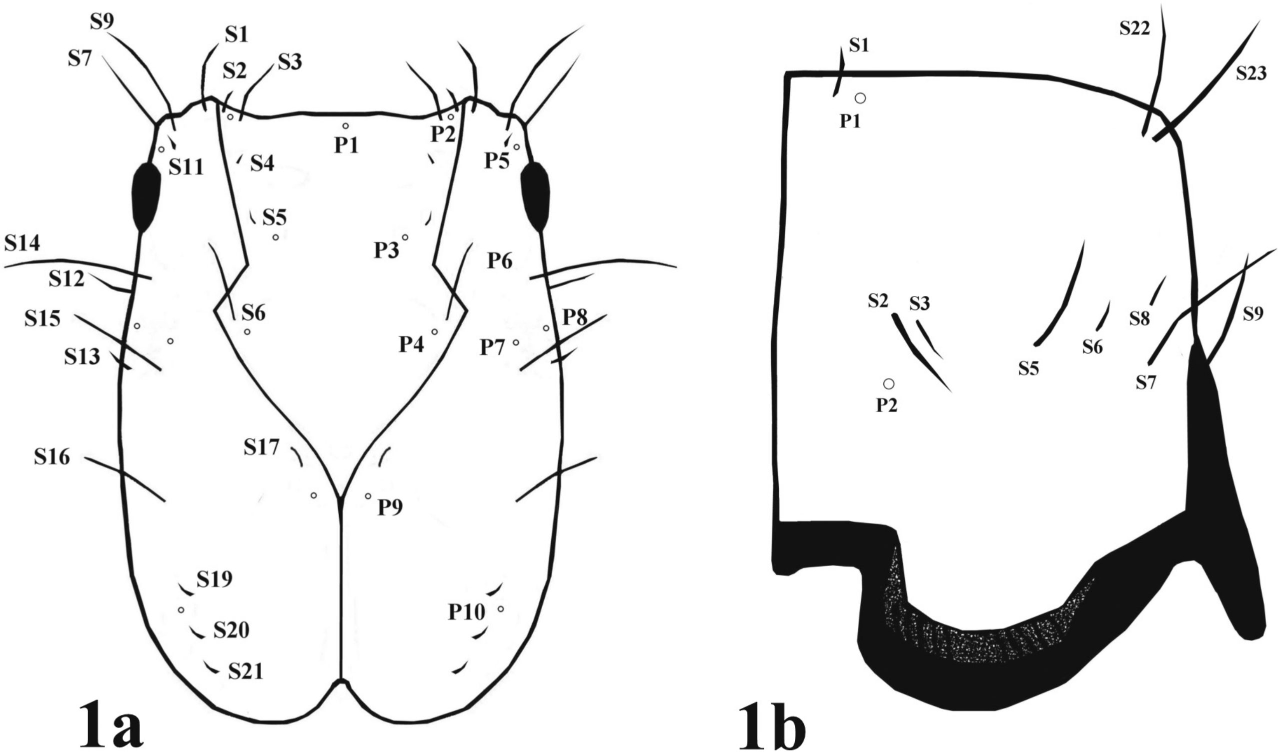


FIGURE 1. Setae (S) and sensory pits (P) of the head and thorax of *Rhyacophila* larvae. 1a, head, dorsal; 1b, prothorax, right sclerite, dorsal (from Williams and Wiggins, 1981).

DNA Extraction, Amplification, and Sequencing. Muscle tissue of legs and thorax from adults, pupae, and larvae was dissected. Samples were fully immersed in 0.5 ml digestion buffer (Gilbert *et al.* 2007) and incubated overnight at 55°C with gentle agitation. The buffer consisted of 5 mM $CaCl_2$, 2% sodium dodecyl sulphate (SDS), 40 mM dithiotreitol (DTT), 250 mg/ml proteinase K, 10 mM Tris buffer pH 8.0, 2.5 mM EDTA (Ethylene-Diamine-Tetra-Acetic acid) pH 8.0, and 10 mM $NaCl_2$ (final concentrations). After incubating, nucleic acids were extracted from the digestion buffer using a Qiaquick PCR purification kit (QIAGEN, Alda *et al.* 2007). A 660-bp region of the mitochondrial COI gene (Cytochrome Oxidase I) was amplified with the primers C1-J-1718 (5’-GGAGGATTTGGAAATTGATTAGTTCC-3’) and C1-N-2329 (5’-ACTGTAAATATATGATGAGCTCA-3’) (Folmer *et al.* 1994; Simon *et al.* 1994). Three microlitres of the DNA solution were used as a template. Other components of the 25 µl PCR reaction were: 1x of the corresponding buffer [75 mM Tris HCl, pH 9.0; 50 mM KCl and 20 mM $(NH_4)_2SO_4$], 2 mM $MgCl_2$, 10 mM dNTPs mix, 0.1 µM of both primers, 0.02% BSA, and 0.125 units AmpliTaq Gold® DNA Polymerase (Applied Biosystems). Five microlitres of PCR products were electrophoresed through a 1.5% agarose gel and visualized with SYBR Safe™ DNA Gel Stain (Invitrogen) under ultraviolet light. PCR products were purified by treatment with ExoSAP-IT (USB Amersham, Buckinghamshire, UK) and incubated at 37 ºC for 15 min, followed by 80 ºC for 15 min to inactivate the enzyme. Purified PCR product was then used to sequence in both directions using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems Inc., Foster City, USA) in a 10 µL volume, containing 15–20 ng of purified product and 3 pmol of primer. To verify that the sequences obtained came from a *Rhyacophila* specimen, they were compared with sequences from GenBank using Blast (Altschul *et al.* 1997). The alignment of all *Rhyacophila* COI gene sequences generated in our lab was performed and edited manually using MEGA X (Kumar *et al*. 2018). Fine adjustments were made by eye, as the COI does not present any gaps.

A total of 99 DNA unique haplotype sequences were selected and used for the study: 80 were prepared in laboratory, 17 were downloaded from GenBank (2020) (accession numbers in Table 1) and BOLD (2021) and 2 sequences were reported directly by Valentina Stamenković and Jelena Hinić. The complete list of DNA sequences obtained is available from Valladolid *et al.* (2022).

Phylogenetic analyses were carried out using Maximum Likelihood (ML) and Bayesian inference (BI) methods. The CIPRES Science Gateway v.3.3 online server (Miller *et al.* 2010) was used to run jModeltest v.2.1.9 (Darriba *et al*. 2012; Guindon & Gascuel 2003) which was used to calculate the nucleotide substitution model and the ML with RAxML-HPC BlackBox (Stamatakis 2014), using 10,000 bootstrap replicates to assess node support. BI analyses were run in MrBayes-3.2.7 (Ronquist *et al.* 2012). We used the substitution model space with the option lset nst=mixed rates=invgamma, for 1.5×106 generations, discarding the first 25% generations as burn-in and synthesizing the resulting. The best tree was visualized using FigTree v1.4.4. (Rambaut 2019), and the nodes were labeled with posterior probabilities. Distances among and within species were calculated using the maximum composite likelihood and p-distance models with MEGA X. Nucleotide sequences were deposited in GenBank (2022) (accession numbers and Tissues and DNA collection numbers in Table 1).

Geographical distribution. The analysis of the species distribution was based on the records from the Global Biodiversity Information Facility page (GBIF 2020), Malicky (2005), and our own sampling points. The distribution map was drawn using ArcGIS 10.3 (ESRI 2011).

Results

Rhyacophila fasciata Hagen 1859

Description of the imago. A complete description of the species is included in the publication by Valladolid *et al.* (2021). Here we describe small differences from the original description of *R. fasciata* found in male and female specimens from Kosovo.

Male body length: 8.06–10.68 mm ($\bar{x}$ = 9.44, n = 22); each forewing: 10.11–12.52 ($\bar{x}$ = 11.32, n = 22); each hind wing: 8.94–10.91 mm ($\bar{x}$ = 9.87, n =22).

Female body length: 8.92–12.99 mm ($\bar{x}$ = 10.75, n = 7); each forewing: 10.57–12.67 mm ($\bar{x}$ = 11.98, n = 7); each hind wing: 9.21–11.31 mm ($\bar{x}$ = 10.42, n = 7).

Colour: Specimens preserved in ethanol reddish brown dorsally, yellowish ventrally, spurs reddish, setae golden brown, females generally darker than males; small black or dark brown spots in dorsal area; in lateral view, some specimens with large black spots marking border between dorsal and ventral areas. Forewings (Figs 8a, b) dark with small pale spots, two short transversal dark bands (fasciae) in Kosovo specimens (Fig. 8b), pale in preserved specimens, darker in females, hind wings pale (Fig. 8c). In specimens from Kosovo (Fig. 8b), forewings each with dark thick pterostigma area on leading edge between apices of subcostal (Sc) and first radial (R_1) or second radial (R_2) veins, each hind wing with pale thick pterostigma area between apices of subcostal (Sc) and second radial (R_2) veins, but not reaching second radial veins in some specimens; each forewing hyaline in irregular or triangular zone connecting medial (M) and medio-cubital (*m-cu*) veins and in distal area (arculus) of second cubital vein (Cu_2); in each hind wing (Fig. 8c) proximal segment of medial veins and connection area (M_{1-4}) not conspicuous, second cubital (Cu_2) veins not conspicuous proximally, proximal portion of cubital vein (Cu) and distal portions of first cubital vein (Cu_{1a1b}) thicker.

Male genitalia (Fig. 15): Apical segment of each inferior appendage in lateral view (Fig. 15a; Valladolid *et al.* 2021, fig. 12A) with basal and distal edges diverging, dorsal and ventral edges diverging, posterior edge slightly concave dorsally and slightly convex ventrally, ventral edge slightly concave; apicodorsal vertex slightly angular (~117°), apicoventral angle rounded, projecting as thick lobe tapering to apex.

Parameres in ventral view curved posteromesad in apical half (Fig. 15c; Valladolid *et al*. 2021, fig. 12BV). In lateral view (Fig. 15d; Valladolid *et al*. 2021, fig. 12BL) each constricted at short distance beyond base, dilated at middle (~38% as thick as length of paramere), with almost parallel posterodorsal and anteroventral margins in central area (S-shape), two files of long and thick spines on midventral margin, pointing outward (some Kosovo specimens with two files of sparse spines); midlateral surface covered by sparse fine spicules or setae, from middle anteroventral to posterodorsal edges and covering most of apex, absent in middle anteroventral and dorsal edges.

Aedeagus (phallicata) in lateral view (Valladolid *et al*. 2021, fig. 12 CL) with anterodorsal margin concave and posterior corner of this concavity hooked anterad, posterior edge of aedeagus straight, apicoventral tip projected posterodorsad, subapically concave dorsally and convex ventrally. Ventral lobe of aedeagus triangular in ventral view (Fig. 15c; Valladolid *et al*. 2021, fig. 12BV), lateral edges slightly convex; lateroventral lobes of phallus straight, posterior edges strongly convex, subapicolateral margins slightly convex (Valladolid *et al*. 2021, fig. 12CD).

Apicodorsal lobe of segment IX slightly dilated subapicolaterally, almost round in dorsal view (Fig. 15b; Valladolid *et al*. 2021, fig. 12DD), with apicomesal excision in some specimens; preanal appendages round, shorter than apicodorsal lobe. In ventral view (Valladolid *et al*. 2021, fig. 12DV), apical band V-shaped, nearly as wide as long, non-sclerotized inside area with posteromesal excision, posterior edges rounded, anal sclerites triangular.

Female genitalia (Fig. 17): In lateral view (Fig. 17L, Valladolid *et al*. 2021, figs 13La; 13Lb-1, -2, -3), posterodorsal margin of segment VIII valves on each side irregular (in Kosovo specimens concave), with subdorsal projection, convex on ventral edge, posterolateral margin irregular, with 1 or 2 posterior projections, ventral projection more conspicuous, in some specimens slightly indented posteriorly, in Kosovo specimens only one ventral projection, rounded apically.

In dorsal view (Fig. 17D; Valladolid *et al*. 2021, fig. 13Da), with indentation between segment VIII valves, and rounded apical and subapical corners, delimiting oval space, small projection in apical corner in some specimens.

In ventral view (Fig. 17V; Valladolid *et al*. 2021, fig. 13V) segment VIII valves forming two elongate sclerites, narrowly separate basally, basal 1/4 of mesal edges parallel, distal 3/4 of mesal edges irregular, slightly concave, diverging; intersegmental membrane with pair of dark, ovoid or slightly elongate sclerites, ovoid in Kosovo specimens.

Rhyacophila macedonica **Karaouzas, Valladolid & Ibrahimi (n. sp.)**

This species has been found in continental Greece, North Macedonia, Kosovo and Serbia.

Etymology. Named after the Greek region Macedonia, where the new species was first collected and identified.

Type material. Holotype ♂: GREECE. Serres, Elaionas (41.142N, 23.566E, 556 m a.s.l.), 20/ix/2018 (I. Karaouzas) (KPC) (GenBank accession number MZ645045).

Paratypes: 5 ♂♂P + 2 ♀♀P + 2 IP + 1L, same locality as holotype, 20/ix/2018 (I. Karaouzas) (KPC). 2 ♂♂P + 1 ♀P + 1L, Serres, Agios Ioannis, 01/v/2013 (I. Karaouzas) [1 ♂P MNCN_Ent 296080 (MNCN); 1 ♂P + 1 ♀P (KPC); 1L MNCN_Ent 296081 (MNCN)]; 1 ♂P + 1 IP + 1 L, 21/ix/2018 (I. Karaouzas) (KPC). 2 ♂♂P + 4 ♀♀P + 2 IP + 3L, Drama, 21/ix/2018 (I. Karaouzas) [1 ♀P MNCN_Ent 296082 (MNCN); 2 ♂♂P + 4 ♀♀P + 2 IP + 3 L (KPC)].

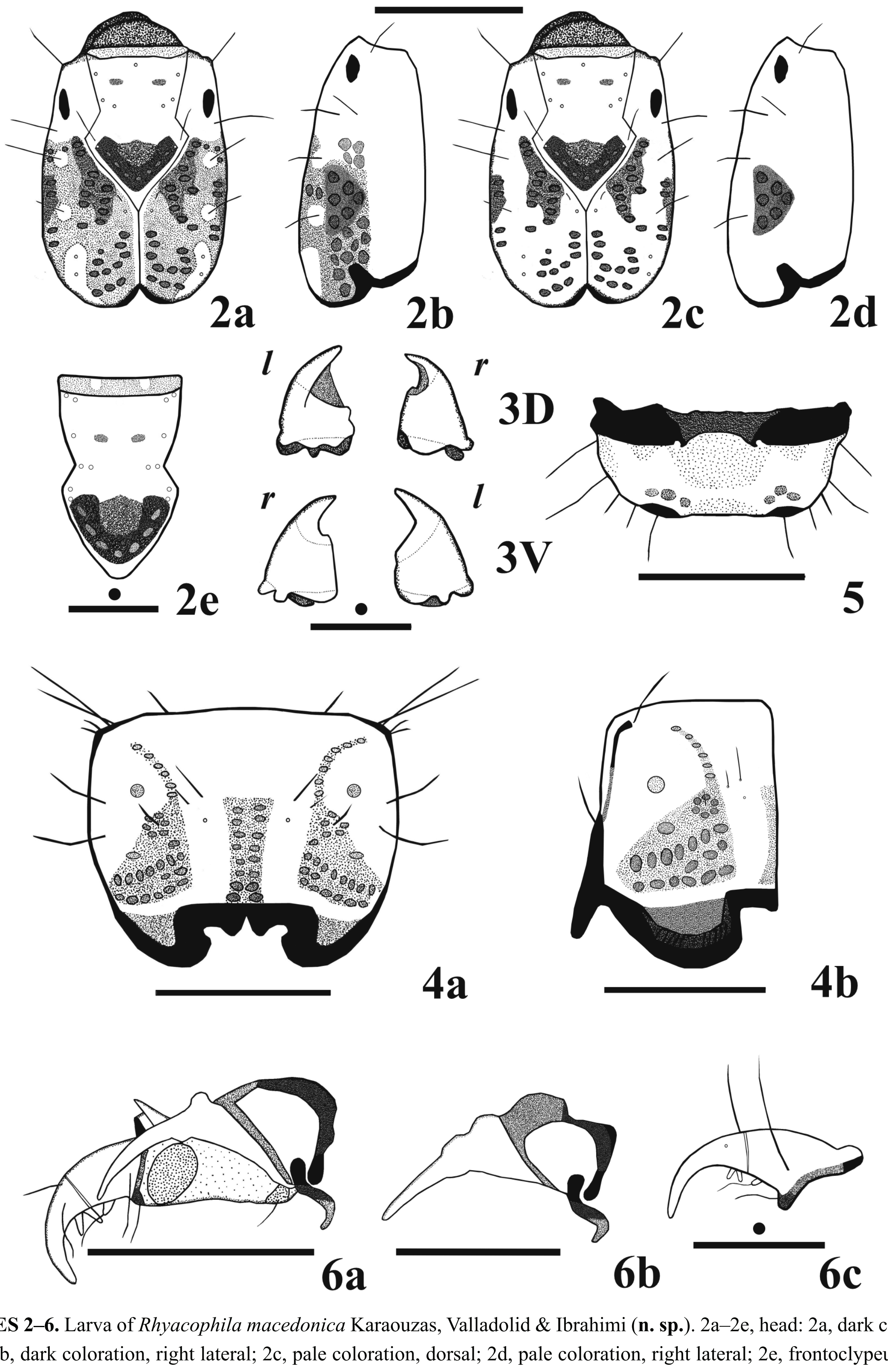
REPUBLIC OF NORTH MACEDONIA. 1 ♂, Banjaska Reka river, above Banjane village, 11/viii/2018 (J. Hinić & V. Slavevska Stamenković) [NMK_SCG005, (MNCI)].

KOSOVO. 1 ♂, Mjak, 17/v/2017 (A. Bilalli & M. Musliu) (KTC). 1 ♂ + 2 ♀♀, Samakovë, 21/v/2017 (H. Ibrahimi) [1♀ MNCN_Ent 296085 (MNCN); 1 ♂ + 1 ♀ (KTC)]. 3 ♂♂, Lugu i Kopilaqës, 3/xi/2019, (H. Ibrahimi) [1 ♂ MNCN_Ent 296083 (MNCN); 2 ♂♂ (KTC)]. 1 ♂ + 1 ♀, Malishevë, Gjilan, 16/vii/2020, (H. Ibrahimi) (GJTC). 4 ♂♂, Kamenicë, 19/ix/2020 (A. Bilalli & H. Ibrahimi) (GOTC). 2 ♂♂, Kamenicë municipality, 19/ix/2020 (H. Ibrahimi) (GOTC). 2 ♂, Shushtë, 17/vii/2017 (A. Bilalli, H. Ibrahimi & M. Musliu) [1 ♂ MNCN_Ent 296084 (MNCN); 1 ♂ (KTC)]. 1 ♂, Dubovë, 20/v/2020 (H. Ibrahimi) (BNTC).

SERBIA. 1♂ + 1 ♀, Jastrebac, 25/ix/2020 (A. Bilalli & M. Musliu) (STC).

Description of the larva (Figs 2–6, 12.)

Biometrics of last instar larva and prepupa: Length: 12.02–18.41 mm ($\bar{x}$ = 14.61, n = 7). Maximum width of larva at metathorax: 2.53–3.38 mm ($\bar{x}$ = 3.04, n = 7). Specimens preserved in ethanol reddish brown, dorsum dark, on thorax two pale dorsolateral areas; on abdomen, two stripes of anterolateral circular pale spots, central stripe of irregular pale spots. Sclerotized areas of head and thorax pale with dark areas and spots.



FIGURES 2–6. Larva of *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**). 2a–2e, head: 2a, dark coloration, dorsal; 2b, dark coloration, right lateral; 2c, pale coloration, dorsal; 2d, pale coloration, right lateral; 2e, frontoclypeus, dorsal. 3D–3V, mandibles from last instar larval exuviae, left (*l*) and right (*r*): 3D, dorsal; 3V, ventral. 4a–4b, prothorax: 4a, dorsal; 4b, left hemisclerite, left lateral. 5, abdominal tergite IX. 6a–6c, details of anal prolegs: 6a, right anal claw, right lateral; 6b, detail of basolateral plate with sword process and basoventral hook, right lateral; 6c, detail of right claw, right lateral. Scale bars: unlabelled = 1 mm; ● = 0.5 mm.

Head (Figs 2, 12a). Length: 1.73–1.95 mm ($\bar{x}$ = 1.82, n = 7). Width: 1.11–1.28 mm ($\bar{x}$ = 1.21, n = 7). Maximum width at posterior third of head, lateral margins slightly converging anteriorly, rounded posteriorly. Cephalic capsule (Figs 2a–2e), dorsally and dorsolaterally light brown, except anterior third, areas adjacent to frontoclypeal and anterior coronal sutures and anterior dorsolateral areas paler (Fig. 2a), in lighter specimens (Fig. 2c) pale in general, only posterior frontoclypeus and small spotted areas brown; ventral side pale; anteroventral edge and submentum reddish brown, posterior edge black or brown with black areas; laterally (Fig. 2b) with dark band in posterodorsal half with darker spots, triangular brown area with dark spots in middle, in lighter specimens (Fig. 2d) with only triangular brown area. Frontoclypeal apotome (Fig. 2e) pale; pair of oval pale brown submesal muscle attachment spots in anterior third, between setae #4; dark pigmentation in posterior third somewhat heart-shaped, with truncate anterior edges, central area paler brown, pointed anteriorly, with 4–6 oval darkly bordered brown muscle attachment spots, dark area including pair of pits #4 but not setae #6. Two-thirds of posterior dorsal area of head light brown, or pale in lighter specimens, marked with darker muscle attachment spots; at midlength one pair of light brown patches, wing-shaped, outside and paralleling posterior edge of frontal suture, in anterior extension, 2 or 3 spots parallel to suture, in middle of each patch two rows of spots perpendicular to suture; light circular area on juncture of frontal and coronal sutures, 3 or 4 pairs of spots aligned on both sides of coronal suture, two groups of spots on each side of posterior area. Three pair of pale spots around bases of setae #15, #16, and #19–21 in darker specimens, one pale stripe in lighter specimens. Dorsal head pits with dark borders. Posterior occipital foramen black at midheight, dark brown surrounding remainder of foramen.

Mandibles (Figs 3D, 3V) asymmetrical, as in other rhyacophilids; left mandible slightly larger than right one. Inner blade at base of left mandible slightly convex; right mandible inner blade at base straight with small tooth in middle. Maxillolabium and labrum as in other *Rhyacophila* species. Labrum with small transparent area in middle of anterior edge.

Thorax (Figs 4, 12b): Anterior half of pronotum pale or very light coloured. Sinuous posterior margin bordered completely by dark band, reddish brown anterior of dark band at midheight of each half. Posterior half of pronotum (Fig. 4a) with three dark areas separated by light zones; (1) central dark region tapered posteriorly and reaching posterior edge of sclerite, anteriorly truncate near midlength of pronotum, wider than posteriorly, lateral edges concave, pair of submesal lines of dark brown spots inside this central dark region reaching posterior edge, several dark brown spots along posterior edge; (2) pair of lateral dark areas almost triangular, each with one long edge parallel to and as long as central dark region, one parallel to and slightly longer than posterior reddish brown region, and third edge diagonal between anterior subdorsal edge and ventral subposterior edge; (3) pair of circular spots anterolateral of anterior corners of triangular lateral dark areas. Each lateral dark area with two to three transverse rows of brown circular-oval spots with dark borders parallel to posterior border; four–six brown spots around setae #5, oval spot near diagonal edge of triangular dark area and curved row of pale dots from setae #5 extending toward anterolateral corner of pronotum, curving around circular spot (spot #3) on anterior half in darker specimens (Figs 4a, 4b); each side with narrow black band in anterolateral angle, sometimes extending posterad as fine brown band laterally to posterolateral margins; anterior margin light brown, with several brown spots near anterior angle (Fig. 12b).

Legs: Similar to those of other *Rhyacophila* species. Colour yellowish, proximal regions of coxae, femora and distal regions of trochanters of fore- and midlegs black or dark brown, tibiae and tarsi light reddish; each inner and outer face of each femur with circular spots. Forelegs each ventrally with row of spicules, extending along from distal half of trochanter, and proximal half of femur, distal half of tibia and length of tarsus. Setae black or dark brown, some transparent in ventral area.

Abdomen (Figs 5, 6, 12c, 12d): Similar to that of other species of *Rhyacophila*. In preserved larvae reddish brown dorsally, two stripes of anterolateral circular pale spots, central stripe of irregular pale spots, star shaped, ventral side pale. Widths of anterior abdominal segments similar, narrower in posterior segments. Lateral abdominal gills in tufts of 30–35 fine filaments. Dorsal sclerite of segment IX (Figs 5, 12c) light coloured, with continuous dark band on anterior edge and pair of discontinuous black bands on posterior edge; band of anterior edge black in lateral areas, brown in middle, with conspicuous pits in both sides; in darker specimens with semicircular coloured area in anterior middle; some dark spots near black bands on posterior edge, semicircular hyaline zone in middle posterior edge. Anal prolegs typical of group, each with long sword process (Figs 6a, 6b, 12d). Anal claws each with three or four teeth on ventral edge (Figs 6a, 6c), basoventral hook black basally and reddish brown apically (Figs 6b, 12d).

Morphological characters diagnosing larvae of *R. macedonica* and *R. fasciata*

Head: In *R. macedonica* the pair of dark brown patches outside the posterior edge of the frontal sutures is more or less triangular; inside each patch there are two rows of dark spots aligned perpendicular to the suture. In *R. fasciata* the pair of dark brown patches outside the posterior edge of the frontal sutures is in the shape of open wings, inside each patch there are two rows of with dark spots aligned parallel to the suture.

In *R. macedonica* each side of the head has a band with dark spots and a triangular darker area extending two-thirds of the length of the head from the posterior edge, or only a triangular darker area in lighter specimens. In *R. fasciata*, each side of the head has laterally an oval dark area with darker spots, extending half the distance from the posterior edge.

In *R. macedonica* the left mandible has its inner blade straight or slightly concave. In *R. fasciata* the left mandible has its inner blade slightly convex.

Thorax: In *R. macedonica* on the posterior half of the pronotum, the median dark stripe is truncate anteriorly and with its lateral edges concave; each triangular posterior area has two or three transverse rows of oval brown spots with dark borders and a bigger oval spot near the diagonal anterolateral edge. In *R. fasciata* on the posterior half of the pronotum the median dark stripe is convex anteriorly and with its lateral edges slightly convex; each triangular posterior area has two or three transverse rows of circular brown spots with dark borders but no bigger spot near the diagonal anterolateral edge. In *R. macedonica* the anterior margin of the pronotum has several brown spots near each anterolateral angle; the narrow black band in the anterolateral angle sometimes extends posterad as a brown band laterally to the posterolateral margins; the posterior marginal band at midheight is black posteriorly (reddish brown anteriorly). In *R. fasciata* the anterior margin of the pronotum has a black spot near each anterolateral angle; the narrow black band in each anterolateral angle extends posterad laterally to the posterolateral margin, and the posterior marginal band at midheight is dark brown or black anterior and posteriorly.

Abdomen: In *R. macedonica* tergite IX is light coloured and in darker specimens has a semicircular coloured area in the anterior middle; the band of the anterior edge is black laterally, brown in the middle. In *R. fasciata* tergite IX is uniformly coloured, the band of the anterior edge has two extensions surrounding pit #1 and is connected with dark stripes in darker larvae.

Description of the pupa (Figs 7l, 7r.)

Biometrics of pupa and cocoon. Pupal length (total): 8.23–12.64 mm ($\bar{x}$ = 10.28, n = 17) [male pupa: 8.23–10.49 mm ($\bar{x}$ = 9.42, n = 10); female pupa: 10.28–12.64 mm ($\bar{x}$ = 11.52, n = 7)]. Cocoon length (total): 11.04–16.84 mm ($\bar{x}$ = 13.72, n = 20) [male cocoon length: 11.04–14.22 mm ($\bar{x}$ = 12.72, n = 9); female cocoon length: 12.73–16.71 mm ($\bar{x}$ = 14.53, n = 6)].

Head: Mandibles pale, each with distal half reddish brown, left mandible with two preapical teeth, distal tooth large; right mandible with three teeth, distal tooth large and two proximal teeth small, both mandibles with numerous fine teeth on inner blade. Antennae of variable length, reaching posterior edge of abdominal segment IV to posterior edge of segment VIII (from posterior edge of segment V to posterior edge of segment VIII in males, posterior edge of segment IV to posterior edge of segment V in females).

Thorax: Tubercles of prothorax each with 4 or 5 setae. Hind wing pads variously reaching posterior edge of abdominal segment III to posterior edge of segment V.

Abdomen: Paired anterior hook plates pedunculate, almost circular and present on abdominal terga IV to VII (Fig. 7), each with 15–20 spines and 20–30 spines (segment IV and segments V–VII, respectively); also tergum III with pair of small flat anterior hook plates each with 0–5 spines. Paired posterior hook plates sessile on terga III–V oblong transversally (length: width = 1: 1.5), each with less than 30 spines.

Morphological characters diagnosing pupae of *R. macedonica* and *R. fasciata*

Abdomen: In *R. macedonica* the paired anterior hook plates are almost circular and the pair of small flat anterior plates on tergum III have 0–5 spines; the paired posterior hook plates are oblong transversally (length: width = 1: 1.5). In *R. fasciata* the paired anterior hook plates are palmate and the pair of small flat anterior plates on tergum III are without spines; the paired posterior hook plates are only slightly oblong transversally (length: width = 1: 1.3).

Description of the imago

Holotype male: Length (distance from front of head to distal edge of segment IX): 7.33 mm, each forewing: 9.47 mm, each hind wing: 7.81 mm.

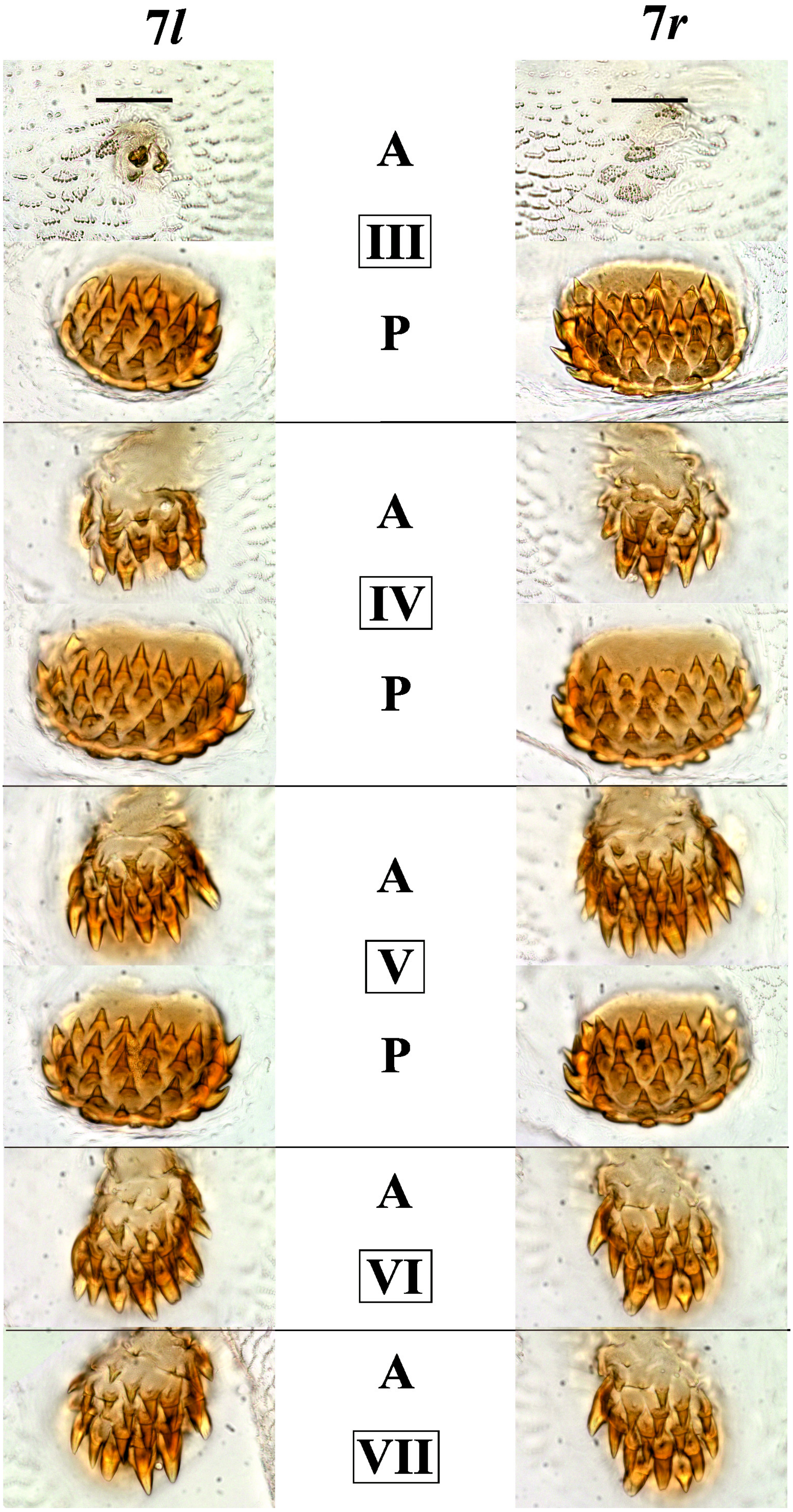
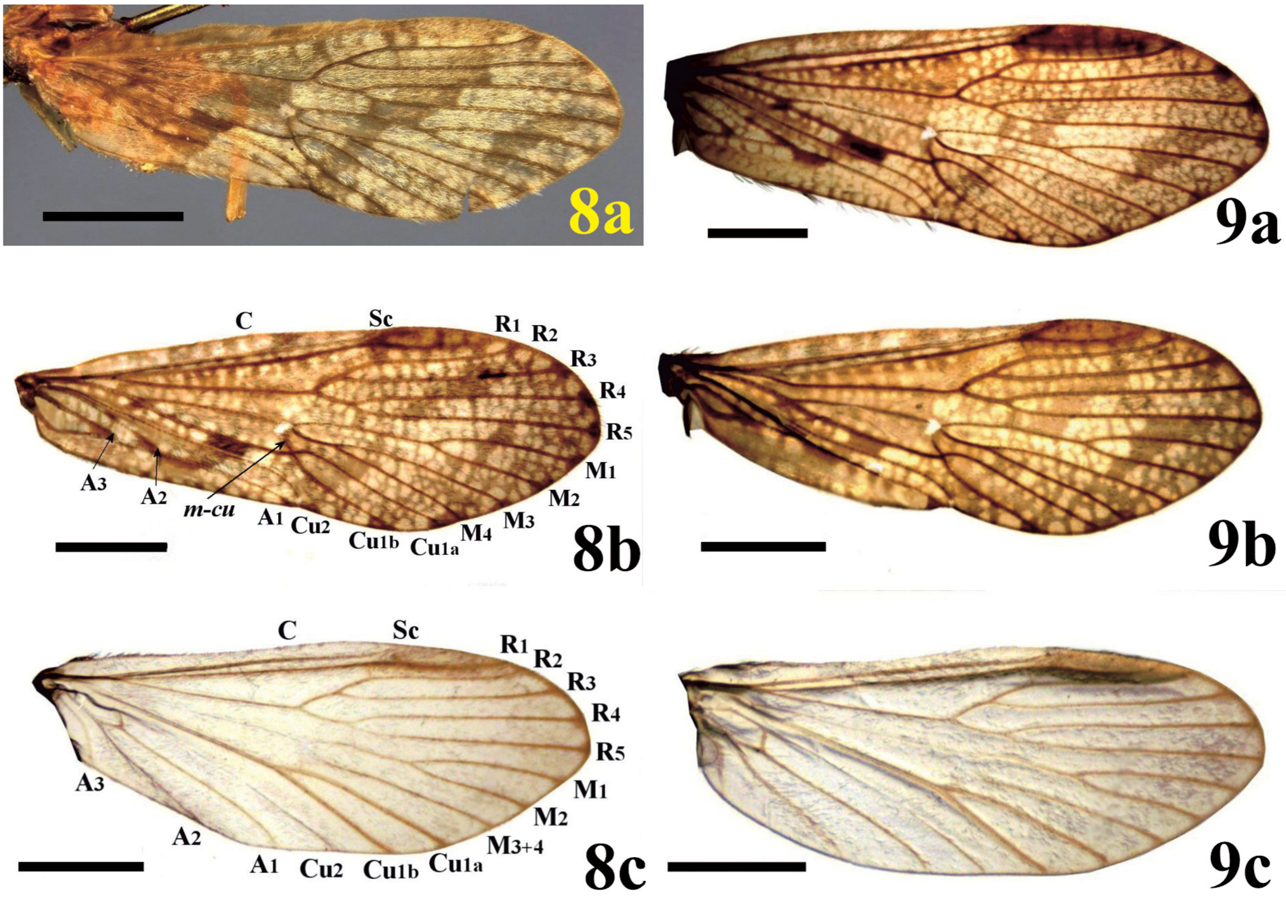


FIGURE 7. *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**), paired abdominal hook plates of male pupa. 7*l* and 7*r*, left and right hook plates, respectively. A = anterior hook plates, dorsal, P = posterior hook plates, dorsal; III–VII = abdominal terga III through VII, dorsal. Scale bars: 50 µm.

Male body length: 7.33–11.95 mm ($\bar{x}$ = 9.26, n = 20) each forewing 9.47–12.56 ($\bar{x}$ = 11.24, n = 20) each hind wing 7.81–11.23 mm ($\bar{x}$ = 9.76, n = 20).

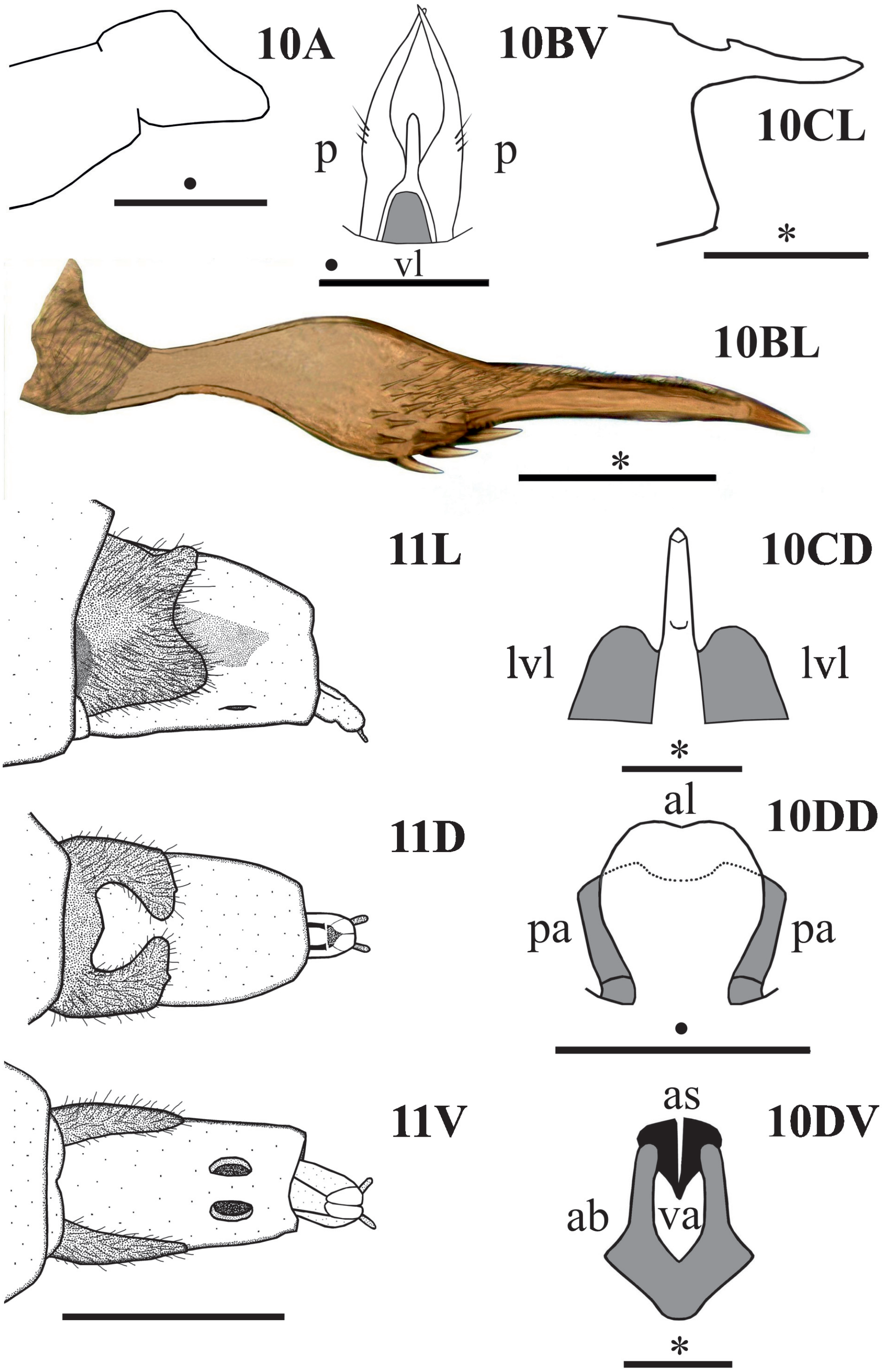
Female body length (distance from front of head to distal edge of segment VIII): 9.98–11.06 mm ($\bar{x}$ = 10.52, n = 4), each forewing 11.80–12.97 mm ($\bar{x}$ = 12.34, n = 4), each hind wing 10.36–11.55 mm ($\bar{x}$ = 10.86, n = 4).

Colour: Specimens preserved in ethanol reddish brown dorsally, yellowish ventrally, spurs reddish, setae reddish brown, females generally darker than males; small black or dark brown spots in dorsal area; in lateral view, some specimens with large elongate black spots marking border between dorsal and ventral areas. Forewings (Figs 9a, 9b) dark with small pale spots, one or two short transversal dark bands (fasciae) in some specimens, the distal band more marked, hind wings (Fig. 9c) pale: each with dark thick stigma area on leading edge, between subcostal (Sc) and first radial (R_1) veins in forewing and between subcostal (Sc) and second radial (R_2) in hind wing; in each forewing, additional vein connecting costal (C) and subcostal (Sc) veins in some Kosovo specimens, crossveins connecting R_1+R_2 (r) and fifth radial (R_5) and first medial (M_1) (r-m) present, hyaline circular zone in connection area of medial (M) and major cubitus (Cu) veins (Fig. 8b; Holzenthal *et al.* 2007); in each hind wing, proximal segment of medial vein (M) not conspicuous; median vein connecting M_2 and M_{3+4} veins missing; crossvein between the radial sector R_5 and median vein M_2 missing (Fig. 8c; Holzenthal *et al.* 2007).

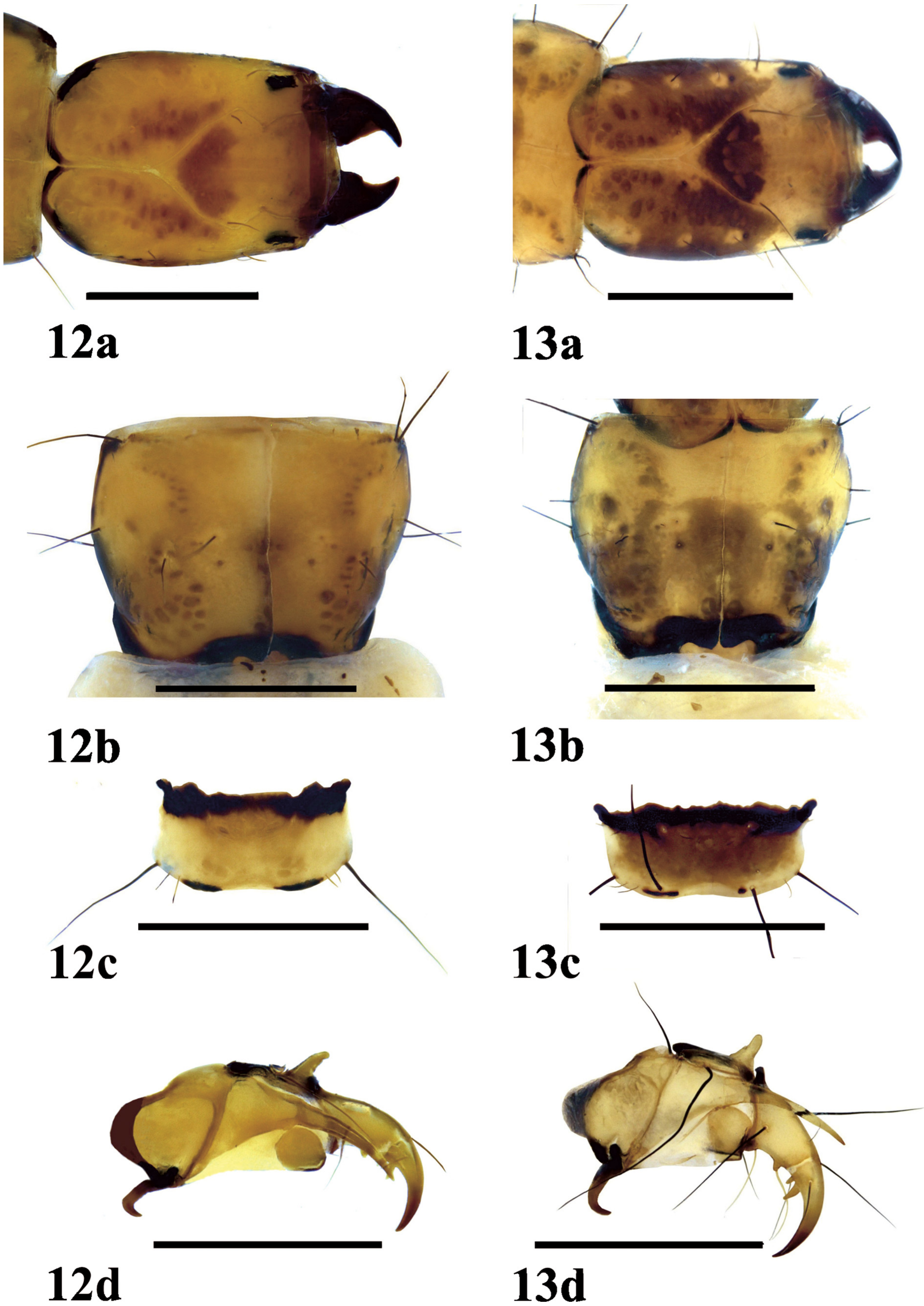


FIGURES 8, 9. Wings of *Rhyacophila fasciata* Hagen 1859 and *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**). 8a, forewing of *R. fasciata*, lectotype [Museum of Comparative Zoology of Harvard University, Cambridge, MA, USA (MCZH), with permission]; 8b, forewing of *R. fasciata* male from Kosovo; 8c, hind wing of *R. fasciata* male from Kosovo. 9a, 9b, forewings of *R. macedonica* males from Kosovo; 9c, hind wing of *R. macedonica* male from Kosovo. Major veins: C = costa, Sc = subcosta, R_1–R_5 = radius, M_1–M_4 = media, Cu_1–Cu_2 = cubitus, A_1–A_3 = anal, *m-cu* = crossvein between medial and cubital veins (from Holzenthal *et al.* 2007). Scale bars: 2 mm.

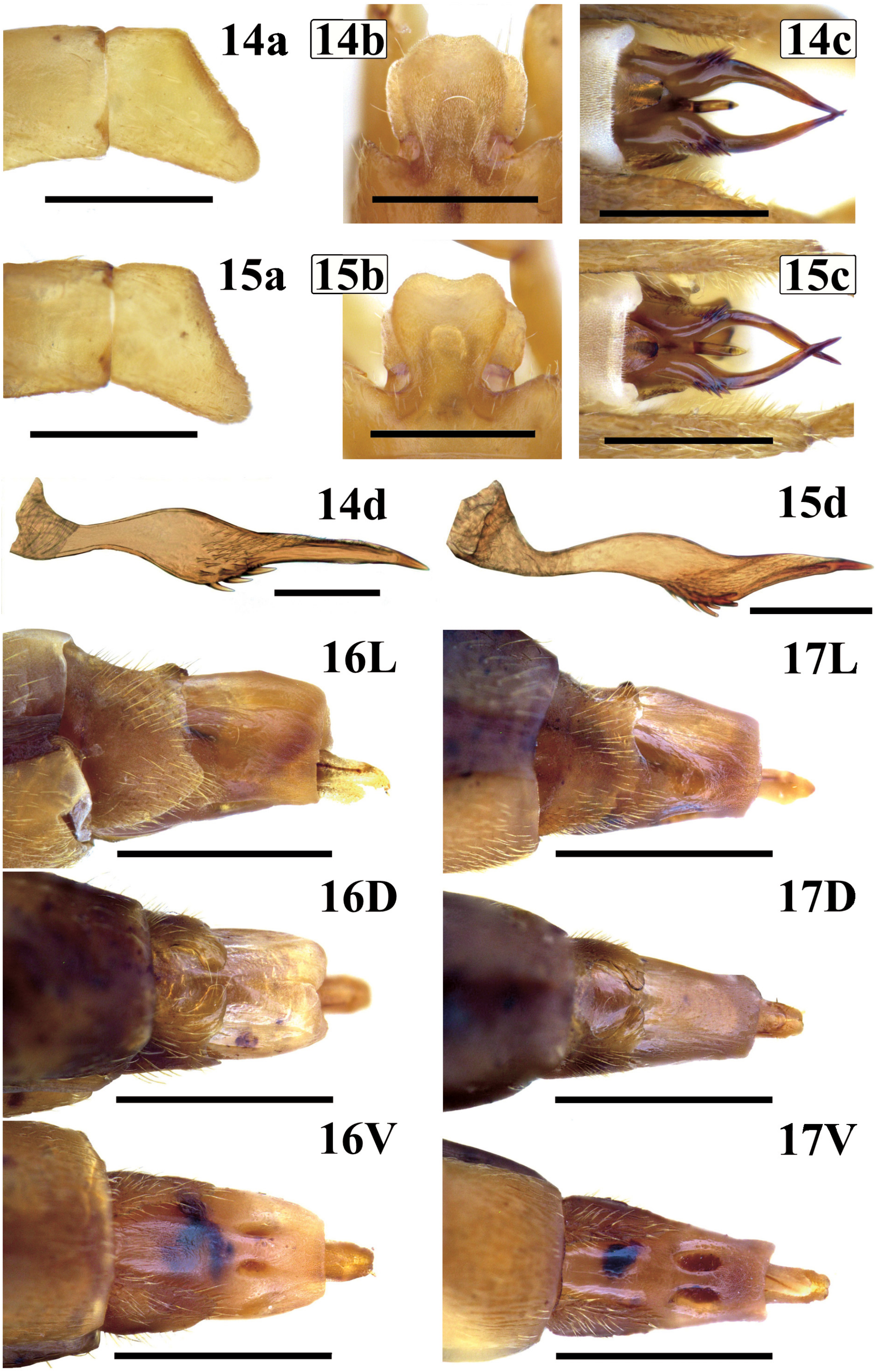
Male genitalia (Figs 10, 14): Apical segment of each inferior appendage (Figs 10A, 14a) with basal and distal edges diverging, dorsal and ventral edges diverging, posterior edge of 2nd segment of each inferior appendage straight or concave, ventral edge straight or slightly convex, 2 times longer than dorsal edge. Apicodorsal vertex angular (~124°), apicoventral angle rounded, projecting as thick lobe tapering to apex.



FIGURES 10, 11. Male and female genitalia of *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**), respectively. 10, male: 10A, 2nd segment of left inferior appendage, left lateral; 10BV, 10BL, parameres: 10BV, parameres (p) and ventral lobe of aedeagus (phallicata) (vl), ventral; 10BL, left paramere, left lateral. 10CL, 10CD, aedeagus (phallicata) and lateroventral lobes: 10CL, aedeagus and ventral lobe, left lateral; 10CD, aedeagus and its lateroventral lobes (lvl), dorsal. 10DD, apicodorsal lobe of segment IX (al) and preanal appendages (pa); 10DV, segment X, ventral, ab= apical band, as= anal sclerites, va= non-sclerotized ventral area. 11, female segments VIII–XI: 11L, left lateral; 11D, dorsal; 11V, ventral. Scale bars: unlabelled = 1 mm; ● = 0.5 mm; * = 200 µm.



FIGURES 12, 13. Larvae of *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**) and *Rhyacophila fasciata* Hagen 1859. 12, *R. macedonica*: 12a, head, dorsal; 12b, prothorax, dorsal; 12c, abdominal tergite IX, dorsal; 12d, left anal proleg, left lateral. 13, *R. fasciata*: 13a, head, dorsal. 13b, prothorax, dorsal; 13c, abdominal tergite IX, dorsal; 13d, left anal proleg, left lateral. Scale bars: 1 mm.



FIGURES 14–17. Males and females of *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**) and *Rhyacophila fasciata* Hagen 1859 from Kosovo. 14, male of *R. macedonica*: 14a, 2nd segment of left inferior appendage, left lateral; 14b, apicodorsal lobe of segment IX and preanal appendages, dorsal; 14c, parameres and ventral lobe of aedeagus (phallicata), ventral; 14d, left paramere, left lateral. 15, male of *R. fasciata*: 15a, 2nd segment of left inferior appendage, left lateral; 15b, apicodorsal lobe of segment IX and preanal appendages, dorsal; 15c, parameres and ventral lobe of aedeagus (phallicata), ventral; 15d, left paramere, left lateral. 16, female segments VIII–XI of *R. macedonica*: 16L, left lateral; 16D, dorsal; 16V, ventral. 17, female segments VIII–XI of *R. fasciata*: 17L, left lateral; 17D, dorsal; 17V, ventral. Scale bars: Figures 14a–c, 15a–c = 0.5 mm, Figures 14d, 15d = 200 µm, Figures 16, 17 = 1 mm.

Parameres in ventral view curved posteromesad in apical half (Figs 10BV, p; 14c). In lateral view (Figs 10BL, 14d) each constricted at short distance beyond base, dilated at middle (~43% as thick as length of paramere), with rounded dorsal margin; two rows with three long, thick spines on midventral margin, pointing outward; midlateral surface covered by sparse fine spicules or setae from middle anteroventral to posterodorsal edges, sometimes also in posterior middle anteroventral area.

Aedeagus (phallicata) in lateral view (Fig. 10CL) with dorsal margin straight or slightly concave and posterior corner of concavity hooked anterad, posterodorsal edge of aedeagus straight or slightly concave, projected posterodorsad to small truncate apex; ventral lobe of aedeagus in ventral view triangular, truncate at apex (Fig. 10BV, vl). Lateroventral lobes of phallus in dorsal view converging posterad, posterior edges strongly convex, apicolateral margins semicircular (Fig. 10CD, lvl).

Apicodorsal lobe of segment IX in dorsal view (Figs 10DD, al; 14b) slightly dilated subapicolaterally, pentagonal in shape in some specimens, with small apicomesal excision in some specimens; preanal appendages (Fig. 10DD, pa) rectangular, shorter than apicodorsal lobe. In ventral view, apical band V-shaped (Fig. 10DV, ab), 1.2 times as long as wide, non-sclerotized inside area with posteromesal excision (Fig. 10DV, va), posterior arms convergent posterad, rounded apically; anal sclerites triangular (Fig. 10DV, as).

Female genitalia (Figs 11, 16): In lateral view (Figs 11L, 16L), posterodorsal margin of segment VIII valves on each side round, in some specimens dorsolateral projection with very small extension, inconspicuous, posterolateral margin concave or nearly straight, convex or nearly straight on ventral edge, posteroventral projection triangular, its posterior edge more or less rounded.

In dorsal view (Figs 11D, 16D), with indentation between segment VIII valves nearly enclosed by pair of dorsolateral subtriangular projections, with rounded apical and subapical corners, delimiting oval space, in some specimens very small, inconspicuous projection, in middle distal edge.

In ventral view (Figs 11V, 16V), segment VIII valves forming two elongate sclerites, widely separated basally, slightly concave mesally, diverging; intersegmental membrane with pair of dark, ovoid or slightly elongate sclerites.

Morphological characters diagnosing males of *R. macedonica* and *R. fasciata*

Genitalia: In *R. macedonica*, the posterior edge of the 2nd segment of each inferior appendage is straight or slightly concave, the ventral edge is straight or slightly convex. In *R. fasciata,* the posterior edge of the 2nd segment of each inferior appendage is slightly concave dorsally and convex ventrally, the ventral edge is slightly concave (Fig. 15a).

In *R. macedonica* the parameres in lateral view (Fig. 10BL) each have two rows of long, thick spines on the midventral margin, each row with three spines pointing laterad and ventrad; the midlateral surface has sparse thick spicules in addition to fine setae, from the middle of the anteroventral edge to the posterodorsal edge. In *R. fasciata* the parameres in lateral view (Fig. 15d; Valladolid *et al*. 2021, fig. 12BL) each have two rows of long, thick spines on the midventral margin, each row with more than three spines pointing laterad; the midlateral surface has sparse fine setae from the middle of the anteroventral edge to the posterodorsal edge, covering most of apex, but absent from the middle anteroventral and posterodorsal edges.

In *R. macedonica* posterior edge of aedeagus straight or slightly concave, projected posteroventrad in small ventral apex, truncate; ventral lobe of aedeagus triangular, truncate at apex, lateroventral lobes of phallus converging posterad, posterior edges strongly convex, apicolateral margins semicircular. In *R. fasciata* posterior edge of aedeagus straight, projected posteroventrad and rounded at posteroventral apex; ventral lobe of aedeagus triangular (Fig. 15c), lateral edges nearly straight; lateroventral lobes of phallus straight, posterior edges convex, apicolateral margins rounded.

In *R. macedonica,* the apicodorsal lobe of segment IX in dorsal view (Figs 14b; 10DD, al) is dilated subapicolaterally, pentagonal in shape in some specimens, sometimes with a small apicomesal excision; the preanal appendages are rectangular, nearly straight sided; the apical band in ventral view (Fig. 10DV, ab) is 1.2 times as long as wide, its posterior arms close together and slightly converging posterad; the non-sclerotized area between these arms (Fig. 10DV, va) is oval, with a narrow posteromesal excision; the anal sclerites (Fig. 10DV, as) are convex apically. In *R. fasciata* the apicodorsal lobe of segment IX in dorsal view (Fig. 15b; Valladolid *et al.* 2021, fig. 12DD, al) is dilated subapicolaterally, almost round, with a small apicomesal excision in some specimens; the preanal appendages (Valladolid *et al.* 2021, fig. 12DD, pa) are round, convex laterally; the apical band in ventral view (Valladolid *et al.* 2021, fig. 12DV, ab) 1.8 times as long as wide, its posterior arms widely separate and straight; the non-sclerotized area between these arms (Valladolid *et al.* 2021, fig. 12DV, va) is heart-shaped and with a wider posteromesal excision; the anal sclerites (Valladolid *et al.* 2021, fig. 12DV, as) are truncated apically.

Morphological characters diagnosing females of *R. macedonica* and *R. fasciata*

Genitalia: In *R. macedonica* the dorsal and ventral margins of each lateral valve of segment VIII in lateral view (Figs 11L, 16L) are similar in length and in some specimens the dorsolateral projection has a very small, inconspicuous extension; the posterolateral margin of each valve is concave or nearly straight, the single posteroventral projection is triangular and more or less rounded apically, and the ventral margin is convex or nearly straight. In *R. fasciata* the dorsal margin of each lateral valve of segment VIII in lateral view (Fig. 17L; Valladolid *et al.* 2021, fig. 13La) is 3/4 as long as the ventral margin and has a prominent dorsolateral projection; the posterolateral margin of each valve is irregular, with 1 or 2 posterior projections, the posteroventral projection is more conspicuous, and the ventral margin is slightly concave sub-basally and straight or convex distally, in some specimens slightly indented posteriorly.

In *R. macedonica* the indentation between the segment VIII valves in dorsal view (Figs 11D, 16D) is nearly enclosed by a pair of sub-triangular dorsolateral projections delimiting a bean-shaped membranous space, in some specimens there is a very small, inconspicuous projection in the middle of the anterior edge. In *R. fasciata* the indentation between the segment VIII valves in dorsal view (Fig. 17D; Valladolid *et al.* 2021, fig. 13Da) is nearly enclosed by a pair of thick posterolateral projections delimiting an oval membranous space; a small projection in the middle of the anterior edge of some specimens.

In *R. macedonica* the segment VIII valves in ventral view (Figs 11V, 16V) form two elongate sclerites that are widely separated basally, slightly concave on their mesal margins, and slightly diverging ~22°. In *R. fasciata* the segment VIII valves in ventral view (Fig. 17V; Valladolid *et al.* 2021, fig. 13V) form two elongate sclerites that are narrowly separated basally, the basal third of their proximal edges are parallel, and their distal edges are irregular, straight, and more conspicuously diverging ~47°.

Genetic analysis

For the study, we have included 99 unique haplotype sequences of specimens from the *Rhyacophila fasciata* Group used in previous publications and from newly prepared material: 43 sequences of *R. fasciata*, as well as 12 sequences of *R. macedonica*, 2 sequences of *R. septentrionis* (resurrected to species by Valladolid *et al.* 2021), 3 sequences of *R. denticulata* (promoted to species by Valladolid *et al.* 2018), 10 sequences of *R. sociata* from Spain and France (resurrected to species by Valladolid *et al.* 2018), 4 sequences of *R. kykladica* (promoted to species by Valladolid *et al.* 2019), 21 sequences of *R. fasciata delici* (in Valladolid *et al.* 2020), 3 sequences of *R. fasciata viteceki* (in Valladolid *et al.* 2020), and 1 sequence of *R. cf. obliterata* McLachlan 1863 from Poland (see Table 1 for references).

The tree (Fig.18) shows clear differences between *R. fasciata* and the other species included, that previously were considered as *R. fasciata* or subspecies of *R. fasciata*. ML (bootstrap support, BS) and BI (posterior probabilities, PP) phylogenetic analyses recovered similar topologies, supporting two major clades: monophyletic *R. kykladica* (BS = 100, PP = 1) (Fig. 18b) and the rest of species of the *R. fasciata* Group (BS = 93, PP =1). Inside this last clade, we found seven well supported groups: paraphyletic *Rhyacophila macedonica* (Fig. 18c) and monophyletic *R. fasciata* (BS = 76, PP = 1) (Fig. 18d) as sister species (BS = 75, PP = 1), monophyletic *R. denticulata* (BS = 100, PP = 1) (Fig. 18e) and monophyletic *R. sociata* (BS = 100, PP = 1) (Fig. 18f) also as sister species (BS= 95, PP = 1), those groups and paraphyletic *R. fasciata viteceki* (Fig. 18g) and monophyletic *R. fasciata delici* (BS = 89, PP = 1) (Fig. 18h) as an unresolved polychotomous group (BS= 73, PP = 0.81), and paraphyletic *R. septentrionis* as sister species to all other groups in this clade (BS = 93, PP = 1) (Fig. 18i).

Intraspecific distances (maximum composite likelihood model) are lower than 1% (Table 2a), being the lowest in *R. septentrionis, R. f. viteceki*, and *R. kykladica* (0.0012, 0.0026, and 0.0030, respectively) while interspecific distances are higher than 1% (Table 2b) among all of these species. The lower distances are between *R. septentrionis* and *R. f. viteceki* (0.0103) and between *R. fasciata* and *R. macedonica* (0.0173).

TABLE 1. Data for specimens analyzed by DNA. Species numbers refer to sequences included in the study. Sex: A= adult (sex not specified), M = male, F = female, MP = male pupa, FP = female pupa, L= larva. N, E = latitudinal and longitudinal geographical coordinates, respectively. Alt = altitude (m a.s.l.). Accession numbers from GenBank and Tissues and DNA Collection of Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas (MNCN, CSIC).

Species	Sex	Locality	N	E	Alt	GenBank	MNCN Collection
macedonica 3	MP	Serres, Agios Ioannis (Greece)	41.092	23.560	60	MZ645043	MNCN:ADN 94021
macedonica 10	IP	Serres, Elaionas (Greece)	41.142	23.566	556	MZ645050	MNCN:ADN 102667
macedonica 11	FP	Serres, Elaionas (Greece)	41.142	23.566	556	MZ645051	MNCN:ADN 102668
macedonica 12	FP	Serres, Elaionas (Greece)	41.142	23.566	556	MZ645052	MNCN:ADN 102669
macedonica 14	MP	Serres, Elaionas (Greece)	41.142	23.566	556	MZ645054	MNCN:ADN 102671
macedonica 17	MP	Serres, Elaionas (Greece)	41.142	23.566	556	MZ645057	MNCN:ADN 102674
macedonica 32	M	Banjaska River (Republic of North Macedonia)	42.130	21.380	640	MZ645072	MNCN:ADN 110373
macedonica 34	M	Samakovë (Kosovo)	42.254	21.348	630	MZ645074	MNCN:ADN 110375
macedonica 35	F	Samakovë (Kosovo)	42.254	21.348	630	MZ645075	MNCN:ADN 110376
macedonica 36	M	Dubovë (Kosovo)	42.720	20.356	500	MZ645076	MNCN:ADN 110378
macedonica 38	M	Kamenicë municipality (Kosovo)	42.624	21.687	615	MZ645078	MNCN:ADN 110382
macedonica 41	F	Malishevë, Gjilan (Kosovo)	42.450	21.504	480	MZ645081	MNCN:ADN 110385
fasciata 2		Trøgstad, Indre Østfold (Norway)	59.746	11.272	115	KX293282	
fasciata 3	F	Finnmark, Alta, Gargiaveien (Norway)	69.822	23.480	95	KX293192	
fasciata 9	A	Hessen, Schlitz, Breitenbach (Germany)	50.662	9.624	280	KX294688	
fasciata 10	A	Hessen, Schlitz, Breitenbach (Germany)	50.662	9.624	280	KX292344	
fasciata 12		Limburg, Veurs, Voeren (Belgium)	50.749	5.814	130	KX142884	
fasciata 26	M	Trondheim, Nidelva (Norway)	63.339	10.442	70	KX291512	
fasciata 28	L	Paltamo, Vaarainjoki (Finland)	64.472	27.630	175	KX143671	
fasciata 29	L	Mustjala, Moeldrioja (Estonia)	58.490	22.303	5	KX294243	
fasciata 33		Limburg, Veurs, Voeren (Belgium)	50.749	5.814	130	KX142016	
fasciata 35	F	Tampere, Tiikonoja (Finland)	61.720	24.031	120	MT816342	MNCN:ADN 92790
fasciata 37	M	Tampere, Tiikonoja (Finland)	61.720	24.031	120	MT816344	MNCN:ADN 92792
fasciata 39	F	Tampere, Tiikonoja (Finland)	61.720	24.031	120	MT816346	MNCN:ADN 92794
fasciata 42	M	Tampere, Tiikonoja (Finland)	61.720	24.031	120	MT816349	MNCN:ADN 92797
fasciata 53	A	Hesse, Breitenbach (Germany)	50.662	9.624	280	KY261298	
fasciata 57	MP	River Kumiela, Elblag (Poland)	54.158	19.414	15	MT816350	MNCN:ADN 94323
fasciata 62	FP	River Wałsza (Poland)	54.236	20.128	70	MT816355	MNCN:ADN 94335
fasciata 64	FP	River Gizela, Gaznoty (Poland)	53.534	19.901	190	MT816357	MNCN:ADN 94341
fasciata 70	F	Bílý potok, Bohemia (Czech Republic)	50.925	14.401	330	MT816363	MNCN:ADN 94392
fasciata 72	M	Hluboký potok, Bohemia (Czech Republic)	50.236	12.604	540	MT816365	MNCN:ADN 94394
fasciata 73	F	Hluboký potok, Bohemia (Czech Republic)	50.236	12.604	540	MT816366	MNCN:ADN 94395

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TABLE 1. (Continued)

Species	Sex	Locality	N	E	Alt	GenBank	MNCN Collection
fasciata 74	M	Brook W Nové Strašecí (Czech Republic)	50.145	13.827	430	MT816367	MNCN:ADN 94396
fasciata 78	F	Tributary of Bystrá (Slovakia)	49.560	19.510	1200	MT816371	MNCN:ADN 94404
fasciata 82	M	River Bosna (Bosnia and Herzegovina)	44.550	18.105	170	MT559341	MNCN:ADN 109896
fasciata 83	F	River Bosna (Bosnia and Herzegovina)	44.550	18.105	170	MT559342	MNCN:ADN 109897
fasciata 84	F	River Bosna (Bosnia and Herzegovina)	44.550	18.105	170	MT559343	MNCN:ADN 109898
fasciata 86	L	Bruchanicha str., Ryjiy Ovrag, (Russia)	56.036	36.440	270	MT816372	MNCN:ADN 109948
fasciata 87	L	Bruchanicha str., Ryjiy Ovrag, (Russia)	56.036	36.440	270	MT816373	MNCN:ADN 109949
fasciata 88	L	Stream near Volgusha river (Russia)	56.242	37.405	150	MT816374	MNCN:ADN 109952
fasciata 89	L	Stream Medvejiy, Murmansk (Russia)	67.136	32.702	150	MT816375	MNCN:ADN 109954
fasciata 92	L	Spring brook near Moskva river (Russia)	55.627	36.386	170	MT816378	MNCN:ADN 109957
fasciata 94	F	Kuhmo, Viiksimonjoki (Finland)	64.311	30.299	195	MT816380	MNCN:ADN 110210
fasciata 99	L	Kuusamo, Uopajanpuro brook (Finland)	66.339	29.520	180	MT816385	MNCN:ADN 110216
fasciata 105	M	River Trnovska (Republic of North Macedonia)	42.258	22.361	820	MW703665	
fasciata 106	M	River Kriva (Republic of North Macedonia)	42.231	22.363	710	MW703664	
fasciata 107	M	River Kamenica (Serbia)	44.048	20.045	540	MW703663	MNCN:ADN 110312
fasciata 108	M	Samakové (Kosovo)	42.246	21.348	630	MZ645085	MNCN:ADN 110377
fasciata 109	M	Kamenicë municipality (Kosovo)	42.658	21.733	690	MZ645086	MNCN:ADN 110380
fasciata 111	F	Jastrebac (Serbia)	43.416	21.377	630	MZ645088	MNCN:ADN 110387
fasciata 120	M	Samakovë (Kosovo)	42.246	21.348	630	MZ645097	MNCN:ADN 110438
fasciata 121	M	Dubovë (Kosovo)	42.720	20.356	500	MZ645098	MNCN:ADN 110439
fasciata 122	M	Mjak (Kosovo)	42.259	21.343	620	MZ645099	MNCN:ADN 110440
fasciata 123	M	Kamenicë municipality (Kosovo)	42.624	21.687	615	MZ645100	MNCN:ADN 110441
fasciata 125	F	Jastrebac (Serbia)	43.416	21.377	630	OM666592	MNCN:ADN 110669
septentrionis 1	M	Nothumberland National Park (United Kingdom)	55.317	-2.137	240	KY225476	
septentrionis 2	FP	Afon Marsiandwr (United Kingdom)	53.290	-3.241	110	MW703657	MNCN:ADN 110304
f. delici G1	M	River Gacka, Otočac (Croatia)	44.787	15.368	475	MT559345	MNCN:ADN 96483
f. delici V1	F	Vukovića spring, river Cetina (Croatia)	43.965	16.413	375	MT559347	MNCN:ADN 96485
f. delici V2	M	Vukovića spring, river Cetina (Croatia)	43.965	16.413	375	MT559348	MNCN:ADN 96486
f. delici D1	F	River Dretulja, spring (Croatia)	45.074	15.343	390	MT559349	MNCN:ADN 96489
f. delici D2	M	River Dretulja, spring (Croatia)	45.074	15.343	390	MT559350	MNCN:ADN 96490
f. delici C1	M	River Čabranka, spring (Croatia)	45.601	14.640	560	MT559351	MNCN:ADN 96491
f. delici C2	M	River Čabranka, spring (Croatia)	45.601	14.640	560	MT559352	MNCN:ADN 96492
f. delici C4	M	River Čabranka, spring (Croatia)	45.601	14.640	560	MT559354	MNCN:ADN 103407
f. delici BI1	M	River Bijela, NP Plitvice Lakes (Croatia)	44.833	15.557	750	MT559355	MNCN:ADN 103408

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TABLE 1. (Continued)

Species	Sex	Locality	N	E	Alt	GenBank	MNCN Collection
f. delici R1	F	Roški slap (waterfall), National Park Krka (Croatia)	43.906	15.975	80	MT559356	MNCN:ADN 103409
f. delici R2	M	Roški slap (waterfall), National Park Krka (Croatia)	43.906	15.975	80	MT559357	MNCN:ADN 103410
f. delici BR1	F	Brkljača channel, Udovičić (Croatia)	43.656	16.732	290	MT559358	MNCN:ADN 103411
f. delici CT1	M	River Cetina, tributary, Civljani (Croatia)	43.948	16.401	375	MT559359	MNCN:ADN 103412
f. delici BT1	F	River Butišnica, Golubić (Croatia)	44.111	16.227	350	MT559360	MNCN:ADN 103413
f. delici BU1	L	Brušane (Gospić) (Croatia)	44.505	15.236	620	MT559361	MNCN:ADN 109891
f. delici B1	L	River Bosna, Vrelo Bosne (Bosnia and Herzegovina)	43.816	18.273	560	MT772015	
f. delici B2	L	River Bosna, Vrelo Bosne (Bosnia and Herzegovina)	43.816	18.273	560	MT772017	
f. delici M1	L	River Miljacka, Dariva (Bosnia and Herzegovina)	43.858	18.442	580	MT765286	
f. delici M2	L	River Miljacka, Dariva (Bosnia and Herzegovina)	43.858	18.442	580	MT765287	
f. delici M4	L	River Miljacka, Dariva (Bosnia and Herzegovina)	43.858	18.442	580	MT772020	
f. delici RK	M	River Raska (Serbia)	43.133	20.435	560	MW703662	MNCN:ADN 110309
denticulata 1	M	River Balameth, Bethmale (France)	42.865	1.092	1000	MF347380	MNCN:ADN 49487
denticulata 2	F	River Balameth, Bethmale (France)	42.865	1.092	1000	MF347381	MNCN:ADN 49488
denticulata 3	M	River Balameth, Bethmale (France)	42.865	1.092	1000	MF683825	MNCN:ADN 96497
kykladica 1	M	Platanistos, South Euboea (Greece)	38.013	24.511	214	MK422501	MNCN:ADN 94016
kykladica 2	M	Platanistos, South Euboea (Greece)	38.013	24.511	214	MK422502	MNCN:ADN 94017
kykladica 3	L	Platanistos, South Euboea (Greece)	38.013	24.511	214	MK422503	MNCN:ADN 94018
kykladica 8	MP	Platanistos, South Euboea (Greece)	38.013	24.511	214	MK422508	MNCN:ADN 94428
sociata 1	M	River Oja, La Rioja (Spain)	42.547	-2.915	490	MF347382	MNCN:ADN 57431
sociata 2	F	River Oja, La Rioja (Spain)	42.547	-2.915	490	MF347383	MNCN:ADN 57433
sociata 6	F	River Oja, La Rioja (Spain)	42.547	-2.915	490	MF347387	MNCN:ADN 57442
sociata 9	M	River Oja, La Rioja (Spain)	42.953	-2.915	490	MF347390	MNCN:ADN 57445
sociata 10	M	River Oja, La Rioja (Spain)	42.953	-2.915	490	MF347391	MNCN:ADN 57434
sociata 11	M	River Lez, Audressein (France)	42.930	1.023	500	MF347392	MNCN:ADN 49486
sociata 12	M	River Aude, Languedoc-Roussillon (France)	42.954	2.449	500	KX141525	
sociata 14	M	River Cares, Asturias (Spain)	43.193	-4.908	570	MF347393	MNCN:ADN 92806
sociata 15	M	River Cares, Asturias (Spain)	43.193	-4.908	570	MF347394	MNCN:ADN 92807
sociata 16	F	River Correc de la Sogueda, Sorède (France)	42.500	2.958	390	MF683826	MNCN:ADN 96499
f. viteceki N1	M	River Neretva, Bačevići (Bosnia and Herzegovina)	43.254	17.825	35	MT559365	MNCN:ADN 96493
f. viteceki B1	M	River Bunica (Bosnia and Herzegovina)	43.243	17.855	30	MT559368	MNCN:ADN 96496
f. viteceki S1	M	River Sturba, Livno (Bosnia and Herzegovina)	43.762	17.030	755	MT559362	MNCN:ADN 94430
cf. obliterata 1	FP	Kudowski Potok stream (Poland)	50.482	16.337	840	MT816391	MNCN:ADN 94336

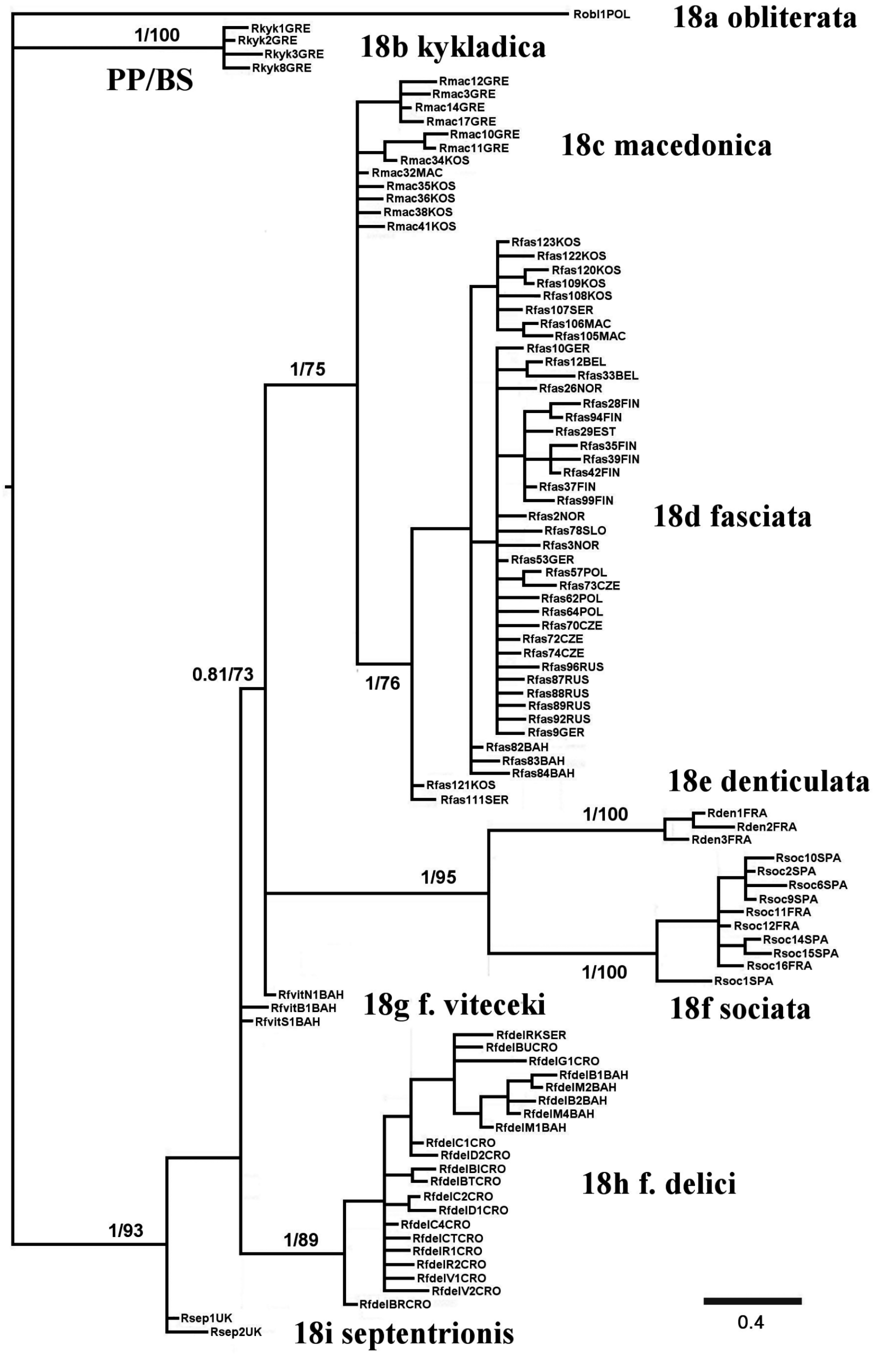

FIGURE 18. Phylogenetic relationship among the species of the *Rhyacophila fasciata* Group included in this study. Combination of bayesian phylogenetic and maximum likelihood trees based on COI. Support for each node is represented by the posterior probabilities (PP) resulting from the Bayesian inference analysis and the bootstrap support values (BS) obtained for the maximum likelihood tree (PP/BS). *Rhyacophila cf. obliterata* (Robl) (18a), outgroup; Rhyacophila *fasciata* Group: *Rhyacophila kykladica* Malicky & Sipahiler 1993 (Rkyk) (18b), *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**) (Rmac) (18c), *Rhyacophila fasciata* Hagen 1859 (Rfas) (18d), *Rhyacophila denticulata* McLachlan 1879 (Rden) (18e), *Rhyacophila sociata* Navás 1916 (Rsoc) (18f), *Rhyacophila fasciata viteceki* Valladolid & Kučinić 2020 (in Valladolid *et al.* 2020) (Rfvit) (18g), *Rhyacophila fasciata delici* Kučinić & Valladolid (in Valladolid *et al.* 2020) (Rfdel) (18h), *Rhyacophila septentrionis* McLachlan 1865 (Rsep) (18i). Data for specimens are summarized in Table 1. Countries: BEL = Belgium, BAH = Bosnia and Herzegovina, CRO = Croatia, CZE = Czech Republic, EST = Estonia, FIN = Finland, FRA = France, GER = Germany, GRE = Greece, KOS = Kosovo, MAC = North Macedonia, NOR = Norway, POL = Poland, RUS = Russia, SER = Serbia, SLO = Slovakia, SPA = Spain, UK = United Kingdom. Scale bar: mean number of nucleotide substitutions per site or nucleotide position on the respective branch.

TABLE 2. Genetic distances (maximum composite likelihood model). 2a: intraspecific distances. *R. cf. obliterata* = not computed (1 specimen only). 2b: interspecific distances. In **bold**: distance between *Rhyacophila macedonica* and the species/subspecies of the *Rhyacophila fasciata* Group.

TABLE 2A

	Intraspecific
R. f. delici	0.0086
R. denticulata	0.0053
R. fasciata	0.0070
R. kykladica	0.0030
R. macedonica	0.0053
R. septentrionis	0.0012
R. sociata	0.0057
R. f. viteceki	0.0026
R. cf. obliterata	n/c

TABLE 2B

Interspecific	*R. f. del.*	*R. dent.*	*R. fas.*	*R. kyk.*	***R. mac.***	*R sep.*	*R. soc.*	*R. f. vit.*
R. denticulata	0.0580							
R. fasciata	0.0433	0.0621						
R. kykladica	0.0553	0.0674	0.0671					
R. macedonica	**0.0319**	**0.0541**	**0.0173**	**0.0551**				
R. septentrionis	0.0260	0.0519	0.0297	0.0437	**0.0206**			
R. sociata	0.0622	0.0431	0.0686	0.0795	**0.0594**	0.0559		
R. f. viteceki	0.0200	0.0476	0.0248	0.0515	**0.0169**	0.0103	0.0488	
R. cf. obliterata	0.1061	0.1035	0.1021	0.0957	**0.0976**	0.0854	0.1155	0.0881

Discussion

The use of the combination of selected characters in males (e.g., inferior appendages, dorsal lobes, parameres, and aedeagus) and females (shape of the sclerotized lateral valves of segment VIII in dorsal, ventral and lateral views) has allowed the description of new subspecies of the *R. fasciata* Group (Valladolid *et al.* 2019), the resurrection of species previously considered synonyms of *R. fasciata* (Valladolid *et al.* 2018, 2021), and changes of status from subspecies to species (Valladolid *et al.* 2018, 2019). In this study, these characters show the presence of a new species of this group, *Rhyacophila macedonica* **n. sp.**

Mitochondrial DNA (mtDNA) sequences can be useful as a first indicator in species delineation, and, combined with careful morphological, behavioural, distributional, and ecological analyses, can help to establish accurate species boundaries (Bickford *et al.* 2007; Dasmahapatra & Mallet 2006; Galtier *et al.* 2009). Most recent studies show the importance of mtDNA in the creation of new species through mitochondrial-nuclear (mitonuclear) coevolution. Among the mitochondrial DNA, the nucleotide sequence of the gene cytochrome c oxidase subunit 1 has been established as a highly effective DNA barcode for diagnosing the species boundaries of animals (Bucklin *et al.* 2011; Dasmahapatra & Mallet 2006; Hebert *et al*. 2003b; Hill 2016). In a majority of metazoan populations, there is a correspondence between the species designated by COI and species previously described morphologically (Tavares & Baker 2008; Tavares *et al.* 2011) and discovery of barcode gaps often leads to the recognition of new species upon further study (Bickford *et al.* 2007; Hebert *et al.* 2004; Hill, 2016).

The corresponding differences observed at genetic and morphological levels corroborate the hypothesis that *R. fasciata* and *R. macedonica* are two different species: The intraspecific distances were always less than 1% divergence, and interspecific distances, although lower than in other pairwise comparisons, were greater than 1% (Table 2). This result is similar to what we found in specimens of *R. denticulata* and *R. sociata* (Valladolid *et. al.*

2018), *R. kykladica* (Valladolid *et al.* 2019), or *R. f. delici* and *R. f. viteceki* (Valladolid *et al.* 2020). Other authors that have analysed the divergence of COI sequences among different species of several taxonomic groups found that more than 98% of the species pairs show divergences greater than 2% (e.g., Hebert *et al*. 2003b), and that the intraspecific divergences usually are less than 1% (Avise 2000). We also agree with the premise of Hebert *et al.* (2003a) that we can find evidence of distinct species by their genetic divergences from known species assemblages. In this case, the species was identified originally as *R. fasciata*. In future studies we intend to prepare Cytochrome b gene sequences for these specimens. This gene, used extensively and particularly for vertebrates, has enabled researchers to resolve relationships among closely related taxa (Tauzt *et al*. 2003), and combined with the COI findings could lead us to a better knowledge of this group of Trichoptera in Europe.

By now, our successive studies of the *Rhyacophila fasciata* Group have been increasing the number of sequences (haplotypes), providing more information about the relation among the species previously described and the possible new species, mainly in those more related due to the existence of a recent common ancestor. Our new findings show that the taxon previously described as *R. f. delici* is in fact a distinct species (Fig. 18h), so we propose to change the status of *Rhyacophila fasciata* ssp. *delici* to *Rhyacophila delici* (*status promotus*).

In this study we found six localities in Kosovo and one in Serbia where *R. fasciata* and *R. macedonica* have been collected together at the same time, demonstrating sympatry and synchrony, evidence of their independent breeding and implying that they are genetically distinct species.

So, based on our morphological and distributional evidence, we propose the creation of a new species: *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi, **n. sp.** from Greece, North Macedonia, Kosovo, and Serbia.

Distribution and ecology

Figure 19 presents the preliminary distribution pattern of *R. macedonica* **n. sp.** in parts of Greece, North Macedonia, Kosovo, and Serbia. In Greece, *R. macedonica* is confined to the northern part of the country, mainly in the Greek regions of Thrace and Macedonia. In North Macedonia, it is currently known from a single northern location near the border with Kosovo. In Kosovo, it has been found in northern, eastern, and southern mountains. Finally, in Serbia we found the species at one locality in the Jastrebac Mountains.

Rhyacophila macedonica **n. sp.** inhabits a wide range of lotic habitats, from lowland to headwater streams (30–1400 m a.s.l.) with moderate to high current speed and low to relatively warm water temperatures (mean 12°C, 6–24°C). Larvae of *R. macedonica* **n. sp.** inhabit substrates consisting of boulders, cobbles, pebbles, and gravel as well as coarse particulate organic matter (i.e., fallen leaves, twigs, etc.) from the woody riparian zone [*Platanus orientalis*, *Salix alba*, *Alnus glutinosa*, *Picea abies*, *Acer* spp., *Nerium oleander*, etc.]. Flight activity occurs usually from mid-April to November. As typical for Rhyacophilidae species, the emergence period is extended and the life history pattern is not synchronized, e.g., several larval instars are always present throughout the year. Other caddisfly species inhabiting the same habitats are as follows: *Hydropsyche incognita* Pitsch 1993a, *Hydropsyche instabilis* (Curtis 1834), *Philopotamus montanus* Donovan 1813, *Plectrocnemia geniculata* McLachlan 1871, *Rhyacophila palmeni* McLachlan 1879, and *Wormaldia subnigra* McLachlan 1865.

In the Republic of North Macedonia, *R. macedonica* **n. sp.** seems to share similar ecological preferences with *R. fasciata*. Currently, the only locality where we have found this new species is in the Banjanska Reka River, situated in the North Macedonian part of Karadak Mountain, close to Kosovo, located at an altitude of around 620 m a.s.l. and characterized by cold water flowing over rocky substrate. The shared habitat preferences between *R. macedonica* **n. sp.** and *R. fasciata*, along with the similarities in the appearance of some of their morphological characteristics may lead to misidentification of the two species. Careful use of the diagnostic characters provided here should result in accurate identifications.

In Kosovo, *R. macedonica* **n. sp.** has been found in streams and streamlets from 1.5 to 4 m width, with rich stream-bank vegetation and substrate composed of gravel, cobble, and boulder substrates, surrounded by fine sediment. The width of the riparian zone is more than 30 m and although human activities and grazing have impacted the areas around the sampling stations only minimally, those activities are considerably evident in other segments of the stream. *Rhyacophila macedonica* **n. sp.** was found in Kosovo in sympatry with *R. fasciata*, *R. loxias* Schmid 1970, *R. polonica* McLachlan 1879, *R. nubila* Zetterstedt 1840, *Psilopteryx montanus* Kumanski 1968, *Micropterna caesareica* Schmid 1959, *M. nycterobia* McLachlan 1875, *M. sequax* McLachlan 1875, *Halessus digitatus* (Schrank 1781), *Glyphotaelius pellucidus* Retzius 1783, and *Odontocerum albicorne* Scopoli 1763.

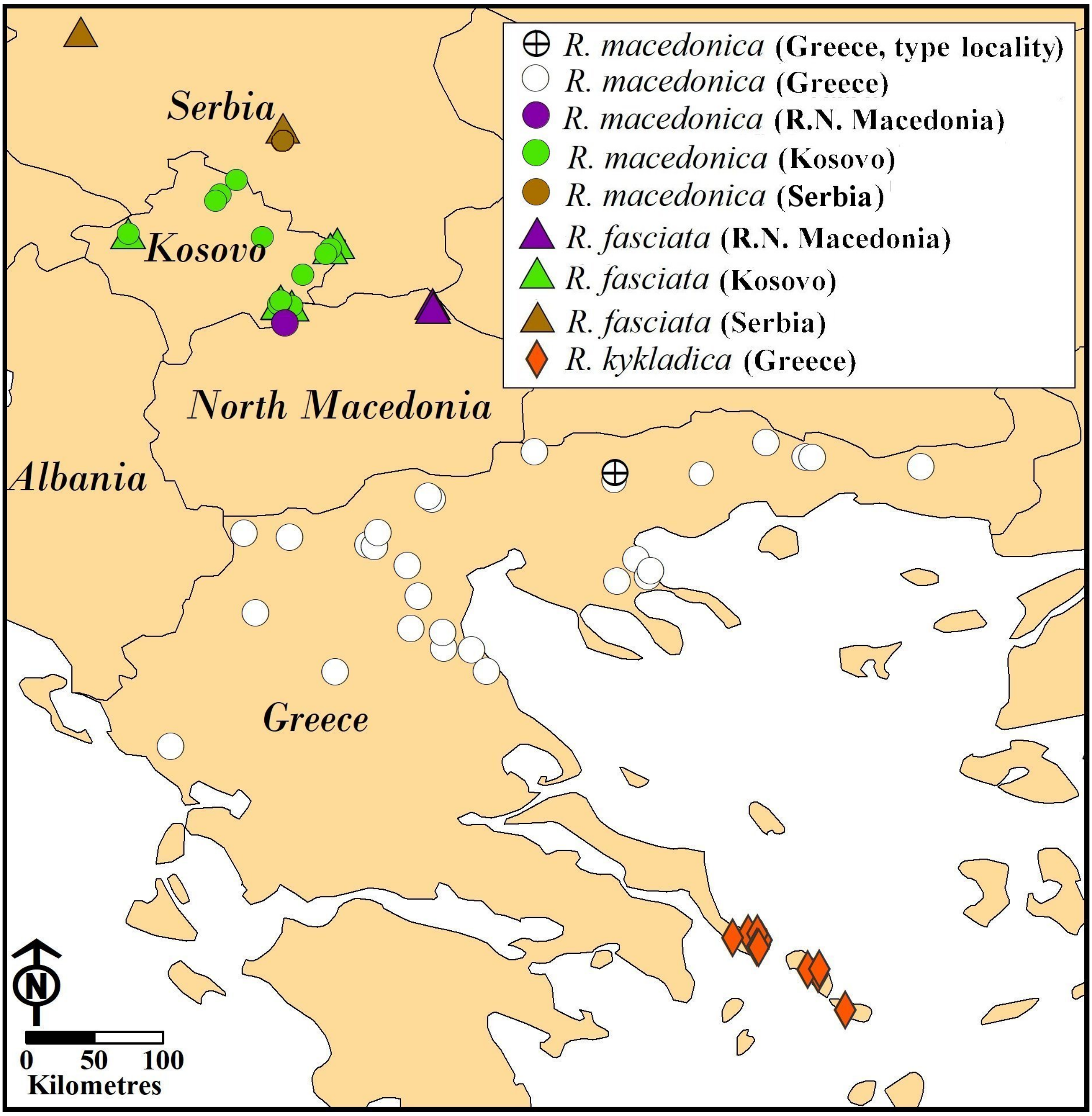

FIGURE 19. Spatial distribution of *Rhyacophila macedonica* Karaouzas, Valladolid & Ibrahimi (**n. sp.**), *Rhyacophila kykladica* Malicky & Sipahiler 1993 and *Rhyacophila fasciata* Hagen 1859 in Greece, Republic of North Macedonia, Kosovo and Serbia. Circle: localities of *R. macedonica*. Triangles: localities of *R. fasciata*. Diamond: localities of *R. kykladica*. White, orange: specimens from Greece. Green: specimens from Kosovo. Purple: specimens from Republic of North Macedonia. Brown: specimens from Serbia.

In Serbia, *Rhyacophila macedonica* was collected at one locality in the Jastrebac Mountains, near a second order stream, surrounded by densely forested vegetation. The substrate of the stream was composed by large stones and pebbles and was dominated by meso- to macrolithal substrate. Adults of this species were collected by UV light trap only and were not observed during daylight sampling with an entomological net.

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