ACTA ENTOMOLOGICA SLOVENICA

LJUBLJANA, DECEMBER 2024 Vol. 32, št. 2: 125–138

MORPHO-ANATOMICAL AND MOLECULAR INSIGHTS INTO *PTILOCEPHALA BIROI* (REBEL, 1909), AND RELATED TAXA (LEPIDOPTERA: PSYCHIDAE)

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Abstract – This study presents a comprehensive morphological and molecular analysis of *Ptilocephala biroi* (Rebel, 1909), comparing it to *P. vesubiella* (Millière, 1872), historically regarded as its closest congener. We also examine *P. biroi* in relation to *P. pyrenaella ducalis* Bertaccini, 2017, which is geographically nearest to the type locality of *P. biroi* in Croatia (Velebit). Our molecular analyses of representative populations from the southern Dinaric Alps (Biokovo) revealed a 2.35% genetic divergence among populations. Notably, both populations showed a closer genetic affinity to *P. pyrenaella* (Herrich-Schäffer, 1852) from its type locality in the Pyrenees than to *P. pyrenaella* populations from other regions.

In light of these findings, we reasses the taxonomic status of *Oreopsyche montenegrina* Gozmány, 1960, which was previously synonymized with *P. pyrenaella* by Bourgogne (1967). We propose reclassifying this taxon as *Ptilocephala biroi* ssp. *montenegrina* (Gozmány, 1960) (status revised). Additionally, we present and describe the females of *P. biroi* for the first time.

Key words: DNA barcoding, genetic divergence, taxonomic revision, biogeography, morphology, Croatia, Montenegro, Dinaric Alps

Izvleček – MORFOLOŠKO-ANATOMSKI IN MOLEKULARNI VPOGLEDI V VRSTO *PTILOCEPHALA BIROI* (REBEL, 1909) IN SORODNE TAKSONE (LEPIDOPTERA: PSYCHIDAE)
Ta študija predstavlja celovito morfološko in molekularno analizo *Ptilocephala biroi* (Rebel, 1909) ter primerjavo s *P. vesubiella* (Millière, 1872), ki je zgodovinsko veljala za njenega najbližjega sorodnika. Prav tako smo preučili *P. biroi* v povezavi s *P. pyrenaella ducalis* Bertaccini, 2017, ki je geografsko najbližja vrsta tipičnemu nahajališču *P. biroi* na Hrvaškem (Velebit). Naše molekularne analize reprezentativnih populacij iz južnega Dinarskega gorstva (Biokovo) so razkrile 2,35 % genetske razlike med populacijami. Zanimivo je, da sta obe populaciji pokazali tesnejšo genetsko sorodnost s *P. pyrenaella* (Herrich-Schäffer, 1852) iz njenega tipičnega nahajališča v Pirenejih kot s populacijami *P. pyrenaella* iz drugih regij.
Ob upoštevanju teh ugotovitev ponovno ocenjujemo taksonomski status *Oreopsyche montenegrina* Gozmány, 1960, ki je bil prej sinonimiziran s *P. pyrenaella* s strani Bourgogne (1967). Predlagamo, da se ta takson ponovno razvrsti v *Ptilocephala biroi* ssp. *montenegrina* (Gozmány, 1960) (status revidiran). Poleg tega prvič predstavljamo in opisujemo samice *P. biroi*.
Ključne besede: DNA barkodiranje, genska analiza, taksonomska revizija, biogeografija, morfologija, Hrvaška, Črna Gora, Dinaridi

Introduction

The genus *Ptilocephala* Rambur, 1858 includes approximately 24 described species from the Palaearctic region (Sobczyk, 2011; Arnscheid & Weidlich, 2017). These species are predominantly distributed on the Iberian Peninsula, in the Pyrenees, the Alps, and the Apennine Peninsula. Fewer species are known from Central Europe, the Balkans, and Anatolia (Arnscheid & Weidlich, 2021).

Ptilocephala biroi (Rebel, 1909) was first recorded as a single male specimen collected in the Velebit Mountains (Croatia), near Raduč, approximately 23 km southeast of Gospić, on July 28, 1893, by the Hungarian entomologist Lajos Biró. This specimen, now preserved in the Hungarian Natural History Museum (HNHM), was formally described by Rebel in 1909 under the name *Oreopsyche biroi*, in honor of its collector. Additional unverified findings of this species have been reported across the Balkans (Kozhanchikov, 1956; Arnscheid & Weidlich, 2017). Nevertheless, the status of this species has remained uncertain for over a century.

Until now, conducting internal morphological and genetic studies on *P. biroi* has been nearly impossible. The available material has been extremely limited, and the age of the specimens has caused significant DNA degradation. Moreover, DNA extraction and internal morphological analysis often involve partially destructive techniques, such as removing body parts, piercing the chitin, or immersing specimens in chemical solutions; methods, that are unsuitable or unfeasible for rare or type specimens.

For the first time, we now have the opportunity to carry out comprehensive morphological and, crucially, genetic comparisons using a sufficient amount of fresh

material reliably attributed to *P. biroi*. This study does not attempt to resolve the complex taxonomic issues surrounding the entire *P. pyrenaella* (Herrich-Schäffer, 1852) complex; rather, it focuses on detailed comparisons between *P. biroi*, *P. b. montenegrina* (Gozmány, 1960), and their close relatives, from both morpho-anatomical and molecular perspectives.

Materials and methods

Collecting and rearing. This work is based on material from the genus *Ptilocephala*, primarily collected by the authors, along with specimens from other private collections and the Hungarian Natural History Museum (Table 1). Most of the material was obtained by collecting young larvae, which were then reared in captivity until they reached the adult stage. The caterpillars are polyphagous and were fed a variety of herbs during their development. All procedures were conducted under external conditions to ensure that the environment closely resembled their natural habitat. Additionally, searching for larval cases proved to be an effective method for sample collection. These cases were kept in breeding boxes that were lightly moistened with water daily until the adults emerged.

Morphology and illustrations. For genitalia preparation, each specimen was boiled in 10% KOH for approximately 5–7 minutes. Photographs of the specimens were taken using a Nikon D3300 digital camera, with high-resolution images captured through a 20 mm f/2.8 D lens and an Auto Extension Tube Set DG. The images were then edited using Adobe Photoshop. All photographs are the work of the first author, except for those depicting the paratype of *O. montenegrina*, for which copyright belongs to the Hungarian Natural History Museum (Lepidoptera collection). Nomenclature follows Arnscheid & Weidlich (2017).

Molecular methods. For the DNA barcoding analyses, one or two legs were removed from each dried specimen and transferred to lysis plates. Molecular studies using mitochondrial DNA (mtDNA) and the COI 5' DNA barcode were conducted at the Canadian Centre for DNA Barcoding in Guelph, Ontario, Canada (CCDB). Sequence divergences within and between species were calculated using the Kimura 2-parameter model, as implemented in the Barcode of Life Data System (BOLD v4, www.barcodinglife.org) (Ratnasingham & Hebert, 2007).

Table 1: List of specimens used in this study, along with corresponding BOLD sequence Ids.

Species	Stadium/Sex	Gen bank Accession	Locality	Legit and collection
Ptilocephala biroi	male	BC_ZSM_Lep_117091	Croatia, Velebit, Visočica, 1470–1500 m, 6.VII.2021	leg. J. Rekelj in coll. Bertaccini
Ptilocephala biroi	male	BC_ZSM_Lep_117092	Croatia, Velebit, Visočica, 1500–1550 m, 12.VII.2021	leg. Ž. Predovnik in coll. Bertaccini
Ptilocephala biroi montenegrina	male	BC_ZSM_Lep_117093	Croatia, Biokovo, Sveti Jure, 1570–1600 m, 3–7.VII.2021	leg. J. Rekelj in coll. Bertaccini

Ptilocephala biroi montenegrina	male	BC_ZSM_Lep_117094	Croatia, Biokovo, Sveti Jure, 1550–1600 m,1.VII.2021	leg. J. Rekelj in coll. Bertaccini
Oreopsyche montenegrina Holotype	male		Montenegro, Durmitor,Jakšiča Katuni, 1800m,ex l. 21.VII.1958	leg. Dr. Gozmány in coll. HNHM
Ptilocephala vesubiella	male	BC_ZSM_Lep_81548	Italy: Liguria, Colla Melosa, (IM), 1800 m, 01.VIII.2013	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella falsevocata	male	BC_ZSM_Lep_84810	Italy: Piemonte, Laghi Clot Foiron (TO), 2140 m, ex l. 25.VII.2014	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella ducalis	male	BC_ZSM_Lep_59255	Italy: Appennino Tosco-Emiliano, Campolino (PT), 1828 m, 29.VII.2016	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella ducalis	male	BC_ZSM_Lep_add_ 00208	Italy: Appennino Tosco-Emiliano, dint. Passo della Vecchia (PT-MO), 1800 m, ex o. 11.V.2016	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella ducalis	larva	BC_ZSM_Lep_91965	Italy: Emilia, dint. Passo della Vecchia (MO), 1800 m, 21.VII.2015	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella ducalis	male	BC_ZSM_Lep_59256	Italy: Appennino Tosco-Emiliano: dint. Passo della Vecchia (PT-MO), 1800 m, ex o. 26.V.2016	leg. E. Bertaccini in coll. Bertaccini
Ptilocephala pyrenaella	male	CWA BC0025	Spain: Lerida, Porto del Canto, 1730 m, 24.VI.2012	leg. W.R. Arnscheid in coll. Arnscheid
Ptilocephala pyrenaella	male	CWA BC0024	Spain: Girona, Nuria, 1900m, 28.VI.2012	leg. W. R. Arnscheid in coll. Arnscheid

Abbreviations

BIN	barcode index number
BOLD	barcode of life data system
COI	Cytochrome C Oxidase subunit I
coll.	collection
DNA	deoxyribonucleic acid
ex l.	ex larva (from the larva)
ex o.	ex ovo (from the egg)
leg.	legit (si.) or legerunt (pl.) from legere in Latin (to collect)
HNHM	Hungarian Natural History Museum
GP	genital preparation

Results

Field observations. In 2019, Croatian lepidopterist Damir Šešok discovered a freshly attached larval case, believed to belong to an unidentified species of Psychidae, near the peak of Visočica in the Velebit mountain range. This discovery renewed interest in *Ptilocephala biroi*, and three years later, new insights into the species' biology and morphology were published based on field observations in Croatia (Predovnik & Rekelj, 2022). Around the same time, additional investigations were conducted in other areas of the Balkan mountains. In 2019, the third author, Ž. Predovnik, found an old male larval case and two old female cases on the wall of a mountain lodge just below the peak of Sveti Jure in Biokovo Nature Park. Subsequent entomological expeditions, carried out in collaboration with the fourth author, J. Rekelj, resulted in the collection of new material in early larval stages, which were successfully reared to adults.

The breeding of both populations (from Visočica and Sveti Jure) in the same season did not reveal significant differences in the behavior of the larvae, despite identical rearing conditions. The larvae are polyphagous; in captivity, both populations were fed with the same food, and they showed a preference for *Trifolium pratense* L., *Taraxacum officinale* Web., and *Plantago lanceolata* L. without noticeable differences.

The only significant difference observed was in the emergence of the adults. The population from Sveti Jure began emerging a week later than the population from Visočica, despite identical conditions. This suggests that these are genotypes adapted to their specific environments and represent genetically fixed ecotypes. This adaptation is justified and further supported by our field observations, which noted that the Biokovo locality is cooler and approximately 100 meters higher in elevation than Visočica. Such adaptation allows the adults to be on the wing later in the summer, when temperatures are more stable.

Genetics. A Neighbor Joining tree is provided for barcodes of *P. biroi* and *P. biroi montenegrina*, together with barcodes of the other related European species *P. pyrenaella*, *P. pyrenaella falsevocata* (Bourgogne, 1979), *P. pyrenaella ducalis* Bertaccini, 2017 and *P. vesubiella* (Millière, 1872), (Fig. 1). Sequences of all 12 specimens used in this study (Table 1) formed six different BINs. Each species or subspecies has its own BIN and is recognizable by its DNA barcode.

Prior to this study, *P. biroi* was considered a closely related species to *P. vesubiella*, as argued by the most authoritative specialists in this field (Rebel, 1909; Kozhanchikov I.V., 1956; Bourgogne, 1967; Arnscheid & Weidlich, 2017). This analysis reveals highly divergent evolutionary lines between *P. biroi* and its two congeners, *P. vesubiella* (with a pairwise distance of 9.77%) and *P. pyrenaella ducalis* (with a pairwise distance of 8.77%).

Comparison *P. biroi* with other species revealed the closest evolutionary lines to *P. pyrenaella*, showing pairwise distances ranging from 4.53% to 4.69%. Additionally, the phenotypes of ssp. *montenegrina* exhibited even more modest distances, falling between 3.46% and 3.84%. While we did not observe major differ-

ences in the morpho-anatomical aspect between the two entities (*biroi* - *montenegrina*), the two populations still exhibit distinct molecular characteristics (minimum pairwise distance = 2.35%), prompting a change in the original taxonomic status. Therefore, *Oreopsyche montenegrina* Gozmány, 1960 which was downgraded to synonymy of *Ptilocephala pyrenaella* by Bourgogne (1967) is now revived at subspecies rank: ***Ptilocephala biroi montenegrina* (Gozmány, 1960), stat. rev.**

Table 2: Matrix of pairwise distances in the *P. biroi* - *montenegrina* complex and its closely related congeners, based on the CO1 gene barcode region and computed using the Kimura 2-parameter method.

No	Sample	1	2
1	*P. biroi* (BC_ZSM_Lep_117091)		
2	*P. biroi montenegrina* (BC_ZSM_Lep_117093)	2,35	
3	*P. pyrenaella* (CWA BC0025)	4,69	3,46
4	*P. pyrenaella* (CWA BC0024)	4,53	3,84
5	*P. pyrenaella ducalis* (BC_ZSM_Lep_59255)	8,77	8,19
6	*P. pyrenaella falsevocata* (BC_ZSM_Lep_84810)	8,21	7,29
7	*P. vesubiella* (BC_ZSM_Lep_81548)	9,77	9,17

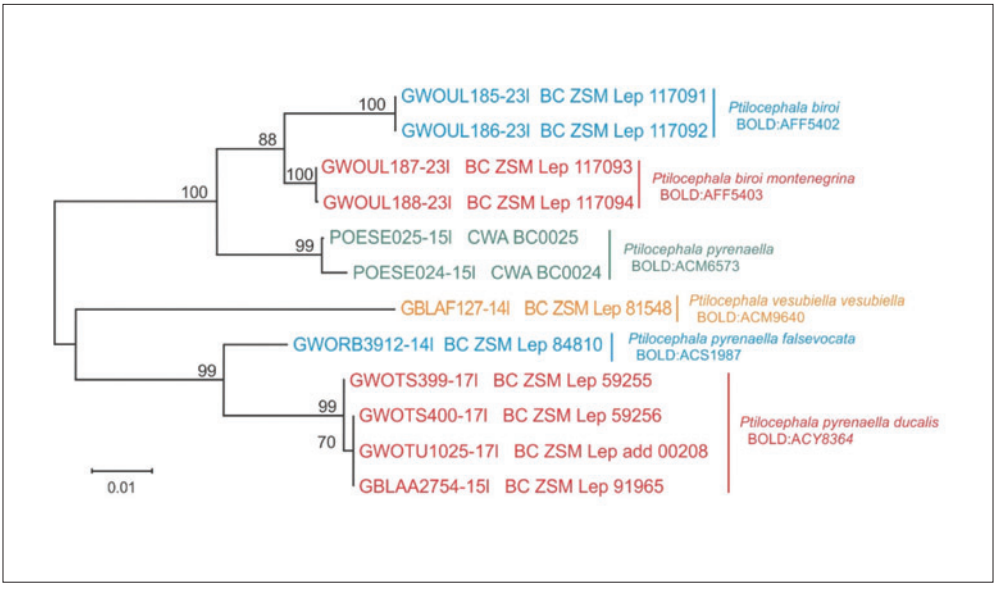

Fig. 1: A neighbor Joining tree of *Ptilocephala biroi* and selected closely related species. Samples are submitted with sequence ID (BOLD), scientific name and barcode index number (BIN). Numbers at the branches represents bootstrap percentages, scale bar represents 1% K2P divergence between sequences.

Morphology. Males. In the table below the actual values of morphological characteristics are presented, observed in various taxa under examination. In Gozmány's (1960) original description, it was noted that the wingspan of males falls within the range of 19–22 mm, while the holotype, which is currently preserved at the HNHM, measures approximately 18 mm.

Table 3: A comparison of morphological characteristics of *biroi-montenegrina-vesubiella* complex.

Complex: ***biroi-montenegrina-vesubiella***					
Male	***biroi*** Croatia, Velebit	***montenegrina*** Montenegro, Durmitor	***montenegrina*** Croatia, Biokovo	***vesubiella*** Italy, Liguria	***ducalis*** Italy, Emilia
Wingspan (objective data)	17-20 mm	19-22 mm 18 (holotype)	17-20 mm	19-27 mm	16-19 mm
Antennalsegments	25-27	26	25-27	26-30	25-27
Larval case ♂ ♀	12-21 16-21mm 12-19 mm	24-28 mm 21mm	17-21 mm	15-22 16-22mm 15-17 mm	12-18 14-18mm 12-16 mm

Females. Females of the nominate species, as well as subspecies, have not yet been described. Only one source mentions females, stating that despite extensive efforts, no females were found among the larger numbers of male larval cases (Vasić et al. 1990). All female members of the genus *Ptilocephala* are very similar in appearance, they are apterous, antennae and legs are fully reduced. Here we provide the first description of the females of *P. biroi* and its subspecies *P. biroi montenegrina* stat. rev.:

Ptilocephala biroi (Rebel, 1909)

Material: Croatia, Srednji Velebit, Visočica, 1500–1550 m: 9♀♀ with larval cases, leg. Ž. Predovnik, 13.VI.2020 (ex l. 14.VI.–12.VII.2021), coll. Ž. Predovnik; 16♀♀ with larval cases, leg. J. Rekelj, 13.VI.2020 (ex l. 14.VI.–12.VII.2021), 13♀♀ coll. J. Rekelj, 3♀♀ coll. Bertaccini. 4♀♀ with larval cases, leg. J. Rekelj, 6.VII.2021, 2♀♀ coll. J. Rekelj, 2♀♀ coll. Bertaccini.

Description of female: Specimens measure 11–12 mm in length and have a diameter of 5–6 mm, with these measurements taken after they were freed from their rigid pupal envelopes (Fig. 18). Head small, shiny, turned below the thoracic segments, directed posteriorly. Rudiments of antennae absent, the eye rudiments appear as two small but clearly visible black dots. Wingless, rudiments of legs absent. The abdomen has a very light yellowish color, both thoracic and abdominal segments covered with dense, short hairs. The thoracic segments strongly sclerotized dorsally and laterally, giving them a shining appearance. Boundaries between segments indis-

tinct. The ovipositor everted. The pupa exhibits a semicircle of rigid spines in the anal area on the dorsal side (Figs. 14, 16).

Ptilocephala biroi montenegrina (Gozmány, 1960)

Material: Croatia, Biokovo, Sv. Jure, 1550m, 9♀♀ with larval cases, leg. J. Rekelj, 14.VI.2020 (ex l. 3–7.VII.2021, 20–22.VIII.2021), 5♀♀ coll. J. Rekelj, 4♀♀ coll. Bertaccini.

Description of female: Specimens measure 10–11 mm in length with a diameter of 4–5 mm (Fig. 19). Other characters are similar to the nominate species. The pupa bears a sinuous line of stiff spines in the anal area on the dorsal side (Figs. 15, 17).

Discussion

This study illustrates the evolutionary relationships and taxonomic status of *Ptilocephala biroi* and its subspecies *montenegrina*. Through DNA barcoding and morphological analyses, we could conclude that *biroi*, along with its subspecies, is more closely related to *pyrenaella* than previously recognized. The observed genetic distances 4.53% between *biroi* and *pyrenaella*, and 3.46% between *montenegrina* and *pyrenaella* suggest that these species share a more recent common ancestor than *biroi* does with *vesubiella*, which exhibits a greater pairwise distance of 9.77%. These findings raise important questions about the historical biogeography of these species. The proximity of the Dinaric Mountains to the Pyrenees suggests potential historical connectivity, possibly during postglacial periods, which could explain the close evolutionary ties between *biroi* and *pyrenaella*.

Historically, the classification of these taxa has relied heavily on morphological characteristics, a common practice in taxonomy. However, the results of this study emphasize that speciation is not always accompanied by significant morphological changes. The lack of major morphological differences between *P. biroi* and *montenegrina* suggests that traditional methods of species delineation may overlook cryptic diversity. This finding aligns with recent discussions in taxonomy, which argue that the true number of biological species is likely greater than currently recognized, primarily due to the reliance on morphological traits that may not accurately reflect genetic divergence (Weidlich and Arnscheid, 2021). In all definitions of subspecies, there is unanimous agreement that subspecies are geographically defined and diagnosable by at least one presumably heritable character. This is clearly evident in our case. The genetic divergence observed between the Velebit and Biokovo populations, with a minimum pairwise distance of 2.35%, indicates that even geographically proximate populations can exhibit distinct molecular characteristics. Differences in the timing of adult emergence further support the hypothesis that a genetically fixed ecotype can become a subspecies if sufficiently pronounced genetic and phenotypic differences develop between it and other populations of the same species. A subspecies is typically defined by stable characteristics inherited by individuals within that group, which may be adaptations to specific environmental conditions. If this ecotype reproduces separately from others and maintains its unique

traits over multiple generations, it can be formally classified as a subspecies. In the future, it would be intriguing to identify and rear other populations for behavioral comparison. This molecular differentiation, reproductive isolation, and specific environmental adaptations, despite minimal morpho-anatomical differences support the recognition of *montenegrina* as a distinct subspecies, thereby reinstating its taxonomic status after previous synonymizing.

Furthermore, our study highlights the unexpected genetic diversity present within the *Ptilocephala* complex, raising concerns about potential regional and taxonomic biases in current diversity estimates. The pronounced genetic divergence observed in geographically distant populations such as those from the Dinaric Alps and the Pyrenees invites further investigation into the evolutionary processes at play. These findings resonate with the insights of Bickford et al. (2007), who noted that our understanding of biodiversity is often clouded by assumptions based on morphological criteria.

Acknowledgements

We would like to extend our heartfelt gratitude to all those who supported us by providing valuable data and borrowing essential materials. A special note of thanks goes to Dr. Wilfried R. Arnscheid from Bochum, Germany, for his valuable advice and for granting us access to some molecular data. We sincerely appreciate the invaluable assistance we received from Zsolt Bálint, Balázs Tóth, and Gergely Katona from the HNHM. Furthermore, we are grateful to the Trustees of the Museum for granting us permission to feature the images of the type specimen of *Oreopsyche montenegrina* from the HNHM. Our deepest gratitude goes to the "Centre for Biodiversity Genomics, University of Guelph" and the individuals Paul Hebert, Evgeny Zakharov, and Sujeevan Ratnasingham for their exceptional work in sequencing the DNA of the samples. Finally, we would like to express our sincere gratitude to Stanislav Gomboc for commenting on the manuscript, Prof. Dr. Radovan Kranjčev from Koprivnica, Croatia, as well as the park administration, for their invaluable assistance in obtaining the research permit for Biokovo Nature Park.

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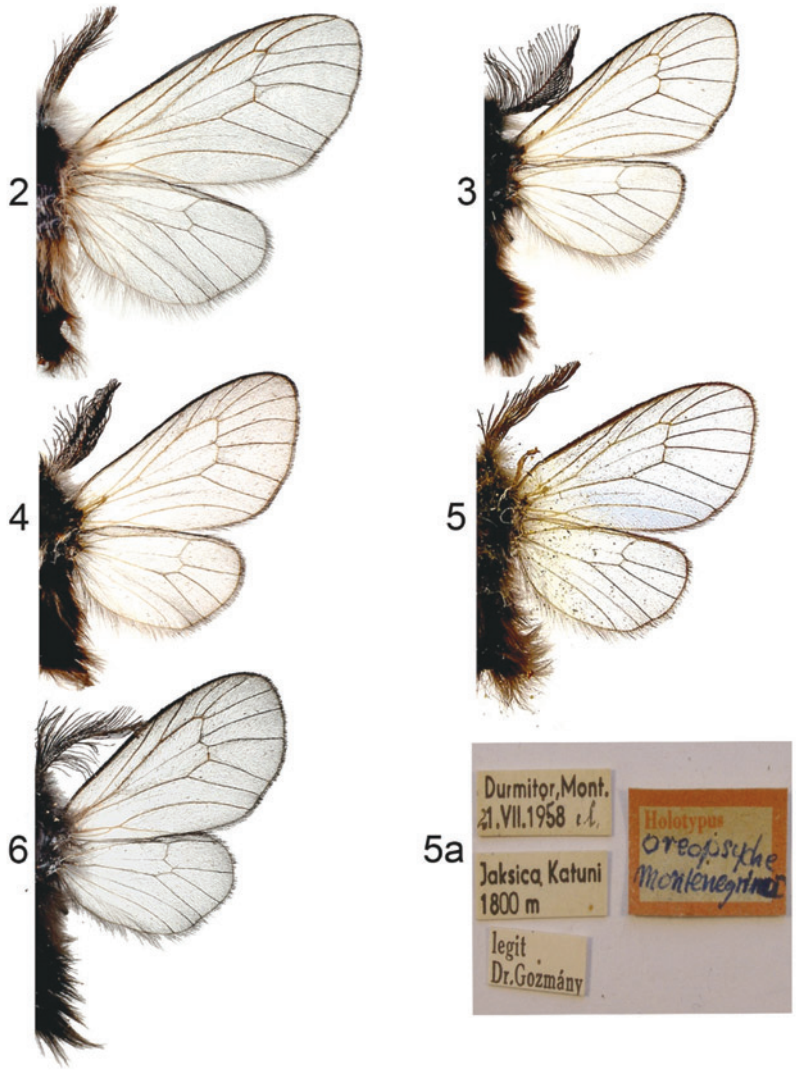
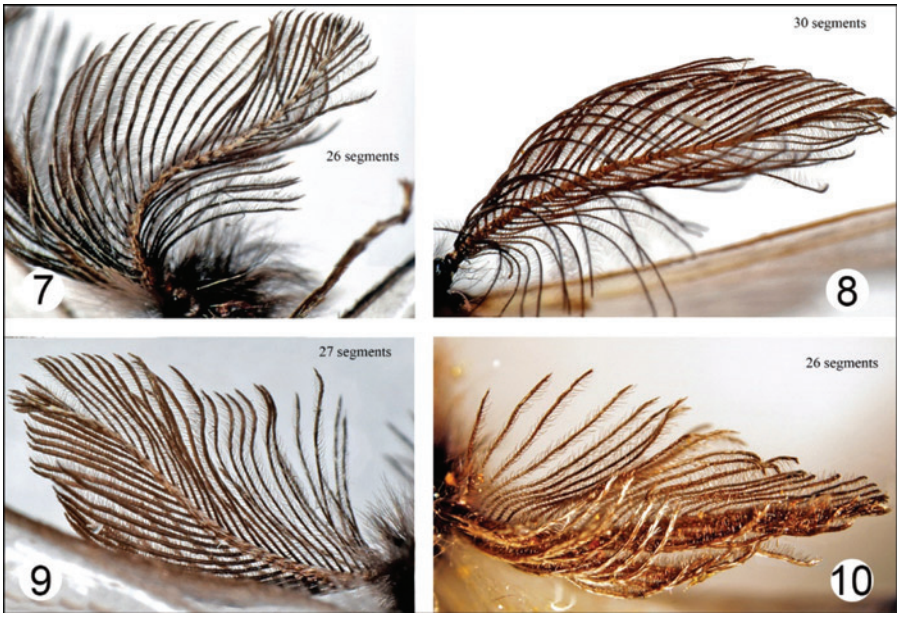
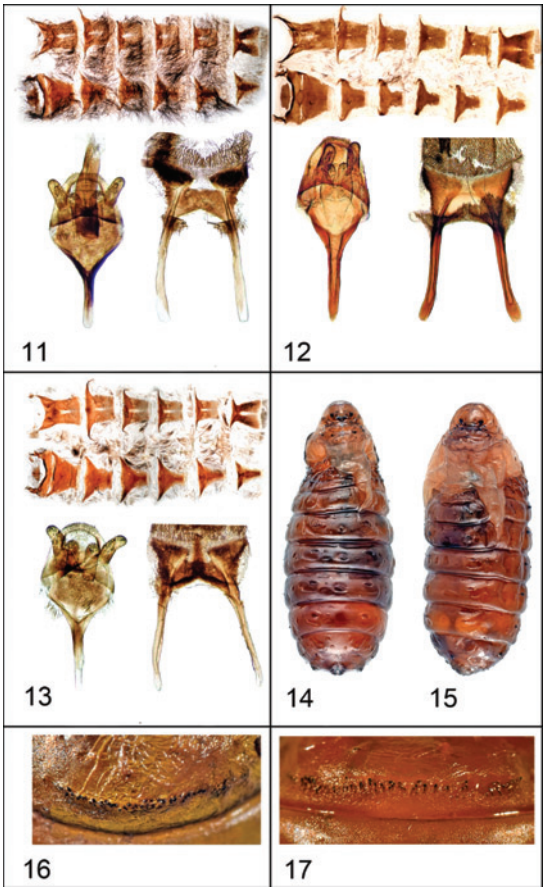


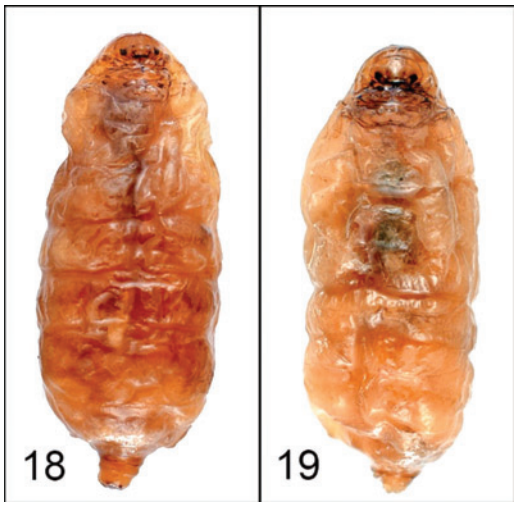
Figs. 2-6: *Ptilocephala* spp., wing venation in males: 2): *Ptilocephala vesubiella*, Italy, Liguria, Colla Melosa (IM), 1650m, 10.VI.2008, ex o., leg. E. Bertaccini in coll. Bertaccini; 3): *P. biroi*, Croatia, Velebit, Visočica, 1470–1500m, 06.VII.2021, leg. J. Rekelj in coll. Bertaccini; 4): *P. biroi montenegrina*, Croatia, Biokovo, Sv. Jure, 1550m, 10.VII.2021, ex l., leg. J. Rekelj in coll. Bertaccini; 5): *P. biroi montenegrina* (*Oreopsyche montenegrina* (Holotypus, HNHM)); 5a): Original labels of *P. biroi montenegrina* (HNHM); 6): *P. pyrenaella ducalis*, Appennino Tosco-Emiliano, dint. Passo della Vecchia (PT MO), 1800 m, 11.V.2016, ex o., leg. E. Bertaccini in coll. Bertaccini.



Figs. 7-10: *Ptilocephala* spp., antennae of males: 7): *Ptilocephala biroi*, Croatia, Velebit, Visočica, 1470–1500m, 06.VII.2021, leg. J. Rekelj in coll. Bertaccini; 8): *P. vesubiella*, Italy, Liguria, Colla Melosa (IM), 1650m, 10.VI.2008, ex o., leg. E. Bertaccini in coll. Bertaccini; 9): *P. biroi montenegrina*, Croatia, Biokovo, Sveti Jure, 1570–1600m 3–7.VII.2021, leg. J. Rekelj in coll. Bertaccini; 10): *P. biroi montenegrina*, Durmitor (Holotypus, HNHM).



Figs. 11-17: *Ptilocephala* spp. morfological characters: 11): *Ptilocephala biroi*, male, abdominal sternites, tergites and genitalia: Croatia, Velebit, Visočica, 1500–1550m, 16.VI–12.VII.2021, ex l., leg. Ž. Predovnik in coll. Bertaccini, (GP № 1100 E. Bertaccini); 12): *P. vesubiella*, male, abdominal sternites, tergites and genitalia: Italy, Liguria, Colla Melosa (IM), 1650m, 10.VI.2008, ex o., leg. E. Bertaccini in coll. Bertaccini, (GP № 694 E. Bertaccini); 13): *P. biroi montenegrina*, male, abdominal sternites, tergites and genitalia: Croatia, Biokovo, Sv. Jure, 1550m, 10.VII.2021, ex l., leg. J. Rekelj in coll. Bertaccini. (GP № 1101 E. Bertaccini); 14): *P. biroi*, female, pupa, Croatia, Velebit, Visočica, 1470–1500m, 06.VII.2021, leg. J. Rekelj in coll. Bertaccini; 15): *P. biroi montenegrina*, female, pupa, Croatia, Biokovo, Sveti Jure, 1570–1600m, 3–7.VII.2021, leg. J. Rekelj in coll. Bertaccini; 16): *P. biroi*, female pupa, dorsal view, detail of the spines on the 7th abdominal segment, Croatia, Velebit, Visočica, 1470–1500m, 06.VII.2021, leg. J. Rekelj in coll. Bertaccini; 17): *P. biroi montenegrina*, female pupa, dorsal view, detail of the spines on the 7th abdominal segment, Croatia, Biokovo, Sveti Jure, 1570–1600m, 3–7.VII.2021, leg. J. Rekelj in coll. Bertaccini.



Figs. 18, 19: 18): *Ptilocephala biroi*, female, Croatia, Velebit, Visočica, 1470–1500m, 06.VII.2021, leg. J. Rekelj in coll. Bertaccini; 19): *P. biroi montenegrina*, female, Croatia, Biokovo, Sveti Jure, 1570–1600m, 3–7.VII.2021, leg. J. Rekelj in coll. Bertaccini.

Fig. 20: Distribution map of *Ptilocephala biroi* and *P. biroi montenegrina*.