ZooKeys 773: 1–55 (2018)
doi: 10.3897/zookeys.773.24878
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

A molecular and conchological dissection of the "scaly" *Georissa* of Malaysian Borneo (Gastropoda, Neritimorpha, Hydrocenidae)

Mohd Zacaery Khalik[1,2,3], Kasper Hendriks[1,4], Jaap J. Vermeulen[1,5], Menno Schilthuizen[1,2,6]

1 *Naturalis Biodiversity Center, Vondellaan 55, 2332 AA Leiden, The Netherlands* **2** *Institute of Biology Leiden, Faculty of Science, Leiden University, 2333 BE Leiden, The Netherlands* **3** *Department of Zoology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia* **4** *Groningen Institute for Evolutionary Life Sciences, Faculty of Mathematics and Natural Sciences, University of Groningen, 9747 AG Groningen, The Netherlands* **5** *JK Art and Science, Lauwerbes 8, 2318 AT Leiden, The Netherlands* **6** *Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia*

Corresponding author: *Mohd Zacaery Khalik* (zacaery12@gmail.com)

Academic editor: *F. Köhler* | Received 7 March 2018 | Accepted 17 May 2018 | Published 9 July 2018

http://zoobank.org/900F9307-844A-4B9A-B428-DA97FF3B4B5C

Citation: Khalik MZ, Hendriks K, Vermeulen JJ, Schilthuizen M (2018) A molecular and conchological dissection of the "scaly" *Georissa* of Malaysian Borneo (Gastropoda, Neritimorpha, Hydrocenidae). ZooKeys 773: 1–55. https://doi.org/10.3897/zookeys.773.24878

Abstract

The Bornean hydrocenids have so far been understudied compared to other non-pulmonate snails in this region. In the present study, we review a first group of minute land snail species belonging to the genus *Georissa* (Gastropoda, Hydrocenidae) from Malaysian Borneo. This group is restricted to the species with conspicuous scale-like sculpture on the shell. Based on materials from recent fieldwork, museums, and personal collections, Malaysian Borneo hydrocenids are more complex and diverse in shell characters than previously anticipated. Here, a molecular, conchological, and biogeographic study of this "scaly group" is presented. We recognise 13 species of which six are new to science, namely *Georissa anyiensis* **sp. n.**, *Georissa muluensis* **sp. n.**, *Georissa bauensis* **sp. n.**, *Georissa silaburensis* **sp. n.**, *Georissa kinabatanganensis* **sp. n.**, and *Georissa sepulutensis* **sp. n.**

Keywords

Gastropods, land snail, limestone karst, Malaysian Borneo, micro-computed tomography, Sabah, Sarawak, species delimitation

Introduction

Over the past 25 years, the microsnail fauna of karst habitats in South East Asia has enjoyed an ongoing surge of attention. Detailed conchological and molecular studies in this region have revealed high allopatric and sympatric diversity (e.g., Liew et al. 2014, Rundell 2008, Tongkerd et al. 2004), which has opened up this fauna for work in the fields of community ecology (Schilthuizen et al. 2005, Schilthuizen 2011), speciation (Schilthuizen et al. 2006, Schilthuizen et al. 2012), and conservation biology (Clements et al. 2006, Clements et al. 2008, Schilthuizen et al. 2005). Although several families of non-pulmonate snails have featured prominently in these studies (in particular the Diplommatinidae and other cyclophoroids), the family Hydrocenidae (Neritimorpha) has so far been understudied. In this paper, we make a start with a first conchological and molecular characterisation of a surprisingly diverse group of species in the genus *Georissa* Blanford, 1864.

The genus *Georissa* Blanford, 1864 is characterised by a calcareous, rounded to ovate concentric, paucispiral operculum, with a calcareous peg emerging from the inner surface (Bandel 2008, Thompson and Dance 1983, Vermeulen et al. 2015). The shell is small, dextral, conical, and frequently presents conspicuous radial and spiral sculpture. The studies by Thompson and Dance (1983) and Vermeulen et al. (2015) showed that the Bornean *Georissa* are between 0.7 and 4.0 mm in adult shell height. The protoconch is usually distinctly hemi-spherically shaped, distinct in microsculpture and distinguishable from the post-embryonic whorls. The internal walls (some would refer these as septa) are resorbed, and the remaining wall ends more than one whorl before reaching the aperture; resorption also leads to excavation of the columella (Thompson and Dance 1983, Bandel 2008). The evolutionary causes for this internal shell restructuring remain to be studied. The snails are often found in moderate to high densities on rocks, especially limestone rocks, where they apparently forage moss, algae, and lichens (Berry 1966). Cave-adapted species may forage on bacterial films (Schilthuizen et al. 2012).

Previous taxonomic treatments of Bornean *Georissa* (Godwin-Austen 1889, Gredler 1902, Haase and Schilthuizen 2007, Smith 1893, 1895, Thompson and Dance 1983, van Benthem-Jutting 1966, Vermeulen and Junau 2007, Vermeulen et al. 2015) revealed that shell shape and size, as well as sculptural patterns on the whorls are important characters for species delimitation. Given the small size of these shells, great benefits can be had from the use of scanning electron microscopy and X-ray microtomography, which are able to show detailed microscopic sculpture patterns and the inner part of the shell.

Since the overview presented by Thompson and Dance (1983), no revisions have been made for the Bornean *Georissa*, although recently, several new Bornean *Georissa* have been described, i.e., *Georissa filiasaulae* Haase & Schilthuizen, 2007, *Georissa pachysoma* Vermeulen & Junau, 2007, *Georissa leucococca* Vermeulen et al., 2015 and *Georissa nephrostoma* Vermeulen et al., 2015. Our new studies of the *Georissa* of Malaysian Borneo reveal additional, previously unrecognized diversity, which warrants a

series of revisions of the various species groups. In the present paper, we first address a group of species that we here call the "scaly group", chiefly consisting of species with conspicuous scale-like sculpture on the shell.

We present detailed species descriptions for a total of 13 Bornean *Georissa* from the "scaly group", of which six species are new to science, namely: *Georissa anyiensis* sp. n., *Georissa muluensis* sp. n., *Georissa bauensis* sp. n., *Georissa silaburensis* sp. n., *Georissa kinabatanganensis* sp. n., and *Georissa sepulutensis* sp. n.

Materials and methods

Materials and fieldwork

We examined collection material from:

RMNH Naturalis Biodiversity Center (previously collection from Rijksmuseum van Natuurlijke Historie), Leiden,
ZMA Naturalis Biodiversity Center (previously collection from Zoological Museum of Amsterdam), Leiden,
NHMUK Natural History Museum, London,
BORN Borneensis Collection, Universiti Malaysia Sabah,
MZU Zoology Museum, Universiti Malaysia Sarawak, and
JJV Jaap Vermeulen (personal collection).

In addition to these available data, we did fieldwork at limestone outcrops in Malaysian Borneo between September 2015 and May 2017. Manual searches were carried out to collect living and empty shells of *Georissa* on limestone walls and rocks, loose organic matter, and on/under living leaves. The living *Georissa* were directly stored in sample tubes containing ~96% ethanol. Ca. 5 litres of soil and leaf litter were sampled at each sampling location to collect empty shells by flotation (Vermeulen and Whitten 1998). The holotypes, paratypes and all of the collected materials were deposited at the Zoology Museum (Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia), Borneensis Collection (Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia), and Naturalis Biodiversity Centre (Leiden, The Netherlands).

Morphological analysis

Microscopy. Shells were observed with a Zeiss SteREO Microscope Discovery V20. The images of examined individuals were captured by AxioCamMRc5, Zeiss PlanApo S 1.0× FWD 60.0mm lenses. A complementary software of the camera AxioVision Special Edition 64-bit version 4.9.1.0 was used for shell measurements, namely, shell height, shell width, aperture height, and aperture width, at 30–60× magnification. The

measurements of "scaly" *Georissa* were carried out following the shell measurement method of Vermeulen and Whitten 1998. **Scanning electron microscopy**. A representative adult shell for each species was cleaned using sodium hypochloride, dried, and sputter-coated with Pd/Pt coating agent before detailed examination with a JEOL JSM-6480LV scanning electron microscope (SEM). We obtained SEM images of the entire shell in top view and apertural view (including clear view of the sculpture), side and top views of the protoconch and the spire. **Micro-computed tomography**. The micro-computed tomography (μCT) scanning was carried out with an Xradia 520 Versa X-ray Microscope using accompanying software Zeiss Xradia Versa (11.1.6315). The X-ray images from the scanning (ca. 950 layers of images in TIF format) were reconstructed into composite 3D images of the shells using software Scout-and-Scan™ Control System Reconstructor (11.1.5707.17179). All shell materials were scanned in air medium at 80/7 voltage/power (kW/P) using objective lens unit 4 in 180° rotation. Detailed scanning parameters for each species are summarized in Suppl. material 1. We used reconstructed 3D images of representative adult shells of each species from μCT scanned data to examine the internal characters, including the operculum and its peg. We conducted 3D image reconstruction to preserve the original structure of the shells and avoiding unintentional shell destruction. The 3D image analysis of the shells was carried out with Avizo ver. 9.2.0, FEI Company.

Molecular analysis

DNA extraction. Genomic DNA was extracted from 127 individuals of *Georissa* using the Qiagen DNeasy Blood and Tissue kit, following the manufacturer's protocol. Prior to the DNA extraction, the shells were removed and the entire soft tissue was used in the DNA extraction procedure. **DNA amplification**. We amplified two mtDNA regions, namely 16S and CO1. DNA amplifications were conducted on a BIO-RAD C1000 Touch Thermal Cycler. For the 16S gene, a fragment of 422-464 bp was amplified using primer pair LR-J-12887 5'-CCGGTCTGAACTCAGATCACGT-3' (forward) and LR-N-13398 5'-CGCCTGTTTAACAAAAAACAT-3' (reverse) (Schilthuizen et al. 2005) in 25.0 μL reaction volume, containing: 1.0 μL undiluted DNA template, 15.0 μL mQ (milli-Q, ultrapure water), 2.5 μL PCR chlorine buffer $10_{\times}$, 2.5 μL $MgCl_2$ 25.0 mM, 0.25 μL BSA 100 mM, 1.0 μL forward primer 10 pmol/μL, 1.0 μL reverse primer 10 pmol/μL, 1.5 μL dNTPs 2.5 mM, and 0.25 μL Taq 5.0 U/ μL. The amplification was carried out with the following cycling protocol: initial denaturation at 95 °C for 5 min, 36 cycles (of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min), and a final extension at 72 °C for 5 min. A 546-603 bp fragment of CO1 was amplified using primer pair LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward) and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse) (Folmer et al. 1994) in 25.0 μL reaction volume, containing: 1.0 μL DNA template, 16.8 μL mQ, 2.5 μL

PCR chlorine buffer 10×, 1.0 µL $MgCl_2$ 25.0 mM, 1.0 µL BSA 100 mM, 1.0 µL forward primer 10 pmol/µL, 1.0 µL reverse primer 10 pmol/µL, 0.5 µL dNTPs 2.5 mM, and 0.25 µL Taq 5.0 U/µL. The amplification was carried out with the following cycling protocol: initial denaturation at 94 °C for 4 min, 40 cycles (of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, extension at 72 °C for 40 s), and a final extension at 72 °C for 5 min. The unsuccessful amplication of CO1 and 16S genes were excluded in further phylogenetic analysis that used concatenated sequence alignment of both genes. **Sequencing**. The PCR products were then Sanger sequenced in both directions at BaseClear B.V. (Leiden, The Netherlands) on the ABI3730XL sequencer from Life Technologies. All new 16S mtDNA sequences used in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and CO1 mtDNA sequences were deposited in GenBank via BOLD (http://boldsystems.org/), under accession numbers as listed in Table 1.

Sequence alignment and phylogenetic analyses

Sequence data and alignement. A total of 12 ingroup taxa of "scaly group" *Georissa* including an outgroup taxon, *Georissa gomantongensis* Smith, 1893, were used for phylogenetic analyses (using a much larger hydrocenid taxon sampling, to be published elsewhere, we confirmed that *G. gomantongensis* indeed branches off basally to the "scaly group"). We added another six 16S mtDNA sequences from GenBank, representing *Georissa saulae* (van Benthem-Jutting, 1966) (GenBank accession no. AY547380, AY547381, AY547384, and AY547385) and *Georissa sepulutensis* sp. n. (GenBank accession no. AY547387 and AY548388). We conducted our phylogenetic analyses based on 128 sequences for 16S and 91 sequences for CO1. The forward and reverse nucleotide reads were assembled using *de novo* Geneious 10.0.7 assembler, manually checked and edited, and later aligned using default settings of MUSCLE alignment (Edgar 2004). **Phylogenetic inference**. For CO1 sequences, we selected the invertebrate mitochondrial genetic code at the second reading frame. Ambiguous nucleotide sequence ends were trimmed and removed prior to further analysis. ModelFinder (Kalyaanamoorthy et al. 2017) was used to select the most appropriate model, based on the corrected Akaike Information Creterion (AICc) for partial 16S and CO1 mtDNA genes. The best fitting models were TIM3+F+I+G4 for 16S and TIM2+F+I+G4 for CO1. **Phylogenetic analysis**. Maximum likelihood analysis was performed using IQ-TREE 1.6.3 (Nguyen et al. 2014) on a concatenated 16S and CO1 sequences of "scaly" *Georissa* using TIM3+F+I+G4 as the nucleotide substitution models with ultrafast bootstrapping (1000 replicates) (Hoang et al. 2017). Bayesian Inference was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) with the next closest nucleotide substitution model, GTR+I+G using the following MCMC settings: Chain length = 1,100,000 generations, heated chain = 4, subsampling frequency = one tree for each 200 generations, burn-in length = 100,000, and chain temperature = 0.2.

Table 1. List of specimens used in molecular analyses.

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
1	*Georissa gomantongensis* Smith, 1893	BOR/MOL 7389	G.gomantongensis_KPH01833.01_Kinabatangan Kinabatangan Valley, Sabah. 05°30.913'N, 118°16.889'E	MG982259	MH033876
2	*Georissa gomantongensis* Smith, 1893	BOR/MOL 7389	G.gomantongensis_KPH01833.02_Kinabatangan Kinabatangan Valley, Sabah. 05°30.913'N, 118°16.889'E	MG982260	MH033875
3	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 2663-2667 (Schilthuizen et al. 2012)	G.saulae_AY547385_Sinobang Batu Sinobang, Sabah. 04°48.040'N, 116°37.035'E	AY547385	n.a.
4	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 2663-2667 (Schilthuizen et al. 2012)	G.saulae_hapA_AY547380_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	AY547380	n.a.
5	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 2663-2667 (Schilthuizen et al. 2012)	G.saulae_hapB_AY547381_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	AY547381	n.a.
6	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 2663-2667 (Schilthuizen et al. 2012)	G.saulae_hapC_AY547384_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	AY547384	n.a.
7	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 3493	G.saulae_ZI003_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982261	n.a.
8	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 3493	G.saulae_KPH00181.02_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982267	n.a.
9	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 12770	G.saulae_Sau-001_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982262	n.a.
10	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 12770	G.saulae_Sau-002_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982263	n.a.
11	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 12770	G.saulae_Sau-003_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982264	n.a.
12	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 12770	G.saulae_Sau-004_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982265	n.a.
13	*Georissa saulae* (van Benthem Jutting, 1966)	BOR/MOL 12770	G.saulae_Sau-005_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982266	n.a.
14	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A001_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982327	n.a.
15	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A002_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982331	MH033908

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
16	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A003_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982330	n.a.
17	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A004_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982329	MH033907
18	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A005_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982328	n.a.
19	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.09	G.hosei_A006_Tongak Bukit Tongak, Bidi, Bau/Jambusan, Sarawak. 01°22.670'N, 110°08.325'E	MG982326	n.a.
20	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C001_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982339	MH033904
21	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C002_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982338	MH033905
22	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C003_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982341	MH033902
23	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C004_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982340	MH033903
24	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C005_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982337	n.a.
25	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C006_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982336	MH033906
26	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C007_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982335	n.a.
27	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C008_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982334	n.a.
28	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C009_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050'N, 110°11.197'E	MG982333	n.a.

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
29	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.04	G.hosei_C0010_Liak Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak. 01°24.050"N, 110°11.197"E	MG982332	n.a.
30	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.08	G.hosei_D001_Siboyuh Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak. 01°22.909'N, 110°11.695'E	MG982346	MH033900
31	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.08	G.hosei_D002_Siboyuh Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak. 01°22.909'N, 110°11.695'E	MG982342	MH033901
32	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.08	G.hosei_D003_Siboyuh Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak. 01°22.909'N, 110°11.695'E	MG982345	MH033898
33	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.08	G.hosei_D004_Siboyuh Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak. 01°22.909'N, 110°11.695'E	MG982344	MH033899
34	*Georissa hosei* Godwin-Austen, 1889	MZU/MOL 16.08	G.hosei_D006_Siboyuh Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak. 01°22.909'N, 110°11.695'E	MG982343	n.a.
35	*Georissa anyiensis* sp. n.	MZU/MOL 17.50	G.anyiensis_BSP2-01_Bukit Sarang Plot 2, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982271	MH033929
36	*Georissa anyiensis* sp. n.	MZU/MOL 17.50	G.anyiensis_BSP2-02_Bukit Sarang Plot 2, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982269	MH033930
37	*Georissa anyiensis* sp. n.	MZU/MOL 17.50	G.anyiensis_BSP2-03_Bukit Sarang Plot 2, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982268	MH033928
38	*Georissa anyiensis* sp. n.	MZU/MOL 17.50	G.anyiensis_BSP2-04_Bukit Sarang Plot 2, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982270	n.a.

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
39	*Georissa anyiensis* sp. n.	MZU/MOL 17.51	G.anyiensis_BSP11-01_Bukit Sarang Plot 11, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	n.a.	MH033926
40	*Georissa anyiensis* sp. n.	MZU/MOL 17.51	G.anyiensis_BSP11-02_Bukit Sarang Plot 11, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982278	MH033927
41	*Georissa anyiensis* sp. n.	MZU/MOL 17.51	G.anyiensis_BSP11-03_Bukit Sarang Plot 11, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982280	MH033924
42	*Georissa anyiensis* sp. n.	MZU/MOL 17.51	G.anyiensis_BSP11-04_Bukit Sarang Plot 11, Bukit Lebik at Bukit Sarang, Bintulu, Sarawak. 02°39.325'N, 113°02.432'E	MG982279	MH033925
43	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-01_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982272	n.a.
44	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-02_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982273	MH033931
45	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-03_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982274	MH033933
46	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-04_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982275	MH033934
47	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-05_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982276	MH033935
48	*Georissa anyiensis* sp. n.	MZU/MOL 17.60	G.anyiensis_BSP22-06_Bukit Sarang Plot 22, Bukit Anyi at Bukit Sarang, Bintulu, Sarawak. 02°39.252'N, 113°02.723'E	MG982277	MH033932

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
49	*Georissa muluensis* sp. n.	MZU/MOL 17.31	G.muluensis_LGG-01_Mulu Plot 1, Lagang Cave, Mulu National Park, Mulu, Sarawak. 04°03.060'N, 114°49.372'E	MG982288	MH033893
50	*Georissa muluensis* sp. n.	MZU/MOL 17.31	G.muluensis_LGG-02_Mulu Plot 1, Lagang Cave, Mulu National Park, Mulu, Sarawak. 04°03.060'N, 114°49.372'E	MG982285	MH033891
51	*Georissa muluensis* sp. n.	MZU/MOL 17.31	G.muluensis_LGG-03_Mulu Plot 1, Lagang Cave, Mulu National Park, Mulu, Sarawak. 04°03.060'N, 114°49.372'E	MG982286	MH033892
52	*Georissa muluensis* sp. n.	MZU/MOL 17.31	G.muluensis_LGG-04_Mulu Plot 1, Lagang Cave, Mulu National Park, Mulu, Sarawak. 04°03.060'N, 114°49.372'E	MG982287	MH033890
53	*Georissa hadra* Thompson & Dance, 1983	MZU/MOL 17.32	G.hadra_LC-01_Mulu Lang Cave, Mulu N.P., Mulu, Sarawak. 04°01.490'N, 114°49.482'E	MG982284	MH033896
54	*Georissa hadra* Thompson & Dance, 1983	MZU/MOL 17.32	G.hadra_LC-02_Mulu Lang Cave, Mulu N.P., Mulu, Sarawak. 04°01.490'N, 114°49.482'E	MG982282	MH033897
55	*Georissa hadra* Thompson & Dance, 1983	MZU/MOL 17.32	G.hadra_LC-03_Mulu Lang Cave, Mulu N.P., Mulu, Sarawak. 04°01.490'N, 114°49.482'E	MG982281	MH033894
56	*Georissa hadra* Thompson & Dance, 1983	MZU/MOL 17.32	G.hadra_LC-04_Mulu Lang Cave, Mulu N.P., Mulu, Sarawak. 04°01.490'N, 114°49.482'E	MG982283	MH033895
57	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.36	G.kobelti_TC-01_Niah Trade Cave, Niah National Park, Niah, Sarawak. 03°49.137'N, 113°46.860'E	MG982296	MH033886
58	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.36	G.kobelti_TC-02_Niah Trade Cave, Niah National Park, Niah, Sarawak. 03°49.137'N, 113°46.860'E	MG982295	MH033889
59	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.36	G.kobelti_TC-03_Niah Trade Cave, Niah National Park, Niah, Sarawak. 03°49.137'N, 113°46.860'E	MG982293	MH033887
60	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.36	G.kobelti_TC-04_Niah Trade Cave, Niah National Park, Niah, Sarawak. 03°49.137'N, 113°46.860'E	MG982294	MH033888

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
61	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.38	G.kobelti_KJ1-01_Baram Plot 1, Bukit Kaijin, Baram, Sarawak. 03°41.753'N, 114°27.555'E	MG982290	MH033882
62	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.38	G.kobelti_KJ1-02_Baram Plot 1, Bukit Kaijin, Baram, Sarawak. 03°41.753'N, 114°27.555'E	MG982289	MH033883
63	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.38	G.kobelti_KJ1-03_Baram Plot 1, Bukit Kaijin, Baram, Sarawak. 03°41.753'N, 114°27.555'E	MG982292	MH033885
64	*Georissa kobelti* Gredler, 1902	MZU/MOL 17.38	G.kobelti_KJ1-04_Baram Plot 1, Bukit Kaijin, Baram, Sarawak. 03°41.753'N, 114°27.555'E	MG982291	MH033884
65	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_PC-01_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982301	MH033965
66	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_PC-02_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982300	MH033878
67	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_PC-03_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982297	MH033877
68	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_PC-04_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982298	MH033954
69	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_GC-01_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982299	MH033879
70	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_GC-02_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982302	MH033880
71	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_GC-03_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982304	n.a.
72	*Georissa niahensis* Godwin-Austen, 1889	MZU/MOL 17.25	G.niahensis_GC-04_Niah Painted Cave, Niah National Park, Niah, Sarawak. 03°48.688'N, 113°47.250'E	MG982303	MH033881

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
73	*Georissa silaburensis* sp. n.	MZU/MOL 17.05	G.silaburensis_SIG3-01_Silabur Plot 3, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982323	MH033949
74	*Georissa silaburensis* sp. n.	MZU/MOL 17.05	G.silaburensis_SIG3-03_Silabur Plot 3, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982324	MH033948
75	*Georissa silaburensis* sp. n.	MZU/MOL 17.05	G.silaburensis_SIG3-05_Silabur Plot 3, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982325	MH033944
76	*Georissa silaburensis* sp. n.	MZU/MOL 17.06	G.silaburensis_SIG4-01_Silabur Plot 4, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982320	MH033945
77	*Georissa silaburensis* sp. n.	MZU/MOL 17.06	G.silaburensis_SIG4-03_Silabur Plot 4, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982321	MH033952
78	*Georissa silaburensis* sp. n.	MZU/MOL 17.06	G.silaburensis_SIG4-06_Silabur Plot 4, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982322	MH033951
79	*Georissa silaburensis* sp. n.	MZU/MOL 17.07	G.silaburensis_SIG5-07_Silabur Plot 5, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982316	MH033946
80	*Georissa silaburensis* sp. n.	MZU/MOL 17.07	G.silaburensis_SIG5-08_Silabur Plot 5, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982317	MH033950
81	*Georissa silaburensis* sp. n.	MZU/MOL 17.07	G.silaburensis_SIG5-09_Silabur Plot 5, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982318	n.a.
82	*Georissa silaburensis* sp. n.	MZU/MOL 17.07	G.silaburensis_SIG5-10_Silabur Plot 5, Gunong Silabur, Serian, Sarawak. 00°57.285'N, 110°30.228'E	MG982319	MH033947
83	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B002_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982306	MH033937
84	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B003_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	n.a.	MH033938
85	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B004_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982309	MH033936

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
86	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B005_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982307	n.a.
87	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B007_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982308	n.a.
88	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B008_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982311	MH033939
89	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B009_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982305	n.a.
90	*Georissa bauensis* sp. n.	MZU/MOL 16.01	G.bauensis_B010_WCave Wind Cave Passage 3, Wind Cave National Park, Bau, Sarawak. 01°24.810'N, 110°08.175'E	MG982310	n.a.
91	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q001_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	MG982313	MH033942
92	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q002_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	MG982312	n.a.
93	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q003_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	MG982314	n.a.
94	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q004_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	n.a.	MH033941
95	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q005_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	n.a.	MH033940

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
96	*Georissa bauensis* sp. n.	MZU/MOL 16.03	G.bauensis_Q006_Ayub Gunong Podam, near Sg. Ayup, Kampung Bogag, Bau, Sarawak. 01°21.158'N, 110°03.577'E	MG982315	MH033943
97	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.11	G.pyrrhoderma_SO3-01_Silabur Plot Outside 3-1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982366	MH033913
98	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.11	G.pyrrhoderma_SO3-02_Silabur Plot Outside 3-1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982364	MH033914
99	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.11	G.pyrrhoderma_SO3-03_Silabur Plot Outside 3-1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982367	MH033915
100	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.11	G.pyrrhoderma_SO3-04_Silabur Plot Outside 3-1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982365	MH033916
101	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.22	G.pyrrhoderma_SIO4-01_Silabur Plot SIO4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982376	MH033918
102	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.22	G.pyrrhoderma_SIO4-02_Silabur Plot SIO4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982377	MH033920
103	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.22	G.pyrrhoderma_SIO4-03_Silabur Plot SIO4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982378	MH033917
104	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.22	G.pyrrhoderma_SIO4-04_Silabur Plot SIO4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982379	MH033919
105	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.22	G.pyrrhoderma_SIO4-05_Silabur Plot SIO4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982380	n.a.
106	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.13	G.pyrrhoderma_SIE1-01_Silabur Plot SIE1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982372	n.a.
107	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.13	G.pyrrhoderma_SIE1-02_Silabur Plot SIE1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982373	MH033922
108	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.13	G.pyrrhoderma_SIE1-03_Silabur Plot SIE1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982374	MH033923

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
109	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.13	G.pyrrhoderma_SIE1-04_Silabur Plot SIE1, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982375	MH033921
110	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.16	G.pyrrhoderma_SIE4-01_Silabur Plot SIE4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982368	MH033910
111	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.16	G.pyrrhoderma_SIE4-02_Silabur Plot SIE4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982369	MH033909
112	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.16	G.pyrrhoderma_SIE4-03_Silabur Plot SIE4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982370	MH033911
113	*Georissa pyrrhoderma* Thompson & Dance, 1983	MZU/MOL 17.16	G.pyrrhoderma_SIE4-04_Silabur Plot SIE4, Gunong Silabur, Serian, Sarawak. 00°57.451'N, 110°30.207'E	MG982371	MH033912
114	*Georissa kinabatanganensis* sp. n.	BOR/MOL 7289	G.kinabatanganensis_KPH01720.01_Pangi Kinabatangan Valley, Pangi, Sabah. 05°32.291'N, 118°18.376'E	MG982348	MH033963
115	*Georissa kinabatanganensis* sp. n.	BOR/MOL 7289	G.kinabatanganensis_KPH01720.02_Pangi Kinabatangan Valley, Pangi, Sabah. 05°32.291'N, 118°18.376'E	MG982347	MH033962
116	*Georissa kinabatanganensis* sp. n.	BOR/MOL 7289	G.kinabatanganensis_KPH01720.03_Pangi Kinabatangan Valley, Pangi, Sabah. 05°32.291'N, 118°18.376'E	n.a.	MH033961
117	*Georissa kinabatanganensis* sp. n.	MZU/MOL 17.26	G.kinabatanganensis_K001_Keruak Keruak, near Kinabatangan river, Sandakan, Sabah. 05°32.291'N, 118°18.376'E	MG982349	MH033959
118	*Georissa kinabatanganensis* sp. n.	MZU/MOL 17.26	G.kinabatanganensis_K002_Keruak Keruak, near Kinabatangan river, Sandakan, Sabah. 05°31.385'N, 118°17.113'E	MG982351	MH033958
119	*Georissa kinabatanganensis* sp. n.	MZU/MOL 17.26	G.kinabatanganensis_K005_Keruak Keruak, near Kinabatangan river, Sandakan, Sabah. 05°31.385'N, 118°17.113'E	MG982350	MH033960
120	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_KPH00176.01_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982357	MH033957
121	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_KPH00176.02_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982356	n.a.
122	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_KPH00181.01_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982359	MH033956

No.	Species	Voucher No.	Species name_sequence origin_location Town/District/Division, State. GPS coordinate	GenBank Accession No.	
				16S	CO1
123	*Georissa sepulutensis* sp. n.	BOR/MOL 12278	G.sepulutensis_Sca-002_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982361	MH033964
124	*Georissa sepulutensis* sp. n.	BOR/MOL 12278	G.sepulutensis_Sca-003_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982360	MH033955
125	*Georissa sepulutensis* sp. n.	BOR/MOL 12278	G.sepulutensis_Sca-004_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982362	MH033953
126	*Georissa sepulutensis* sp. n.	BOR/MOL 12278	G.sepulutensis_Sca-005_Pungiton Sepulut Valley, Gua Pungiton, Sabah. 04°42.410'N, 116°36.040'E	MG982363	n.a.
127	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_ZA004_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982354	n.a.
128	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_ZB003_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982355	n.a.
129	*Georissa sepulutensis* sp. n.	BOR/MOL 39	G.sepulutensis_ZC003_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	MG982358	n.a.
130	*Georissa sepulutensis* sp. n.	RMNH/MOL 333905	G.sepulutensis_ZE003_Simbaluyon Sepulut Valley, Bukit Simbaluyon, Sabah. 04°43.200'N, 116°34.140'E	MG982352	n.a.
131	*Georissa sepulutensis* sp. n.	RMNH/MOL 333905	G.sepulutensis_ZE004_Simbaluyon Sepulut Valley, Bukit Simbaluyon, Sabah. 04°43.200'N, 116°34.140'E	MG982353	n.a.
132	*Georissa sepulutensis* sp. n.	BOR/MOL 39 (Schilthuizen et al., 2005)	G.sepulutensis_hapA_AY547387_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	AY547387	n.a.
133	*Georissa sepulutensis* sp. n.	BOR/MOL 39 (Schilthuizen et al., 2005)	G.sepulutensis_hapB_AY547388_Sanaron Sepulut Valley, Batu Sanaron, Sabah. 04°42.052'N, 116°36.016'E	AY547388	n.a.

Species delimitation and description

For species delimitation, we combined the data of molecular phylogenetic analyses and the assessments of the morphology. We aimed for monophyly in species, allowing paraphyly under certain circumstances (Schilthuizen and Gittenberger 1996), but disallowing polyphyly. Only when we found morphological characters that could distinguish DNA-based clades or paraphyletic groups, did we consider such groups as potential species. Although many forms in *Georissa* are allopatric, we did have a number of cases where two forms occurred sympatrically without forming intermediates, which also aided in determining species status by application of the biological species concept (Mayr 1942). General shell characters were further divided into detailed subcharacters exclusively for the descriptions of the representatives of the "scaly group" of Bornean *Georissa*. The assessed morphological characters follow the descriptions made by Godwin-Austen (1889), Gredler (1902), Haase and Schilthuizen (2007), Smith (1893, 1895), Thompson and Dance (1983), van Benthem-Jutting (1966), Vermeulen and Junau (2007), and Vermeulen et al. (2015). Note that color indications always refer to living or freshly dead specimens, as the color in older specimens usually degrades, with an exception for *Georissa scalinella* (van Benthem-Jutting, 1966), where only old collection specimens were available.

CO1 genetic divergence

In addition to the molecular phylogenetic and morphological assessment in our species delimitation, we conducted divergence analysis of partial CO1 genes to provide additional information to assist in the species delimitation of "scaly" *Georissa*. Several other studies on species delimitation in gastropods have also used CO1 mtDNA successfully (see Liew et al. 2014, Puillandre et al. 2012a, 2012b). Pairwise genetic distances of CO1 sequences from 89 individuals were computed based on Kimura 2-parameter with MEGA v. 7.0.26 (Kumar et al. 2016). These comprised of eleven species, including the six new species.

Web interface species delimitation using 16S mtDNA

We carried out two more approaches of web interface species delimitation to provide more insight in our species delimitation, namely, Automatic Barcode Gap Discovery (ABGD) (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) (Puillandre et al. 2012a), and Poisson Tree Processes (PTP) (http://species.h-its.org/ptp/) (Zhang et al. 2013). ABGD analysis was carried out using 16S mtDNA sequences of the "scaly group" *Georissa* (excluding the outgroup). The parameters were set to default. For PTP analysis, we used the 16S gene tree generated from IQ-TREE (Nguyen et al. 2014). The parameters were set to default. Both ABGD and PTP analyses were conducted using mtDNA

16S sequences and gene tree based on the available data of all studied taxa. ABGD aims to partition the species based on the barcode gap (Puillandre et al. 2012a), while PTP focuses on the branching event of a rooted phylogenetic tree (Zhang et al. 2013).

Results and discussion

Morphology and phylogenetic analyses

Our morphological and phylogenetic studies lead us to conclude that there are at least 13 species of "scaly group" *Georissa* currently existing in Malaysian Borneo (for detailed morphological species descriptions, see the species treatments under the Taxonomy section). For one of these, *Georissa scalinella* (van Benthem-Jutting, 1966), DNA data are yet unavailable. Detailed conchological assessments of the "scaly group" show that at least two species, *Georissa bauensis* sp. n. and *Georissa hosei* Godwin-Austen, 1889, are highly variable (both intra- and inter-populationally) with regard to the "scaly" shell microsculpture characters (see Fig. 1). Due to the high inter- and intraspecific variation of these species, identification based on morphological characters alone could be problematic without prior knowledge of the shell variation within these species. Furthermore, species similar in shell habitus and scale characters, like *Georissa pyrrhoderma* Thompson & Dance, 1983 and *Georissa sepulutensis* sp. n., often have character combinations that overlap with either *G. bauensis* or *G. hosei*. Therefore, for identification of "scaly group" specimens, we found thorough conchological examination of the shells aided with molecular data is most reliable.

Based on the molecular phylogenetic analyses of the "scaly group" *Georissa* we find multiple strongly supported monophyletic groups (bootstrap and posterior output values ranging from 89–100 and 97–100, respectively) which correspond with subtly different conchologies. In contrast, *Georissa kobelti* Gredler, 1902 is paraphyletic, and we treat this as a single species based on the conchological characters that support they are conspecific.

CO1 genetic divergence

Despite geographic proximity for some populations of morphologically highly similar forms, the CO1 divergence analysis shows high genetic divergences (e.g. *G. bauensis* vs. *G. hosei*, genetic divergence = 0.12). For some other species, the interspecific genetic divergence is lower, but such species may be surprisingly distinct in shell sculpture (e.g. *G. hadra* vs. *G. muluensis*, genetic divergence = 0.07). As a consequence, we have sometimes given priority to genetic distinctness, sometimes to morphological distinctness in delimiting species, which means that intraspecific diversity may vary between species. For example, we found that *G. pyrrhoderma*, *G. hosei*, and *G. kobelti* are the three species to have the highest intraspecific divergence (0.06, 0.06 and 0.07, respectively) compared with the rest of the "scaly group", where all other species have an intraspecific divergence equal to or

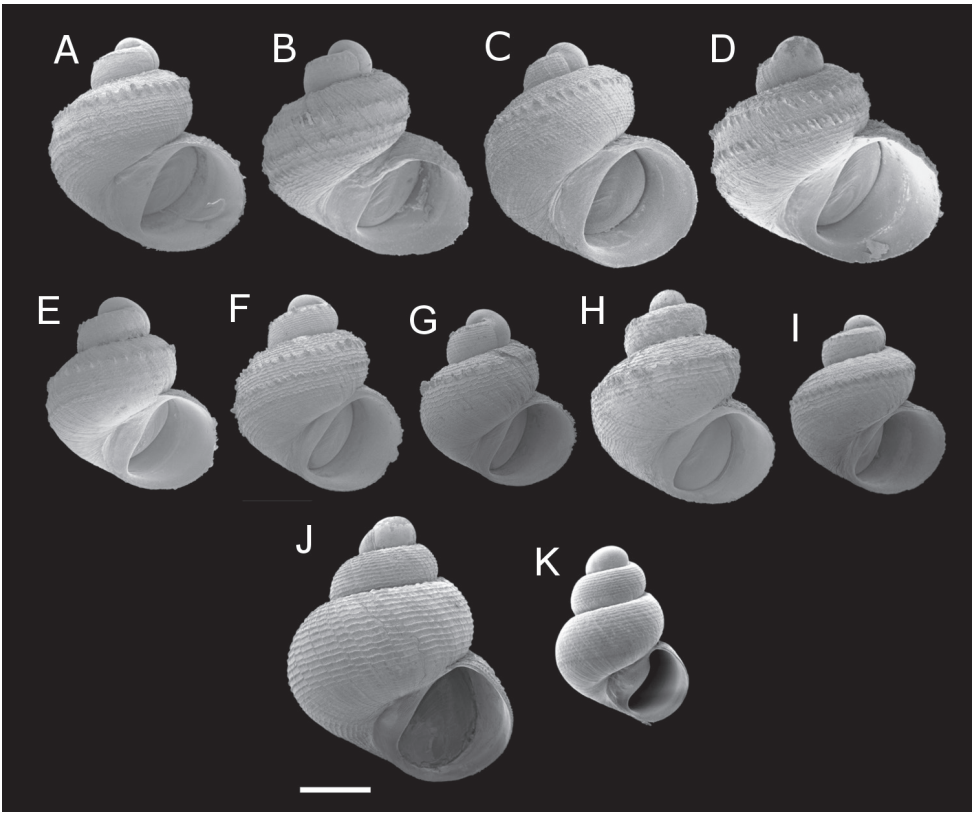


Figure 1. A–D Intraspecific variation in shell shape and sculpture of *Georissa hosei* Godwin-Austen, 1889 **E–G** Intraspecific variation in shell shape and sculpture of *Georissa bauensis* sp. n. **H** *Georissa pyrrhoderma* Thompson & Dance, 1983 **I** *Georissa sepulutensis* sp. n. For comparison with the "scaly group", two additional species are shown that do not belong to the "scaly group", namely: **J** *Georissa gomantongensis* Smith, 1893 and **K** *Georissa nephrostoma* Vermeulen et al., 2015. Localities: **A, B** Gunung Liak/Padang (Jambusan, Sarawak) **C** Bukit Siboyuh (Jambusan, Sarawak) **D** Bukit Tongak (Bau/Jambusan, Sarawak) **E, F** Gunung Podam (Bau, Sarawak) **G** Wind Cave Nature Reserve (Bau, Sarawak) **H** Gunung Silabur (Serian, Sarawak) **I** Batu Sanaron (Sanaron, Sabah) **J** Gua Gomantong (Gomantong, Sabah) **K** Keruak (Kinabatangan, Sabah). Scale bar 500 μm.

lower than 0.05 (see details in Table 2). Our study reveals that within group divergences of "scaly" *Georissa* does not exceed 0.07 for each species, while the divergences between all species pairs exceed 0.10, with the exception of *G. kinabatanganensis* vs. *G. sepulutensis*, *G. bauensis* vs. *G. silaburensis*, *G. hadra* vs. *G. muluensis* and *G. kobelti* vs. *G. niahensis*.

Web interface species delimitation using 16S mtDNA

To test to what extent automated procedures, based on genetic data alone, could reproduce our subjective species delimitation, we carried out ABGD and PTP analyses.

Table 2. Intra- and inter-specific CO1 sequence divergence of eleven species of "scaly" *Georissa*.

		Divergence within group	Number of specimens	*G. kinabatanganensis*	*G. sepulutensis*	*G. bauensis*	*G. silaburensis*	*G. anyiensis*	*G. pyrrhoderma*	*G. hosei*	*G. hadra*	*G. muluensis*	*G. kobelti*	*G. niahensis*
1	*G. kinabatanganensis*	0.05	6											
2	*G. sepulutensis*	0.02	5	0.06*										
3	*G. bauensis*	0.03	8	0.11	0.14									
4	*G. silaburensis*	<0.01	9	0.12	0.13	0.04*								
5	*G. anyiensis*	0.04	12	0.14	0.14	0.12	0.12							
6	*G. pyrrhoderma*	0.06	15	0.10	0.11	0.11	0.11	0.09						
7	*G. hosei*	0.06	11	0.14	0.13	0.12	0.12	0.10	0.12					
8	*G. hadra*	<0.01	4	0.18	0.18	0.16	0.15	0.12	0.15	0.14				
9	*G. muluensis*	<0.01	4	0.17	0.19	0.15	0.15	0.14	0.14	0.14	0.07*			
10	*G. kobelti*	0.07	8	0.11	0.13	0.12	0.12	0.09	0.10	0.09	0.10	0.09		
11	*G. niahensis*	0.04	7	0.13	0.15	0.13	0.14	0.10	0.12	0.10	0.11	0.13	0.05*	

*The average number of net base substitutions per site between species is equal or lower than 0.07, which is the highest number of base substitution per site within a "scaly" species.

ABGD recursive partition divided the "scaly group" *Georissa* into no more than six species at the lowest intraspecific divergence, while the highest divergence grouped all "scaly group" *Georissa* into a single species. The ABGD analysis further showed that partitioning into six species was due mostly to the separation of *G. saulae* into five different species while the rest of "scaly" *Georissa* were considered as a single species. This is possible due to the even higher intraspecific divergence of 16S mtDNA of *G. saulae* compared to the rest of "scaly group" taxa (see Suppl. material 2).

While ABGD analysis underestimated the number of possible species in the "scaly group" of *Georissa*, PTP analysis based on maximum likelihood delimitation results devided the taxa in at least 15 possible species. The results from this species delimitation method therefore more closely match our preferred approach (in which we combined phylogenetic and morphometric assessment). The PTP analysis does, however, differ from our preferred delimitation at several crucial points. *G. saulae*, *G. kinabatanganensis*, *G. hosei*, *G. kobelti*, and *G. niahensis* are each split into two species, whereas the two sets of species composed of (i) *G. hadra* and *G. muluensis*, and (ii) *G. bauensis* and *G. silaburensis*, are each considered as a single species, which make another two species. Otherwise, PTP analysis resolves the same species as in our preferred resolution (see Suppl. material 3).

The results from CO1 barcoding, ABGD, and PTP analyses reveal that objective species delimitation based solely on molecular data will not be successful for "scaly group" *Georissa*, at least if one wishes for the taxonomy to reflect morphology as well. Since most species are allopatric, and therefore the maintenance of species barriers can usually not be tested, we present our taxonomy as a compromise, which remains to be further tested by future workers.

Taxonomy

Systematics and descriptions

Class Gastropoda Cuvier,1797
Family Hydrocenidae Troschel, 1856
Genus *Georissa* Blanford, 1864: 463

"Scaly group"

We here define an informal group of 13 species of *Georissa* from Malaysian Borneo that are characterised by one or more spiral rows of scale-like sculptures. As far as they were known at the time, our "scaly group" corresponds to Thompson and Dance's (1983) "*hosei* group" + "*borneensis* group" p.p.

Conchological description of a generalised "scaly group" representative. *Protoconch*. Color (in living or freshly dead specimens): yellow, orange, red or brown. Sculpture: smooth, meshed, mixed or undefined. *Teleoconch*. Color (in living or freshly dead specimens): yellow, orange, red or brown. First whorl: convex, rounded to flat or

Figure 2. A Phylogeny from ML analysis with ultrafast bootstrapping (1000 replicates) **B** Phylogeny from MrBayes analysis. Analyses were conducted using concatenated sequence alignments of partial CO1 and 16S mtDNA of 133 individuals of "scaly" *Georissa* from Malaysian Borneo, with *Georissa gomantongensis* Smith, 1893 as the outgroup.

Figure 2. Continued.

angular. Subsequent whorls: convex, rounded, concave or tilted at the periphery, or flat, with well-impressed suture. Number of whorls: 2–3 ¼. Shell height (SH) (based on our conchological measurements of available studied materials stated in the methodology): 0.94–2.91 mm. Shell width (SW): 0.98–2.19 mm. Shell index (SI=SH/SW): 0.88-1.37. *Shell sculpture*. Radial sculpture: either absent or present. Growth lines: weak to strong. Spiral sculpture: absent or present; if present then weakly to strongly sculpted, continuous or discontinuous. Scales: between one and four spiral rows of vertical scales (any one of which may be more or less strongly pronounced than the others); scales can be minute to broad, low to acutely projecting. *Columella wall*. Smooth, translucent, and covering the umbilicus region. *Aperture*. Shape: oval to rounded, with straight to concave or convex parietal site, palatal edge either contiguous with or removed from the body whorl. Aperture height (AH): 0.50–1.33 mm. Aperture width (AW): 0.69–1.48 mm. Aperture index (AI=AH/AW): 0.65–1.02. *Peristome*. Simple, thickened inside, sharp toward the edge of the aperture. *Operculum*. Oval to rounded, with a peg facing inward, inner surface of the operculum has a small crater-like hole. Peg: straight or curved. The shell measurement of all measured "scaly group" *Georissa* are summarised in Suppl. material 4.

Anatomy. Haase and Schilthuizen (2007) described the anatomy of two closely related *Georissa*, viz. *G. saulae* and *G. filiasaulae*, and noted interspecific differences in radula, genital anatomy. Anatomical details of other "scaly group" representatives will be the focus of future studies and are not included in the present review.

Habitat and ecology. Members of the "scaly group" of *Georissa* live on limestone rocks, especially in wet and shaded environments. They are also found at lower density on dry limestone rocks, and occasionally on the limestone walls in cave systems (Haase and Schilthuizen 2007).

Distribution. There are at least nine species of this group distributed in Sarawak, and another four are distributed in Sabah (see Figures 3 and 4). In the distribution maps, we combined the geographical coordinates of each species from the known previous fieldwork locations and the available data from the collection repositories. The distribution of "scaly group" *Georissa* was assigned based on the available locality data from the collection from NHMUK, RMNH, ZMA, BOR, ZMU, and JJV. Localities may contain Malay words, namely: Batu = rock; Bukit = hill; Gua = cave; Gunung = mountain. We provide two distribution maps (Figs 3 and 4) to avoid overlapping of species that co-occur at the same or nearby locations.

In the following systematic descriptions of "scaly" *Georissa*, the species are arranged based on the molecular phylogeny. *Georissa scalinella* (van Benthem-Jutting, 1966), for which no genetic data are available, is placed at the top of the list.

For the stacked images of the "scaly" *Georissa* (Figs 5–17A–C), we decided not to remove the periostracum layers of the shells to retain the morphological characters of each species.

Since we needed fresh material to connect the morphology and molecular phylogenetics, we confined our study to Malaysian part of Borneo. We are aware that there might be species or populations in other parts of Borneo (Kalimantan, Indonesian

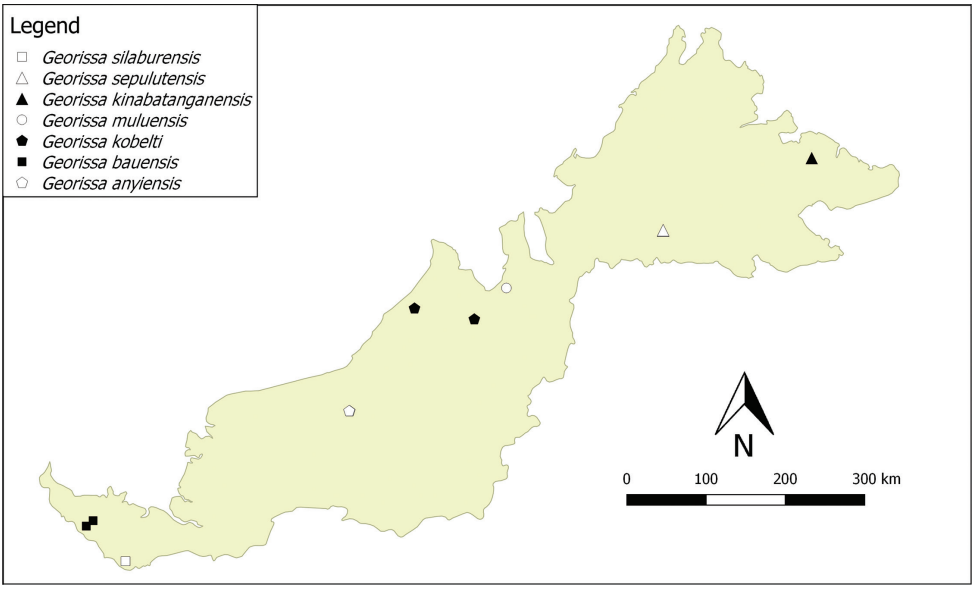


Figure 3. Distribution of seven "scaly group" *Georissa* species in Malaysian Borneo (based on the materials examined from NHM, RMNH, ZMA, BORN, MZU, and JJV).

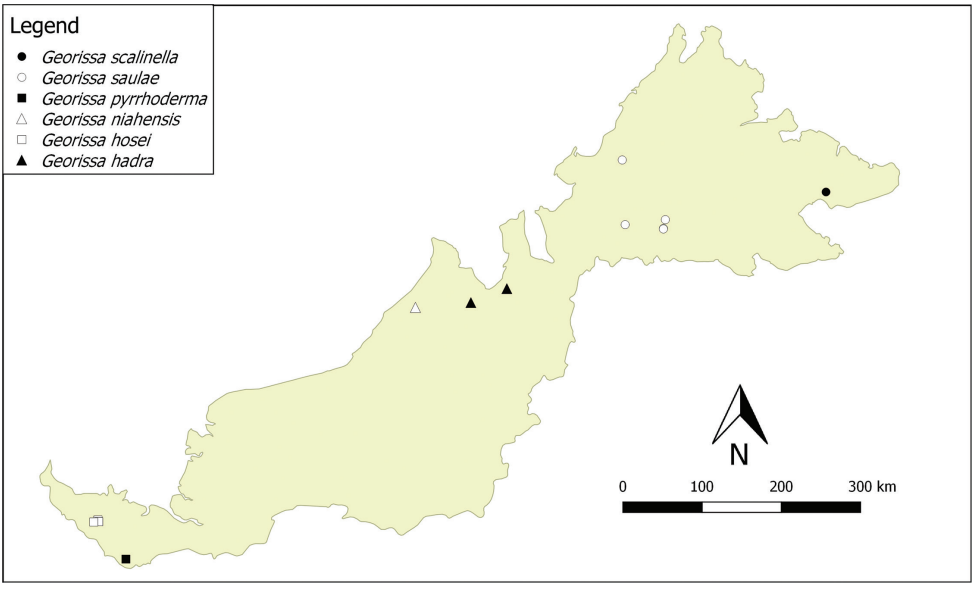


Figure 4. Distribution of five "scaly group" *Georissa* species in Malaysian Borneo (based on the materials examined from NHM, RMNH, ZMA, BORN, MZU, and JJV).

Borneo and Brunei) which belong inside the "scaly group". However, we hope that our study will stimulate colleagues that study *Georissa* in Kalimantan or Brunei to compare their material with our analysis.

Georissa scalinella (van Benthem-Jutting, 1966)

Hydrocena scalinella van Benthem-Jutting, 1966: 39, fig. 1; Saul 1967: 108.
Georissa scalinella (van Benthem-Jutting): Thompson and Dance 1983: 119; Phung et al. 2017: 68, fig. 8B.

Type locality. Lahad Datu Caves on Teck Guan Estate, Sabah.

Type material. *Holotype*. Lahad Datu Caves on Teck Guan Estate, Sabah: ZMA/MOLL 135736 (seen). *Paratypes*. Lahad Datu Caves on Teck Guan Estate, Sabah: ZMA/MOLL 135735 (seen), ZMA/MOLL 315596 (seen).

Description. *Protoconch*. Color: orange to red. Sculpture: smooth to meshed – semi oval mesh to undefined mesh pattern. Mesh width: 7–17 μm. *Teleoconch*. Color: orange. First whorl: flat at the shoulder. Subsequent whorls: flat above, slightly rounded below the periphery. Total number of whorls: 2 ¼-2 ½. SH: 1.56–1.80 mm, SW: 1.46–1.65 mm, SI: 1.03–1.15. *Shell sculpture*. Radial sculpture: absent, only weak to strong growth lines are visible. Spiral sculpture: present, and strongly sculpted, with continuous and discontinuous ribbing. Scales: a series of acute scales, low to highly projected, and regularly spaced. Intercept between growth lines and spiral ribbings form small pointed scale structures throughout the length of the body whorl. *Aperture*. Shape: oval. Basal side: rounded, angular at the columellar region. Parietal side: straight, palatal edge attached to the body whorl. AH: 0.78–0.94 mm, AW: 0.97–1.12 mm, AI: 0.75–0.89. *Holotype dimension*. SH: 1.88 mm, SW: 1.72 mm, AH: 0.84 mm, AW: 1.18 mm.

Cross diagnosis. *Georissa scalinella* has a series of scales at the shoulder. In habitus and scale characters, it resembles *G. pyrrhoderma* from Gunung Silabur, Sarawak. The angular shoulder and small scale-like nodular structure at the intersection of strong spiral ribbings and growth lines are diagnostic for *G. scalinella*.

Distribution. Known only from the type locality, Teck Guan Estate, Lahad Datu, Sabah, and also reported by Phung et al. (2017) at Pulau Tiga, Sandakan, Sabah. However, this may also refer to one of the other "scaly group" species from Sabah.

Discussion. *Georissa scalinella* was first described as *Hydrocena scalinella* van Benthem-Jutting, 1966, before reclassified as *Georissa* by Thompson and Dance (1983). van Benthem-Jutting (1966) described *G. scalinella* as having strong spiral ribbing and multiple lines of scales.

Georissa saulae (van Benthem-Jutting, 1966)

Hydrocena saulae van Benthem-Jutting, 1966: 40, fig. 2; Saul 1967: 109.
Georissa saulae (van Benthem-Jutting): Thompson and Dance 1983: 118, fig. 29, 53–54; Haase and Schilthuizen 2007: 217, fig. 2; Clements 2008: 2762; Schilthuizen et al. 2012: 278; Beron 2015: 181; Phung et al. 2017: 68, fig. 8; Osikowski et al. 2017: 80.

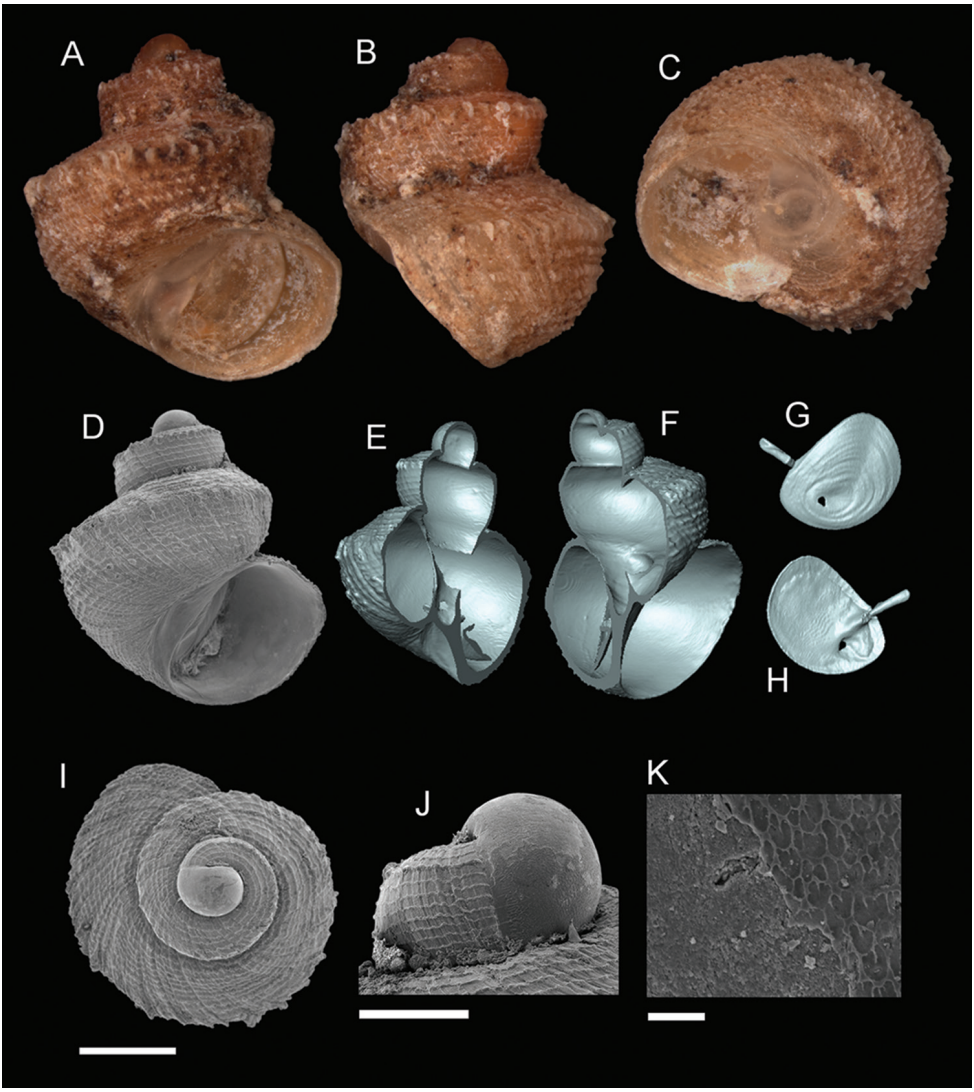


Figure 5. *Georissa scalinella* (van Benthem-Jutting, 1966). **A–C** Holotype: ZMA/MOL/ 135736 **D–K** Paratypes: ZMA/MOLL 135735. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Type locality. Malaysia, Borneo, Sabah, Laying cave, Keningau.

Type material. *Holotype*. Malaysia, Borneo, Sabah, Laying cave, Keningau: ZMA/MOLL 135731 (seen). *Paratypes*. Malaysia, Borneo, Sabah, Laying cave, Keningau: ZMA/MOLL 135598 (seen), ZMA/MOLL 135599 (seen).

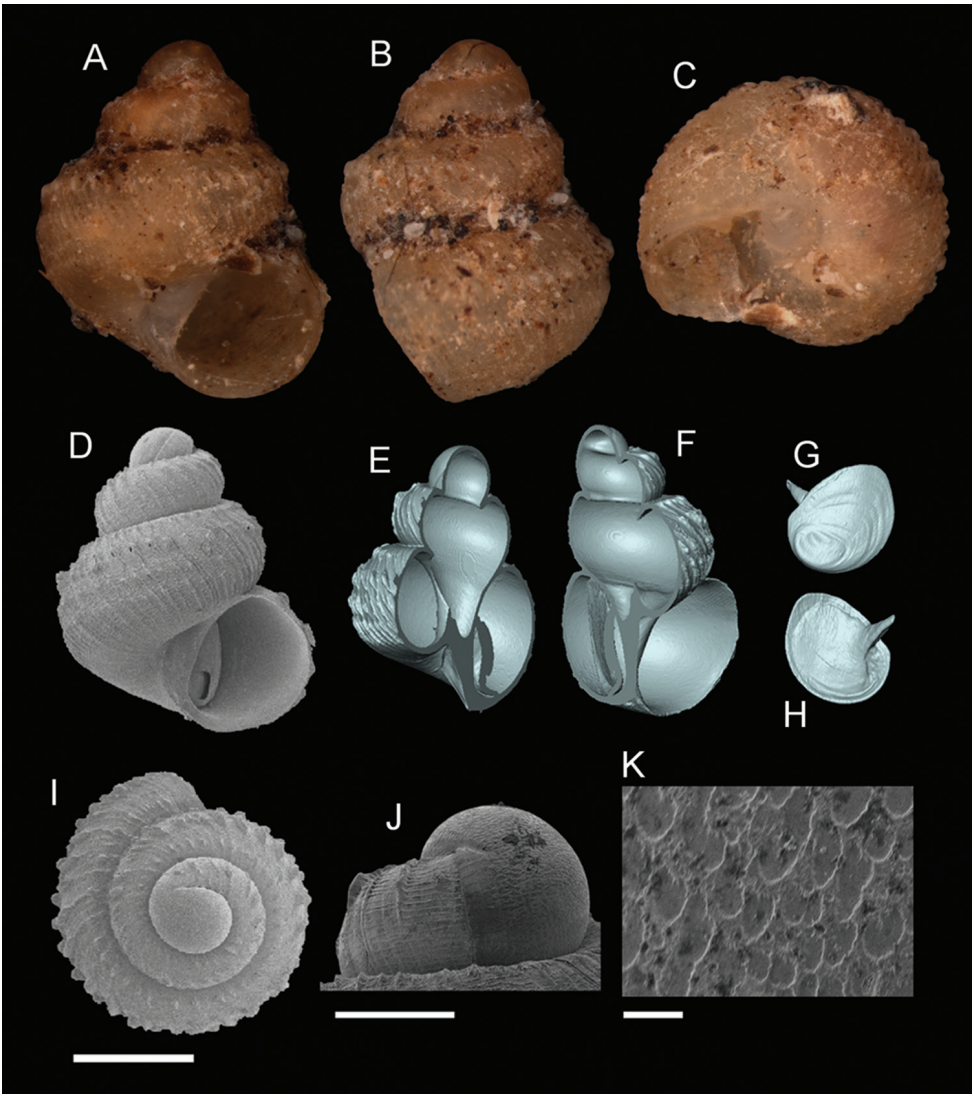


Figure 6. *Georissa saulae* (van Benthem-Jutting, 1966). **A–C** Holotype: ZMA/MOL 135599 **D–K** BOR/MOL 3493. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Other material. Simbaluyon limestone hill, Sabah, Malaysia: RMNH/MOL 333913, RMNH/MOL 333919. Crocker Range National Park, Gua Laing, Keningau, Sabah (05°29.00'N, 116°08.00'E): RMNH/MOL 335180, ZMA/MOLL 315592, ZMA/MOLL 315593, JJV 1119. Sepulut Valley, Gua Pungiton, Sabah (04°42.41'N, 116°36.04'E): BOR/MOL 28, BOR/MOL 12770, JJV 7544. Sepulut valley, Gua Sanaron, Sabah (04°42.05'N, 116°36.01'E): BOR/MOL 29, BOR/MOL 32, BOR/MOL

3493, JJV 7660. Pinangah valley, Batu Urun (Bukit Sinobang), Sabah (04°48.40'N, 116°37.35'E): BOR/MOL 31, JJV 1144, JJV 5632, JJV 7993. Mahua, Sabah: BOR/MOL 33. Pun Batu, Sepulut, Sabah (04°45.00'N, 116°10.00'E): JJV 1268. Sepulut valley, Batu Punggul, Sabah: JJV 1904.

Description. *Protoconch*. Color: red to brown. Sculpture: meshed – ellipsoidal mesh pattern. Mesh width: 29–54 µm. *Teleoconch*. Color: brown to red. First whorl: convex to rounded. Subsequent whorls: convex to rounded. SH: 1.32–1.86 mm, SW: 1.14–1.48 mm, SI: 1.12–1.26. Total number of whorls: 2 ½-3 ¼. *Shell sculpture*. Radial sculpture: often present, when formed by vertical connections between corresponding scales on successive spiral ribs. These vertical connections, especially on the first whorls, form evenly spaced ribs that are raised when crossing a spiral rib. Spiral sculpture: present at the early teleoconch, subsequently becoming weaker, and later only short discontinuous lines are visible in between the radial sculptures. Scales: usually three or four discontinuous series of vertical, low to high-projecting scales, broad to pointed (only if the spiral series of scales are discontinuous). *Aperture*. Shape: rounded to slightly oval. Basal side: rounded, slightly angular before the columellar region. Parietal side: straight, connected to the palatal edge. AH: 0.58–0.83 mm, AW: 0.70–0.94 mm, AI: 0.76–0.92. *Holotype dimension*. SH: 1.60 mm, SW: 1.28 mm, AH: 0.66 mm, AW: 0.80 mm.

Cross diagnosis. *Georissa saulae* possesses clear diagnostic shell characters for distinction from other "scaly" *Georissa* species. *G. saulae* lacks a clear formation of spiral ribbing: although the spiral arrangement of the scales gives the impression of spiral sculpture, no underlying ribs are discernable. *G. scalinella*, *G. kinabatanganensis*, and *G. hosei*, on the other hand, have clear diagnostic spiral ribs. The shell whorls of *G. saulae* are broad but not as rapidly expanding as in *G. hosei*, *G. scalinella* or *G. kinabatanganensis*. It can also be distinguished from *G. scalinella* and *G. hosei* on the basis of a more elongate-conical shell shape and the aperture shape that is more rounded rather than oval.

Distribution. The type locality of *Georissa saulae* is Laying cave, in the Crocker Range, Keningau, Sabah (a misspelling of Laing cave). Otherwise known from limestone outcrops in Sabah's interior, viz., Simbaluyon, Sinobang, Sanaron, and Pungiton, and also has been recorded from Mahua, Sabah, which is not a limestone area. Phung et al. (2017) also report it from Pulau Tiga, Sabah.

Molecular analysis. ML and Bayesian analyses show *Georissa saulae* (16S: n = 11) as a monophyletic group with 100% BS and 100% PP. Schilthuizen et al. (2012) reported that *G. saulae* is a paraphyletic group from which emerges the cave-dwelling species *G. filiasaulae* (Haase and Schilthuizen 2007), a fully unsculptured species that was not included in the present study. *G. saulae* + *G. filiasaulae* are sister to all other species in the "scaly group" (unpublished data).

Discussion. *Georissa saulae* was initially described as *Hydrocena saulae* van Benthem-Jutting, 1966, then assigned to the genus *Georissa* by Thompson and Dance (1983). Thompson and Dance (1983) compared *G. saulae* with *G. scalinella*, and even suggested *G. saulae* might be a subspecies. In contrast, we find that *G. saulae* is a proper

species with very distinct conchological characters, especially the presence of radial ribs on the shell, which makes it easy to identify. In some specimens from the entrance of the Batu Sanaron cave system, the vertical scales are spaced, and radial sculpture is weak. Such individuals presumably represent the hybrid zone with the cave-dwelling *G. filiasaulae* (Haase and Schilthuizen 2007, Schilthuizen et al. 2012).

Georissa hosei Godwin-Austen, 1889

Georissa hosei Godwin-Austen, 1889: 353, fig. 11 plate XXXIX; Smith 1893: 351, fig. 27 plate XXV; Thompson and Dance 1983: 116.

Type locality. Borneo. (Unspecified)

Type material. *Lectotype* (Designated by Thompson and Dance 1983). Borneo: NHMUK 1889.12.7.72 (glued on paper) (seen).

Other material. Jambusan, North Borneo: NHMUK 92.7.20.122, NHMUK 92.7.23.33-4. Gunung Liak/Padang, Kampung Skiat Baru, Jambusan, Sarawak (01°24.05'N, 110°11.19'E): MZU/MOL 16.04, MZU/MOL 16.05, MZU/MOL 16.06, MZU/MOL 16.07. Bukit Siboyuh, Kampung Skiat Baru, Jambusan, Sarawak (01°22.90'N, 110°11.69'E): MZU/MOL 16.08. Bukit Tongak, Bidi, Bau/Jambusan, Sarawak (01°22.67'N, 110°08.32'E): MZU/MOL 16.09.

Description. *Protoconch*. Color: red. Sculpture pattern: smooth. *Teleoconch*. Color: orange to red. First whorl: rounded or shouldered with flat surfaces above and below the shoulder. Subsequent whorls: convex to rounded; number of whorls: 2–2 ¼. SH: 1.06–1.55 mm, SW: 1.09–1.60 mm, SI: 0.94–1.12. *Shell sculpture*. Radial sculpture: absent, only weak growth lines. Spiral sculpture: present, weakly sculpted, continuous ribs, more prominent at the periphery. Scales: two to four series of low and broad vertical scales, regularly spaced, the upper scale series always the strongest, weaker series appear later at the spire, and the spaces between series are irregular. *Aperture*. Shape: oval. Basal side: rounded, angular at the columellar region. Parietal side: straight, palatal edge attached or removed at the body whorl. AH: 0.60–0.95 mm, AW: 0.80–1.16 mm, AI: 0.74–0.88.

Cross diagnosis. *Georissa hosei* has a diagnostic smooth protoconch. It possesses similar shell habitus and scale characters as *G. sepulutensis*, *G. pyrrhoderma*, and *G. kobelti*. However, the scales of *G. hosei* are rarely developed into large and acutely projected scales.

Distribution. Known from Gunung Liak/Padang and Bukit Siboyuh at Kampung Skiat Baru, Jambusan, and Bukit Tongak, in the area of Bau, which is close to Jambusan.

Molecular analysis. ML and Bayesian analyses shows that all *G. hosei* individuals (16S: n = 21; CO1: n = 11) group together in one clade with 100% BS and 100% PP, which is the sister group of all other "scaly group" species, except *G. saulae*.

Discussion. Godwin-Austen (1889), when he described the species, mentioned that the sides of the spire (whorls) are flat, which we find to be the case for the first whorl

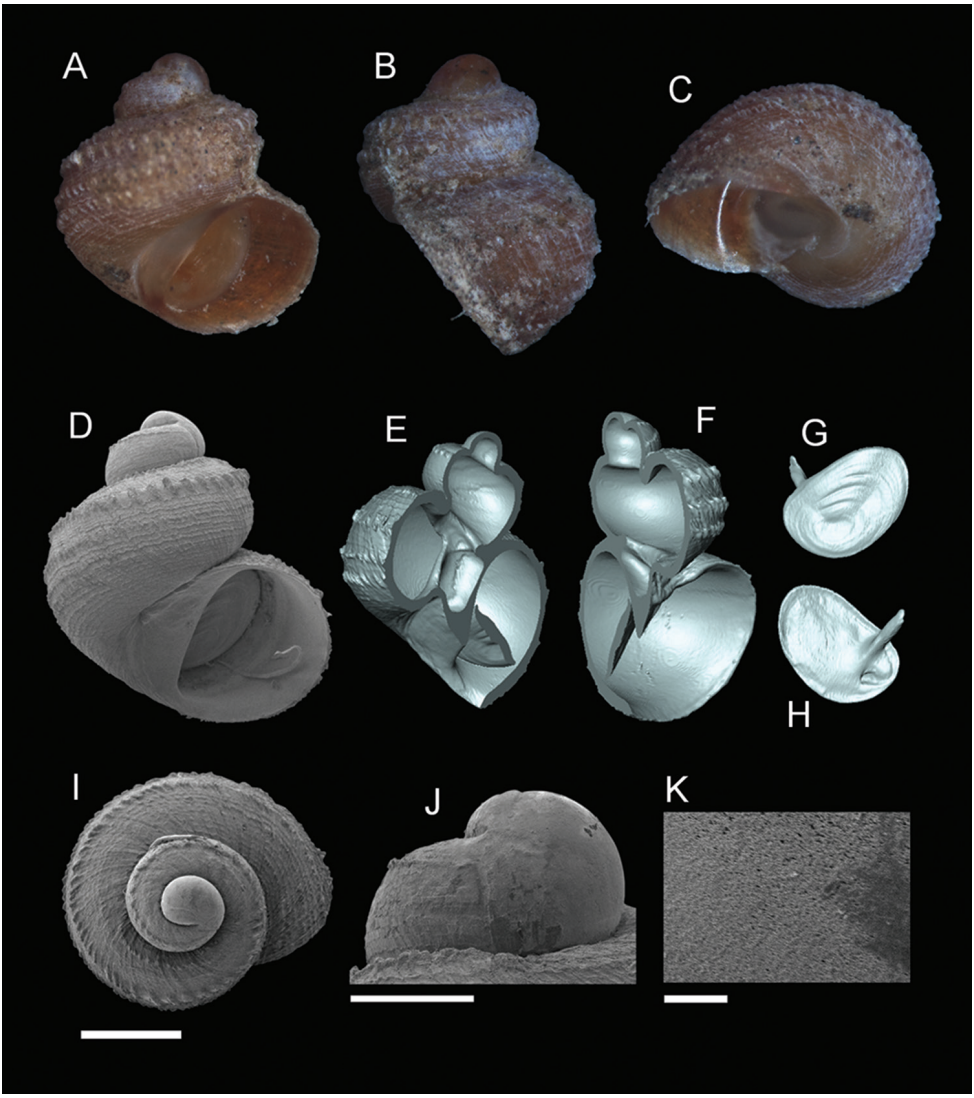


Figure 7. *Georissa hosei* Godwin-Austen, 1889. **A–C** MZU/MOL 16.05 **D–K** MZU/MOL 16.04. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

in our material (as well as in the lectotype). The exact type locality was not specified, but Smith (1893) reported that the specimens of *G. hosei* described by Godwin-Austen (1889) were from Jambusan, Sarawak. It has to be noted that *G. hosei* is highly variable in shell shape and sculpture, even within a local population. For example, material we collected at Gunung Liak/Padang have anything between two and four series of broad and low scales. Material from Bukit Tongak has three to four spiral threads with scales.

Material from Bukit Siboyuh, finally, is brighter in color (orange), with only one or two spiral series of scales. These three limestone outcrops are all within the area of not more than 10 km radius. Thompson and Dance (1983) noted that *G. hosei* is widely distributed in Sarawak, and they give Baram, Marudi, Niah, Tatau, and Bukit Sarang as localities. However, as we elaborate in this paper, many of these populations are not conspecific with *G. hosei*. For example, the image of "*G. hosei*" provided by Thompson and Dance (1983) – UF 35919, from Batu Gading, Baram, appears conspecific to *G. kobelti*. Also, their "*G. hosei*" from Bukit Sarang we here describe as a *G. anyiensis* sp. n.

***Georissa anyiensis* sp. n.**
http://zoobank.org/DD0DD84B-0363-4B68-9A93-877E3602DAE3

Georissa hosei Godwin-Austen: Thompson and Dance 1983: 117, materials from Tatau Valley, Bukit Sarang, Bintulu, Sarawak. (**non** *G. hosei* Goodwin-Austen, 1889)

Type locality. Bukit Anyi at Bukit Sarang, Bintulu, Sarawak, Malaysia (02°39.25'N, 113°02.72'E).

Type material. *Holotype*. Bukit Anyi at Bukit Sarang, Bintulu, Sarawak, Malaysia (02°39.25'N, 113°02.72'E): MZU/MOL 17.90 (leg. MZ Khalik and SK Reduan). *Paratypes*. Bukit Anyi at Bukit Sarang, Bintulu, Sarawak (02°39.25'N, 113°02.72'E): MZU/MOL 17.53, MZU/MOL 17.54, MZU/MOL 17.55, MZU/MOL 17.56, MZU/MOL 17.57, MZU/MOL 17.58, MZU/MOL 17.59, MZU/MOL 17.60, MZU/MOL 17.61, JJV 12840 (40), JJV 12841 (1). Bukit Lebik at Bukit Sarang, Bintulu, Sarawak (02°39.32'N, 113°02.43'E): MZU/MOL 17.50, MZU/MOL 17.51, MZU/MOL 17.52, JJV 12842 (20), JJV 12843 (1). From Thompson and Dance 1983, Bukit Sarang, Tatau valley (20°45'N, 113°02'E): UF 35914, UF 35915, UF 35921 (not seen). Each lot of examined paratypes from MZU are more than 50 individuals.

Etymology. Named after the hill Bukit Anyi at Bukit Sarang, Bintulu, Sarawak, Malaysia, the type locality.

Description. *Protoconch*. Color: yellow to orange. Sculpture pattern: meshed – rounded to ellipsoidal or undefined mesh shape. Mesh width: 8–30 μm. *Teleoconch*. Color: yellow. First whorl: shouldered, cylindrical. Subsequent whorls: convex to rounded, with a deeply impressed suture. SH: 1.39–1.98 mm, SW: 1.32–1.72 mm, SI: 1.05–1.08. Total number of whorls: 2 ¼–2 ¾. *Shell sculpture*. Radial sculpture: absent, only weak to strong growth lines are visible. Spiral sculpture: present, strongly sculpted, continuous ribs, more prominent at the periphery. Scales: at the shoulder a continuous spiral row of highly projecting diagonal crown-like scales; subordinate to that, three to four series of tall, broad or acute diagonal scales, regularly spaced, the uppermost of these always stronger than the lower ones, inter-series pacing irregular. *Aperture*. Shape: oval to rounded. Basal side: rounded, angular at the columellar region. Parietal side: straight. AH: 0.67–0.91 mm, AW: 0.90–1.17 mm, AI: 0.74–0.93. *Holotype dimension*. SH: 1.91 mm, SW: 1.72 mm, AH: 0.90 mm, AW: 1.14 mm.

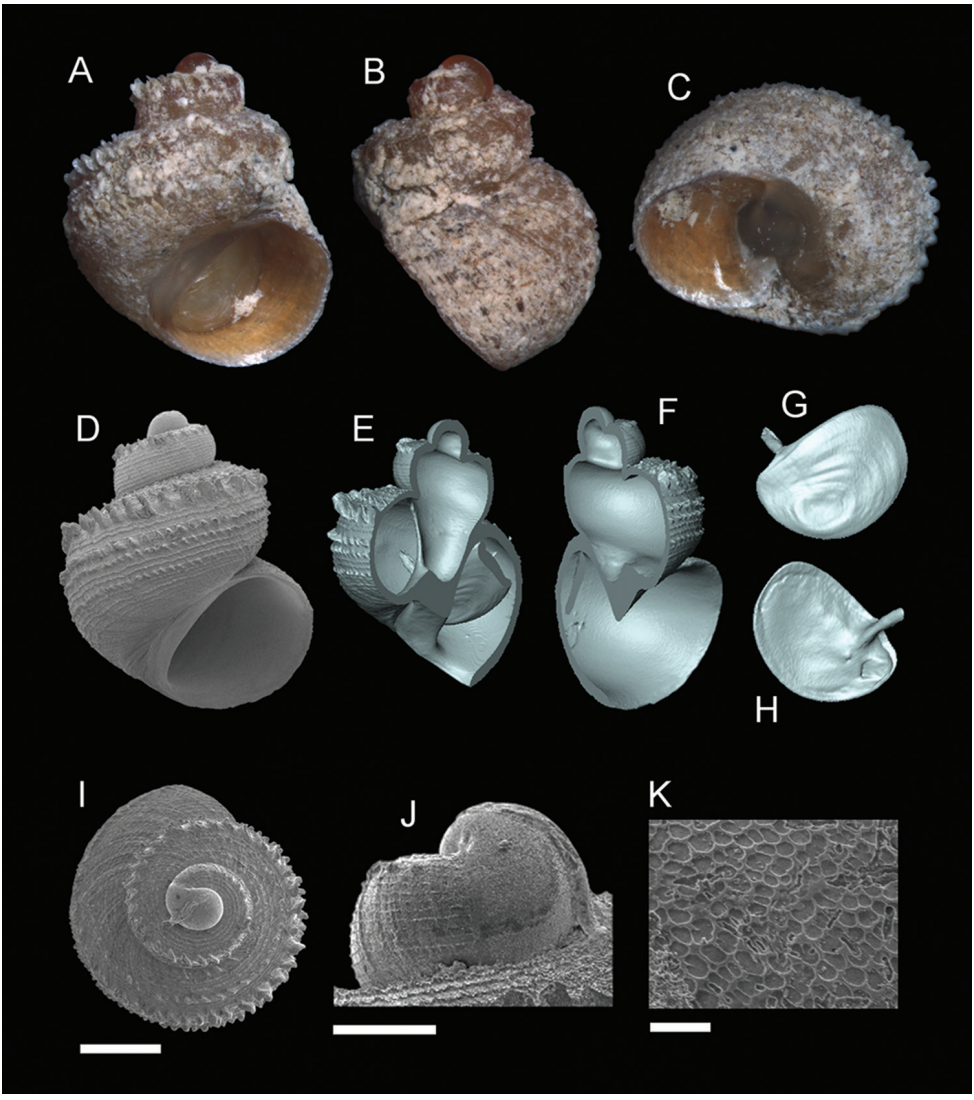


Figure 8. *Georissa anyiensis* sp. n. **A–C** Holotype: MZU/MOL 17.90 **D–K** Paratypes: MZU/MOL 17.55. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Cross diagnosis. In general, *G. anyiensis* has a shell shape that is similar to *G. kobelti*, *G. scalinella*, and *G. muluensis*. However, *G. anyiensis* has an extremely prominent, crown-like spiral series of large scales on the shell periphery, which distinguishes it from other "scaly" *Georissa*.

Distribution. Known from Bukit Anyi and Bukit Lebik, two isolated hills at Bukit Sarang, Bintulu, Sarawak.

Molecular analysis. ML and Bayesian analyses show that the *G. anyiensis* individuals (16S: n = 13; CO1: n = 12) form a monophyletic group with 100% BS and 100% PP, sister group to the four species *G. niahensis* + *G. kobelti* + *G. hadra* + *G. muluensis*.

***Georissa muluensis* sp. n.**
http://zoobank.org/8CAE7706-39F4-47ED-9275-7606DDD5FC26

Type locality. Lagang Cave, Mulu National Park, Mulu, Sarawak, Malaysia (04°03.06'N, 114°49.37'E).

Type material. *Holotype*. Lagang Cave, Mulu National Park, Mulu, Sarawak, Malaysia (04°03.06'N, 114°49.37'E): MZU/MOL 17.86 (leg. MZ Khalik and SK Reduan). *Paratypes*. Lagang Cave, Mulu National Park, Mulu, Sarawak (04°03.06'N, 114°49.37'E): MZU/MOL 17.30 (13), MZU/MOL 17.31 (9).

Other material. Deer Cave, Mulu National Park, Mulu, Sarawak: JJV 10533 (this sample, approximately 120 individuals, also contains specimens of *G. hadra*), JJV 10554 (this sample contains 5 individual of *G. muluensis*, 1 individual *G. hadra*), JJV 10533 (this sample, approximately 150 individuals, also contains specimens of *G. hadra* and *G. kobelti*). Mulu N.P., Mulu, Sarawak: JJV 10527.

Etymology. Named after Mulu National Park, Sarawak, Malaysia, the type locality.

Description. *Protoconch*. Color: yellow. Sculpture pattern: meshed – ellipsoidal mesh shape. Mesh width: 16–26 μm. *Teleoconch*. Color: yellow. First whorl: shouldered, above the shoulder flat, nearly horizontal; below the shoulder flat, cylindrical, but abruptly withdrawn into the deeply incised suture. Subsequent whorls: convex to rounded. SH: 1.67–2.05 mm, SW: 1.57–1.79 mm, SI: 1.08–1.18. Total number of whorls: 2 ¼-3. *Shell sculpture*. Radial sculpture: absent, only weak to strong growth lines are visible. Spiral sculpture: present, consisting of thin, but strongly sculpted and continuous ribs. Scales: two to three series of tall and diagonal scales, regularly spaced, the upper scale series always stronger than the lower ones, weaker series appear later at the spire or consist only of randomly spaced arrays of acute nodules, widely spaced between the first and second scale series, more densely spaced later. *Aperture*. Shape: rounded to slightly oval. Basal side: rounded, angular at the columellar region. Parietal side: straight to slightly curved. AH: 0.82–0.98 mm, AW: 1.03–1.18 mm, AI: 0.77–0.83. *Holotype dimension*. SH: 1.67 mm, SW: 1.53 mm, AH: 0.82 mm, AW: 1.07 mm.

Cross diagnosis. The wide spacing of the major spiral scale series of *G. muluensis* is similar to *G. kinabatanganensis*, but *G. muluensis* has a more elongated shell shape, rather than the more flattened habitus of *G. kinabatanganensis*. In general shell shape and sculpture *G. muluensis* also resembles *G. hadra*, which, however, is larger and more elongated.

Distribution. Known only from the small area of Lagang Cave, Mulu National Park, Mulu, Sarawak, Malaysia.

Molecular analysis. ML and Bayesian analyses show that the individuals of *G. muluensis* (16S: n = 4; CO1: n = 4) form a monophyletic group with 100% BS and 100% PP, which is the sister group of *G. hadra*.

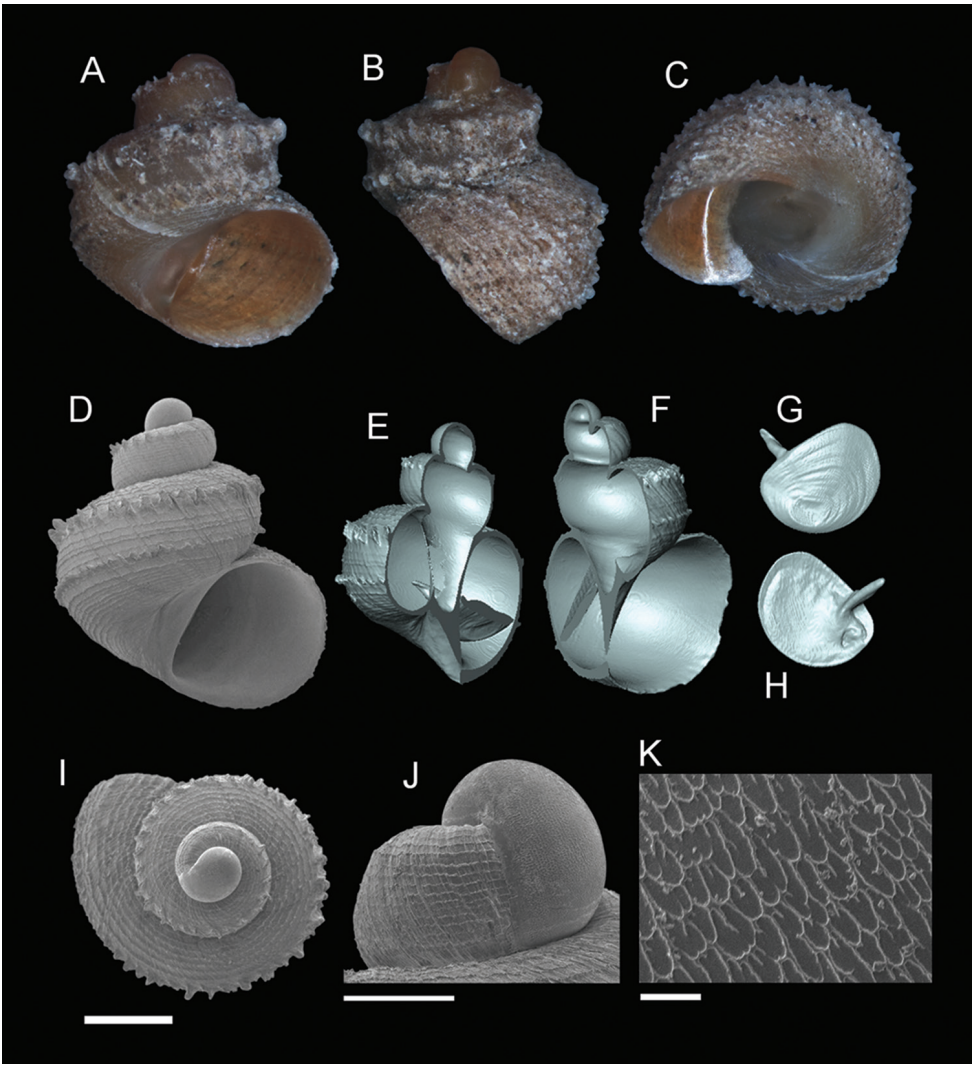


Figure 9. *Georissa muluensis* sp. n. **A–C** Holotype: MZU/MOL 17.86 **D–K** Paratypes: MZU/MOL 17.30. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view. **K**. Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Georissa hadra Thompson & Dance, 1983

Georissa hadra Thompson & Dance, 1983: 115–116, fig. 32, 43–46.

Type locality. Butik Besungai, a small limestone hill 0.5 miles southwest of Batu Gading, and about 4 miles northeast of Long Lama, Baram Valley, Fourth Division, Sarawak. 03°52'N, 114°25'E.

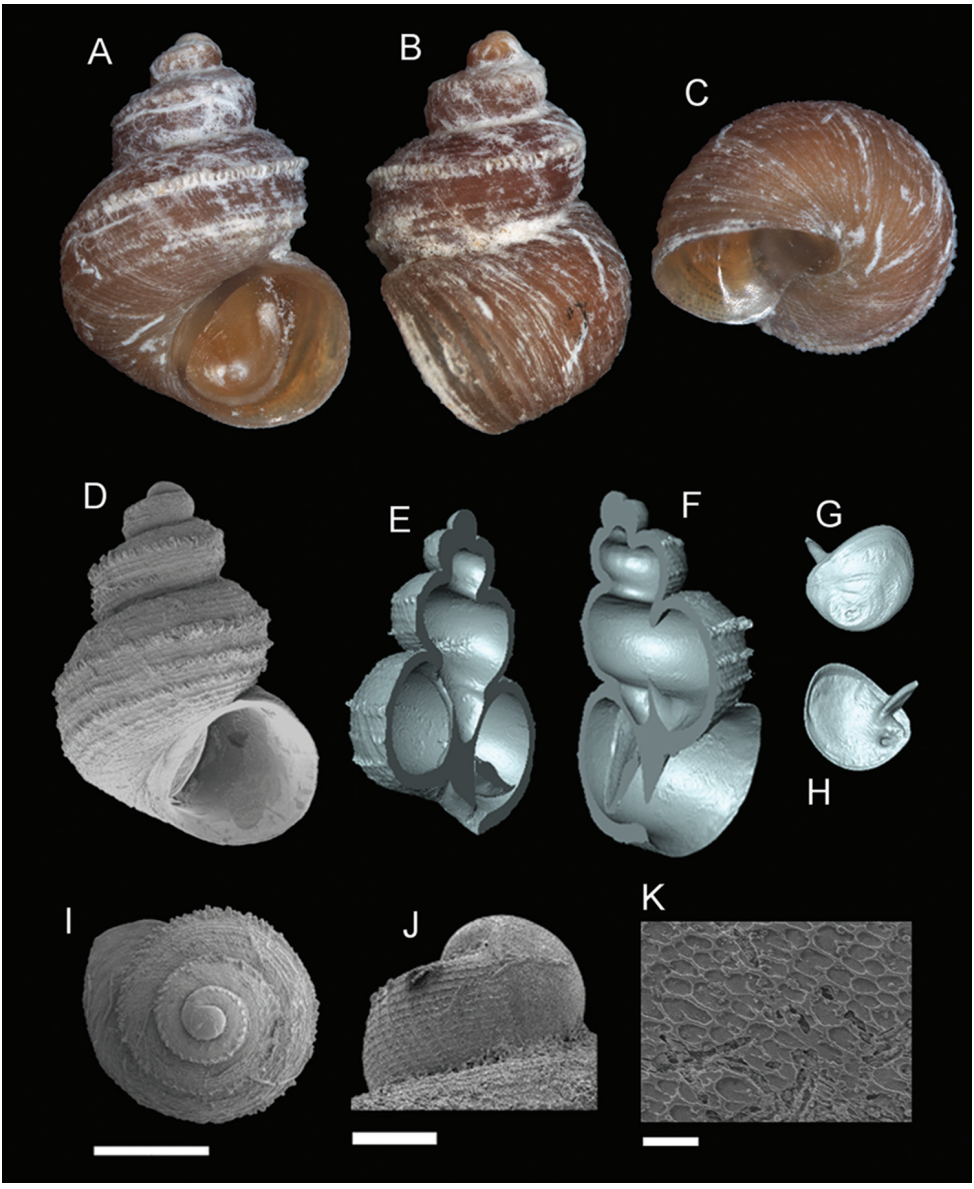


Figure 10. *Georissa hadra* Thompson & Dance, 1983. **A–C** MZU/MOL 17.33 **D–K** ZMA/MOLL 17.32. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 1 mm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Type material. *Holotype*. Butik Besungai, a small limestone hill 0.5 miles southwest of Batu Gading, and about 4 miles northeast of Long Lama, Baram Valley, Fourth Division, Sarawak: UF36107 (not seen). *Paratypes*. Butik Besungai ½ mile SW. of

Batu Gading, 4 miles NE. of Long Lama, Baram Valley, 4th Div., Sarawak, Malaysia: BMNH 1984004 (seen). Baram valley, Long Lama, Bt. Besungai 0.5 m SW of Batu Gading, Sarawak (03°52.00'N, 114°25.00'E): JJV 13421 (seen).

Other material. Lang Cave, Mulu N.P., Mulu, Sarawak (04°01.49'N, 114°49.48'E): MZU/MOL 17.32, MZU/MOL 17.33, MZU/MOL 17.34, MZU/MOL 17.35. Deer Cave, Mulu N.P., Mulu, Sarawak: JJV 10533 (this sample, approximately 120 individuals, also contains *G. muluensis*), JJV 10554 (5 individual of *G. muluensis*, 1 individual *G. hadra*), JJV 10533 (this sample, approximately 150 individuals, also contains *G. muluensis* and *G. kobelti*).

Butik = a misspelling of Bukit, a local name for hill.

Description. *Protoconch*. Color: orange. Sculpture pattern: meshed – rounded to ellipsoidal or undefined mesh shape. Mesh width: 12–24 μm. *Teleoconch*. Color: orange. First whorl: with a distinct shoulder (provided with a series of minuscule scales), above the shoulder flat and tapering towards the suture, below the shoulder flat and cylindrical. Subsequent whorls: distinctly scalariform, with three separate aspects separated by two or more main spiral series of scales: above the uppermost spiral series gently curved towards the suture; in between both spiral series flat and cylindrical; below the lowest spiral series abruptly narrowed towards the deeply impressed suture (on the final whorl these three aspects fuse, forming a uniformly rounded impression). SH: 2.61–2.91 mm, SW: 2.05–2.19 mm, SI: 1.21–1.37. Total number of whorls: 2 ¾-3 ¼. *Shell sculpture*. Radial sculpture: absent, but with strong and unevenly layered growth lines. Spiral sculpture: present, weakly sculpted, continuous to discontinuous. Scales: two to four irregularly spaced series of low to high, and minute to broad diagonal scales, densely spaced, the first scale series always the strongest, weaker series appearing later at the spire. *Aperture*. Shape: rounded, with a tilt below the palatal side. Basal side: rounded, angular at the columellar region. Parietal side: straight to curved. AH: 1.11–1.33 mm, AW: 1.32–1.48 mm, AI: 0.83–1.01.

Cross diagnosis. *Georissa hadra* has scales which are densely arranged, unlike *G. scalinella*, *G. hosei*, *G. muluensis*, *G. anyiensis*, and *G. kobelti*, which have more widely spaced scales. In shell shape, *G. hadra* is similar to the later three species but larger and more distinctly scalariform. *G. hadra* is similar in size to *G. niahensis*, but it has a more slender habitus and a more rounded periphery.

Distribution. The type locality for *G. hadra* is Bukit Besungai, Baram, Sarawak. We also obtained it at Mulu, Sarawak. Currently, therefore, the known distribution range is restricted to Mulu and Baram.

Molecular analysis. ML and Bayesian analyses retrieve *G. hadra* (16S: n = 4; CO1: n = 4) as a single clade with 89% BS and 100% PP, sister to *G. muluensis*.

Discussion. The paratypes of Thompson and Dance (1983) have a very pale orange color, compared to recently collected materials from Mulu; presumably the color has faded.

Georissa kobelti Gredler, 1902

Georissa kobelti Gredler, 1902: 61; Zilch 1973: 265, fig. 11; Thompson and Dance 1983: 117, fig. 28, 50–52.

Georissa hosei Godwin-Austen: Thompson and Dance 1983: 117, fig. 47–49, material from Bukit Besungai at Baram Valley, Niah, Kejin trib. of Baram river (**non** *G. hosei* Goodwin-Austen, 1889).

Type locality. Niah, Baram (Sarawak, Borneo). Unspecified.

Type material. *Lectotype* (Designated by Zilch 1973). Niah, Baram (Sarawak, Borneo): SMF 215893a (not seen).

Other material. Trade Cave, Niah National Park, Niah, Sarawak (03°49.13'N, 113°46.86'E): MZU/MOL 17.36. Great Cave, Niah National Park, Niah, Sarawak: MZU/MOL 17.37. Bukit Kaijin, Baram, Sarawak (03°41.75'N, 114°27.55'E): MZU/MOL 17.38, MZU/MOL 17.39, MZU/MOL 17.40, MZU/MOL 17.41, MZU/MOL 17.42, MZU/MOL 17.43, MZU/MOL 17.44, MZU/MOL 17.45, MZU/MOL 17.46, MZU/MOL 17.47, MZU/MOL 17.48, MZU/MOL 17.49, JJV 10217. Bukit Kasut, Niah N.P., Niah, Sarawak: JJV 10254. Niah N.P., Niah, Sarawak: JJV 1565, JJV 5466, JJV 10306, JJV 10392. Deer Cave, Mulu N.P., Mulu, Sarawak: JJV 10533 (the sample, approximately 150 individuals, also contains *G. muluensis* and *G. hadra*). Tatau river valley, Bukit Sarang, Bintulu, Sarawak: JJV 12551, JJV 12838. From Thompson and Dance 1983, Niah, Baram (Sarawak, Borneo): UF 35919, UF 36179 (not seen).

Description. *Protoconch*. Color: orange to red. Sculpture: meshed – semi-oval mesh shape. Mesh width: 11–22 µm. *Teleoconch*. Color: ranging from red to yellow. First whorl: convex to rounded. Subsequent whorls: convex to rounded. SH: 1.75–2.11 mm, SW: 1.48–1.75 mm, SI: 1.18–1.28. Total number of whorls: 2 ¾-3. *Shell sculpture*. Radial sculpture: absent, only weak growth lines. Spiral sculpture: present with thin but strongly continuous spiral ribs, forming small acute scales near the suture. Scales: three to four spiral rows of tilted, nearly vertical scales, the upper series stronger than the lower ones, scale prominence ranging from high to low and from small and acute to broadly sculpted and ear-like. Scales are regularly spaced, as are the scale series themselves. *Aperture*. Shape: rounded to oval. Basal side: rounded, angular before the columellar region. Parietal side: curved. AH: 0.82–1.04 mm, AW: 1.02–1.17 mm, AI: 0.71–0.90.

Cross diagnosis. The image of the *G. kobelti* lectotype by Zilch (1973) does not provide detailed information about the scale characters of *G. kobelti* as compared to the images of the individual from UF provided by Thompson and Dance (1983), which clearly shows the diagnostic characters of the ear-like scale pattern of this species. In shell habitus, *G. kobelti* is similar to some populations of *G. anyiensis*, *G. saulae*, and *G. hosei*, but these species differ from *G. kobelti* by the pattern of their diagonal scales.

Distribution. The lectotype in Senckenberg (SMF 215893a) was obtained from an unspecified location. As far as known, the species is restricted to the area of Niah to

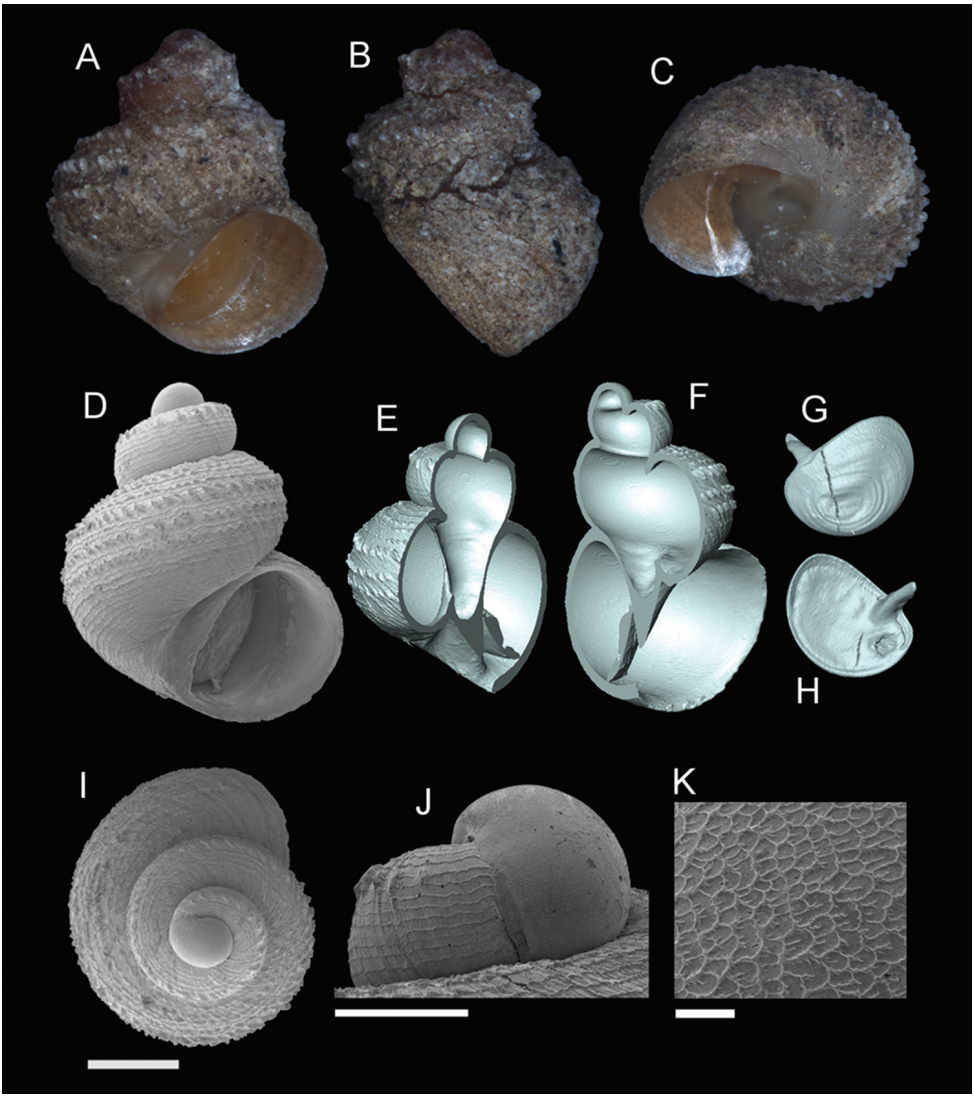


Figure 11. *Georissa kobelti* Gredler, 1902. **A–C** MZU/MOL 17.40 **D–K** MZU/MOL 17.38. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Baram, northern Sarawak. Thompson and Dance (1983) also stated that they examined this species from Beluru, which is located between Niah and Baram.

Molecular analysis. In the ML and Bayesian analyses of *G. kobelti* (16S: n = 8; CO1: n = 8), the Niah and Baram populations form highly supported clades (99% and 100% BS, respectively, and 100% PP for both clades), which are paraphyletic with respect to *G. niahensis*.

Georissa niahensis Godwin-Austen, 1889

Georissa niahensis Godwin-Austen, 1889: 353; Thompson and Dance 1983: 119.

Type locality. Niah Hills, Borneo. (Unspecified)

Type material. *Lectotype* (Designated by Thompson and Dance 1983). Niah Hills, Borneo: NHMUK 1889.12.7.69 (glued on paper) (seen). *Paralectotype*. Niah Hills, Borneo: NHMUK 1889.12.7.70 (glued on paper) (seen).

Other material. Painted Cave, Niah National Park, Niah, Sarawak (03°48.68'N, 113°47.25'E): MZU/MOL 17.25.

Description. *Protoconch*. Color: red. Sculpture pattern: smooth and meshed – ellipsoid to irregular mesh shape. Mesh width: 12–19 μm. *Teleoconch*. Color: orange to red. First whorl: curved above the shoulder, flat and cylindrical below the shoulder. Subsequent whorls: convex, angular at the periphery. SH: 1.81–2.53 mm, SW: 1.51–1.99 mm, SI: 1.10–1.29. Total number of whorls: 3–3 ¼. *Shell sculpture*. Radial sculpture: absent, only strong and unevenly layered growth lines. Spiral sculpture: present, strongly sculpted, continuous to discontinuous, well defined from the first whorl all the way to the peristome. Scales: a single spiral series of low and minute acute scales, regularly spaced at the first whorl, but weaker, grading to imperceptible on the body whorl. *Aperture*. Shape: rounded. Basal side: rounded, angular at the columellar region. Parietal side: straight to curved. AH: 0.85–1.24 mm, AW: 0.92–1.27 mm, AI: 0.83–1.02.

Cross diagnosis. *Georissa niahensis* has a distinctive single series of small scales on the whorl shoulder, close to the suture. *G. niahensis* is one of the largest Bornean *Georissa*, in shell size only matched by *G. hadra* (which, however, is more slender, angular at the shoulder and has a flat to slightly rounded whorls). In general shell shape, *G. niahensis* is closest to *G. kobelti*, but the latter species is more rounded, while *G. niahensis* has a distinctly convex periphery.

Distribution. Known to occur only at Niah, Sarawak.

Molecular analysis. ML and Bayesian analyses of *G. niahensis* (16S: n = 8; CO1: n = 7) showed that all *G. niahensis* specimens form one clade with 100% BS and 100% PP. The sister group is the *G. kobelti* population from Baram (*G. kobelti* is paraphyletic).

Discussion. Both Godwin-Austen (1889) and Thompson and Dance (1983) did not mention anything about the small scale-like nodules close to the suture of *G. niahensis*. Godwin-Austen (1889): "*Shell elongately conoid, solid, imperforate; sculpture a very in-distinct, ill-defined spiral liration, about 20 on the penultimate whorl, upon a rough surface crossed by transverse lines of growth; color ruddy ochre; spire high; apex pointed, finely papillated, minutely lirate; suture impressed; whorls 4 ½ convex; aperture oval, oblique; peristome simple, acute below; columellar margin straight*". Thompson and Dance (1983): "*G. niahensis is similar in sculpture to G. williamsi but is much larger. G. niahensis also shows similarities to the hosei group in the depth of the suture and the*

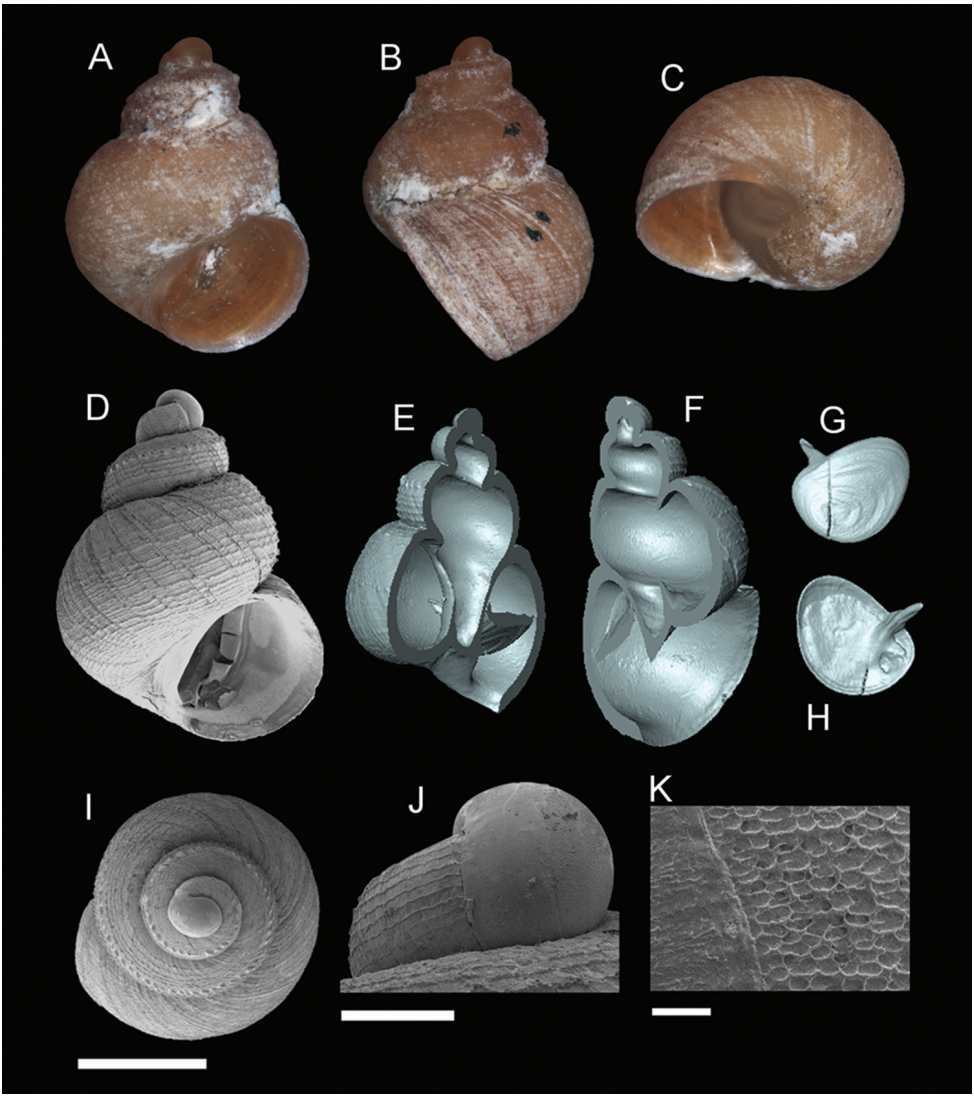


Figure 12. *Georissa niahensis* Godwin-Austen, 1889. **A–K** MZU/MOL 17.25 **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 1 mm (**A–I**); 200 μm (**J**); 10 μm (**K**).

relatively rapid expanding whorls, but it lacks the node-like sculpture found among species of that group." The scales are relatively small which are not very conspicuous among the strong growth lines, and this is the reason why in the previous description of the species the scale characters were lacking. Thompson and Dance (1983) compared *G. niahensis* with what they call the *hosei* group, based on the size and the deeply impressed suture.

***Georissa silaburensis* sp. n.**
http://zoobank.org/E88C99A8-8A0D-4438-9699-9AA86CAEE217

Type locality. Gunong Silabur, Serian, Sarawak, Malaysia (00°57.28'N, 110°30.22'E).

Type material. *Holotype*. Gunong Silabur, Serian, Sarawak, Malaysia (00°57.28'N, 110°30.22'E): MZU/MOL 17.88 (leg. MZ Khalik and SK Reduan). *Paratypes*. Gunong Silabur, Serian, Sarawak, Malaysia (00°57.28'N, 110°30.22'E): MZU/MOL 17.01, MZU/MOL 17.02, MZU/MOL 17.03, MZU/MOL 17.04, MZU/MOL 17.05, MZU/MOL 17.06, MZU/MOL 17.07, MZU/MOL 17.08. Borneo, Sarawak, First Division, western side of Gunong Selabor, Semabang entrance to Lobang Batu Cave (00°55'N, 110°25'E): NHMUK 1984005 (seen). Each lot of examined paratypes from MZU are more than 50 individuals.

Etymology. Named after Gunung Silabur, Serian, Sarawak, Malaysia, the type locality.

Description. *Protoconch*. Color: red. Sculpture pattern: meshed – round to irregular mesh pattern. Mesh width: 8–18 μm. *Teleoconch*. Color: red. First whorl: rounded. Subsequent whorls: convex, number of whorls: 2–2 ¼. SH: 1.59–1.99 mm, SW: 1.50–1.76 mm, SI: 1.06–1.13. *Shell sculpture*. Radial sculpture: absent or weak to strong growth lines. Spiral sculpture: present, thin but strongly sculpted, continuous ribs, more prominent at the periphery. Scales: two to six or more randomly sculpted series of low and broad horizontal scales, or else acute horizontal nodules on the spiral sculpture, scale series irregularly spaced, which series is the most prominent is not consistent across individuals. *Aperture*. Shape: rounded. Basal side: rounded, angular at the columellar region. Parietal side: straight, palatal edge attached to slightly removed from the body whorl. AH: 0.95–1.09 mm, AW: 1.00–1.17 mm, AI: 0.92–0.99. *Holotype dimension*. SH: 1.68 mm, SW: 1.53 mm, AH: 0.95 mm, AW: 1.09 mm.

Cross diagnosis. The shell shape of *G. silaburensis* is distinct compared to other "scaly group" *Georissa*. It has rapid shell expansion like *G. hosei* and *G. scalinella*, but *G. silaburensis* has a different sculpture, consisting of horizontal, rather than vertical or diagonal scales. In addition, the whorls are rounded and convex, with the aperture almost circular, close to *G. saulae*.

Distribution. Known from the inside of the cave system of Gunung Silabur, Serian, Sarawak.

Molecular analysis. ML and Bayesian analyses show that the individuals of *G. silaburensis* (16S: n = 10; CO1: n = 9) form one clade with 95% BS and 98% PP, the sister group of *G. bauensis*.

Discussion. *Georissa silaburensis* was only found inside the cave entrance, with water flowing from the cave roof, and approximately less than 50% light penetration. In shell shape and reduced sculpture, it resembles another cave specialist, *G. filiasaulae*.

***Georissa bauensis* sp. n.**
http://zoobank.org/6E333906-75DD-4055-BD32-578E0D651E1F

Type locality. Wind Cave Passage 3, Wind Cave Nature Reserve, Bau, Sarawak, Malaysia (01°24.81'N, 110°08.17'E).

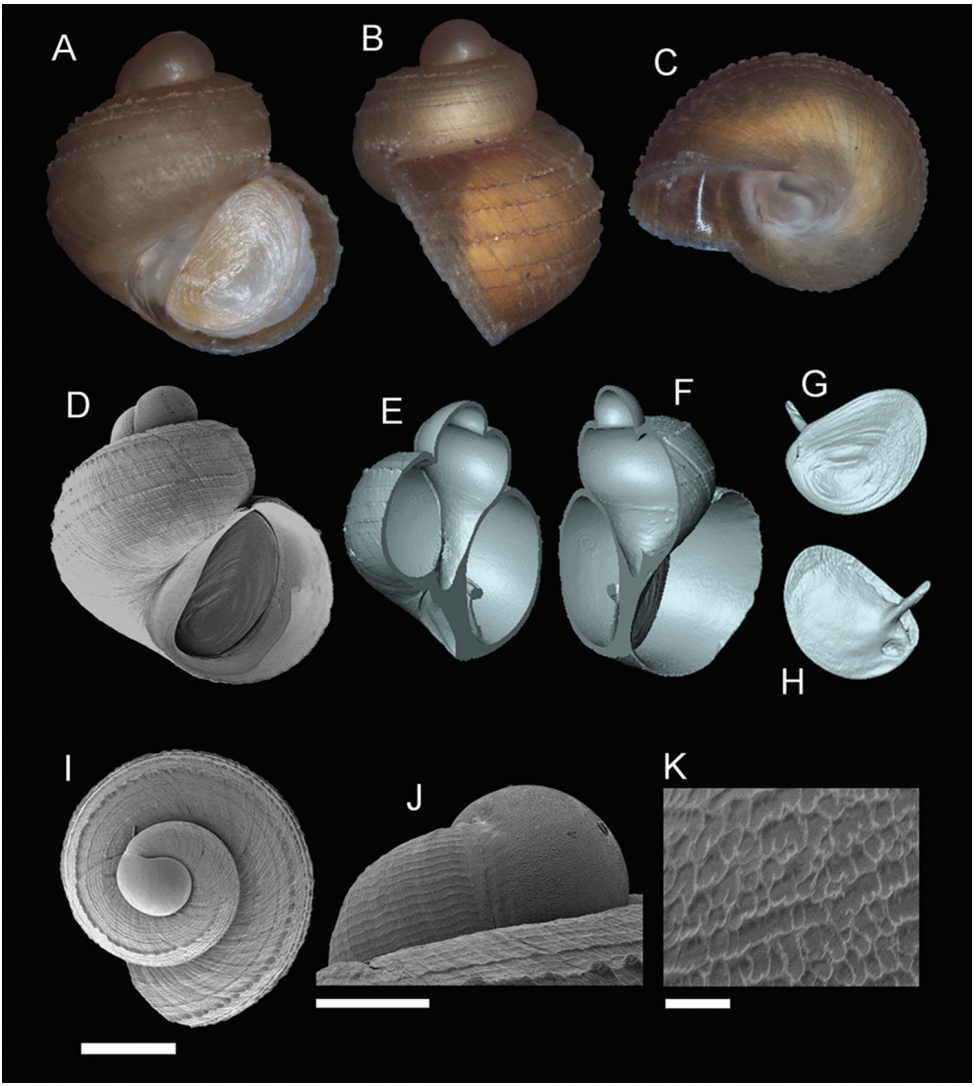


Figure 13. *Georissa silaburensis* sp. n. **A–C** Holotype: MZU/MOL 17.88 **D–K** Paratypes: MZU/MOL 17.04. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Type material. *Holotype*. Wind Cave Passage 3, Wind Cave Nature Reserve, Bau, Sarawak, Malaysia (01°24.81'N, 110°08.17'E): MZU/MOL 17.89 (leg. MZ Khalik). *Paratypes*. Wind Cave Passage 3, Wind Cave Nature Reserve, Bau, Sarawak, Malaysia (01°24.81'N, 110°08.17'E): MZU/MOL 16.01 (25), MZU/MOL 16.02 (>50). Gunung Podam, near Sungai Ayup, Kampung Bogag, Bau, Sarawak, Malaysia (01°21.15'N, 110°03.57'E): MZU/MOL 16.03 (5).

Etymology. Named after the district of Bau, Sarawak, Malaysia, where the type locality Wind Cave Nature Reserve is located.

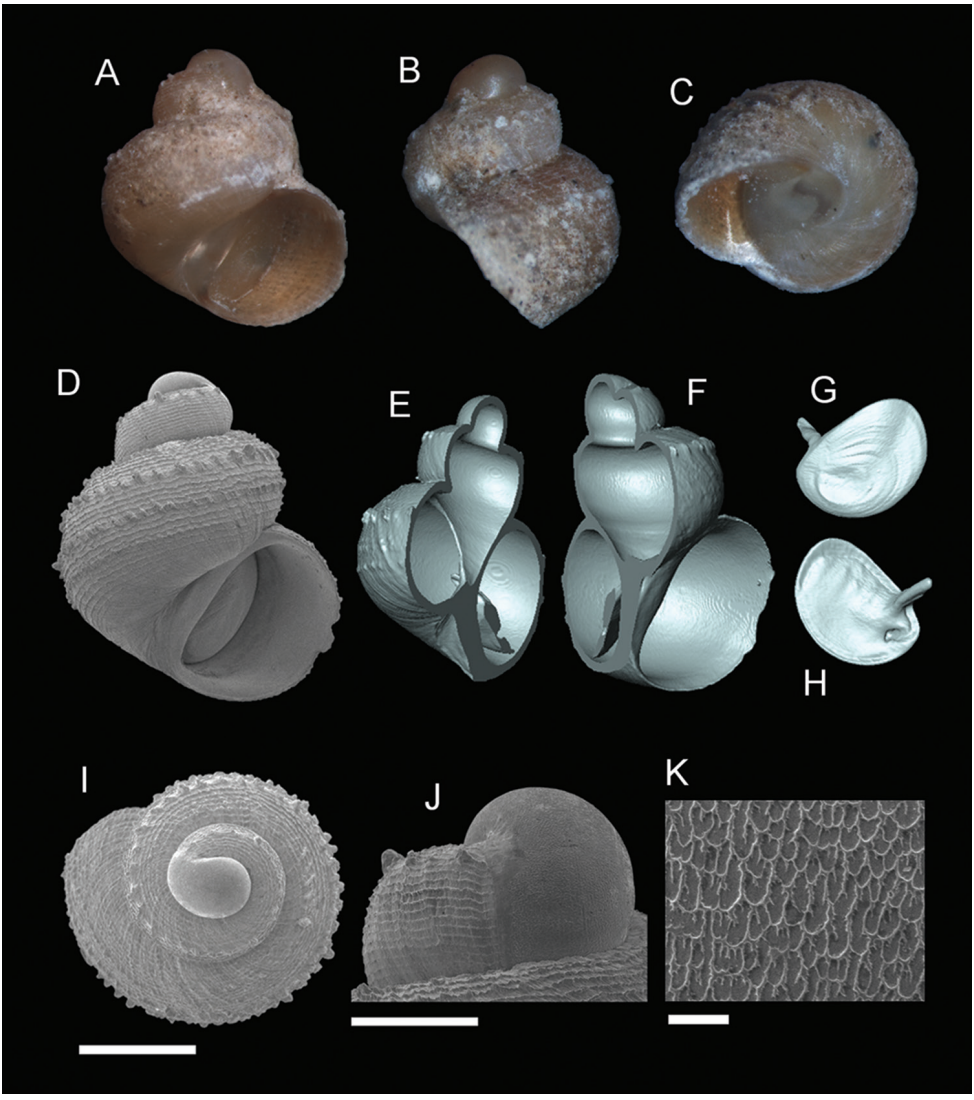


Figure 14. *Georissa bauensis* sp. n. **A–C** Holotype: MZU/MOL 17.89 **D–K** Paratypes: MZU/MOL 16.03. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Description. *Protoconch*. Color: red. Sculpture pattern: meshed – rounded or irregular mesh shape. Mesh width: 12–22 μm. *Teleoconch*. Color: orange to red. First whorl: shouldered, flat both above and below the shoulder. Subsequent whorls: convex shoulder and more rounded at the periphery. SH: 1.16–1.62 mm, SW: 1.06–1.30 mm, SI: 1.02–1.25. Total number of whorls: 2–2 ½. *Shell sculpture*. Radial sculpture: absent, only weak growth lines are visible. Spiral sculpture: present, weakly to strongly

sculpted, continuous to discontinuous ribs, more prominent at the periphery. Scales: two to three major spiral series of low and small diagonal scales, regularly spaced, the upper series always stronger than the lower ones, scale series irregularly spaced. *Aperture*. Shape: rounded and tilted below. Basal side: rounded, angular at the columellar region. Parietal side: straight. AH: 0.57–0.78 mm, AW: 0.69–0.86 mm, AI: 0.74–0.96. *Holotype dimension*. SH: 1.16 mm, SW: 1.06 mm, AH: 0.58 mm, AW: 0.70 mm.

Cross diagnosis. *Georissa bauensis* is very similar to *G. kobelti* (although not closely related phylogenetically), in terms of general shell shape and spiral scale characters. However, *G. bauensis* is sufficiently variable to include specimens that are more similar to *G. hosei* and *G. scalinella*. Furthermore, *G. bauensis* has more strongly sculpted scales than *G. hosei*, and a more rounded and convex shell than *G. scalinella*.

Distribution. Known from Gunung Podam and Wind Cave Nature Reserve, Bau, Sarawak.

Molecular analysis. ML and Bayesian analyses resolve all individuals of *G. bauensis* (16S: n = 13; CO1: n = 8) as a monophyletic group with 99% BS and 100% PP, the sister group of *G. silaburensis*.

Georissa pyrrhoderma Thompson & Dance, 1983

Georissa pyrrhoderma Thompson & Dance, 1983: 123, fig. 64.
Georissa pyrrhoderma van Benthem-Jutting, in Beron 2015: 181.

Type locality. Borneo, Sarawak, First Division, western side of Gunong Selabor, Semabang entrance to Lobang Batu Cave (00°55'N, 110°25'E).

Type material. *Holotype*. Borneo, Sarawak, First Division, western side of Gunong Selabor, Semabang entrance to Lobang Batu Cave: UF36183 (not seen). *Paratypes*. Borneo, Sarawak, First Division, western side of Gunong Selabor, Semabang entrance to Lobang Batu Cave: UF 36184, UF 36185 (not seen).

Other materials. Gunong Silabur, Serian, Sarawak, Malaysia (00°57.45'N, 110°30.20'E): MZU/MOL 17.09, MZU/MOL 17.10, MZU/MOL 17.11, MZU/MOL 17.12, MZU/MOL 17.13, MZU/MOL 17.14, MZU/MOL 17.15, MZU/MOL 17.16, MZU/MOL 17.17, MZU/MOL 17.18, MZU/MOL 17.19, MZU/MOL 17.20, MZU/MOL 17.21, MZU/MOL 17.22, MZU/MOL 17.23, MZU/MOL 17.24.

Description. *Protoconch*. Color: red to brown. Sculpture pattern: smooth to meshed, with ellipsoid mesh shape. Mesh width: 11–26 µm. *Teleoconch*. Color: brown to red. First whorl: shouldered, slightly curved above the shoulder, flat, cylindrical below the shoulder. Subsequent whorls: initially shouldered, but soon grading into uniformly rounded and quickly expanding whorls, with a deeply impressed suture; number of whorls: 2 ¼-2 ½. SH: 1.16–1.31 mm, SW: 1.12–1.20 mm, SI: 1.03–1.09. *Shell sculpture*. Radial sculpture: absent, only weak to strong growth lines are visible. Spiral sculpture: present, strong spiral sculpture. Scales: a single series of low, small and acute, unevenly spaced scales above the periphery, occasionally, in the vicinity of

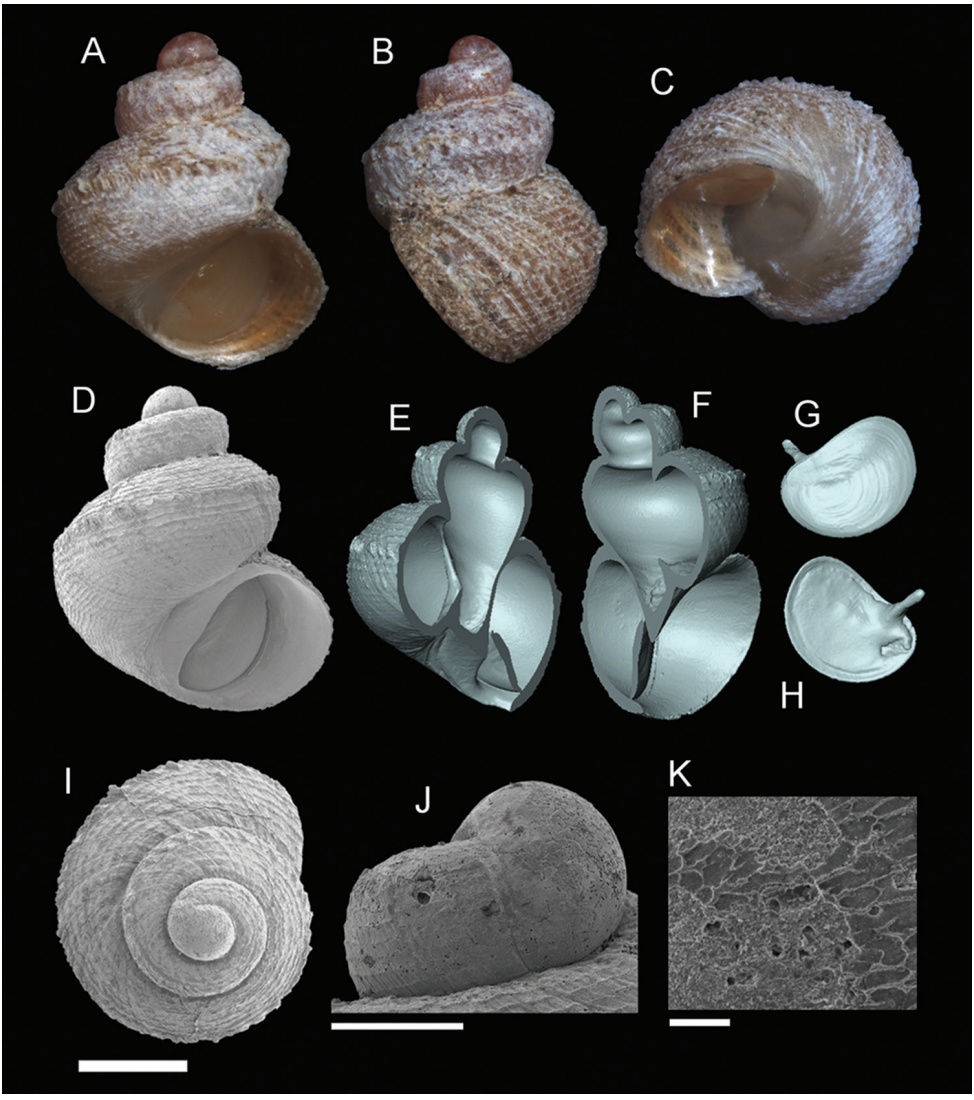


Figure 15. *Georissa pyrrhoderma* Thompson & Dance, 1983. **A–C** MZU/MOL 17.10 **D–K** MZU/MOL 17.09. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

the aperture, subordinate series of minute scales accompany the major series. *Aperture*. Shape: rounded, tilted below the palatal side. Basal side: rounded, strongly angular at the columellar region. Parietal side: straight, palatal edge attached to the body whorl. AH: 0.58–0.63 mm, AW: 0.75–0.85 mm, AI: 0.73–0.81.

Cross diagnosis. *Georissa pyrrhoderma* has a shell habitus that is similar to *G. kobelti*, *G. hosei*, and *G. sepulutensis*. The latter two species are high variable and are

morphologically, especially in sculpture, closely related to *G. pyrrhoderma*. Therefore, *G. pyrrhoderma* is nearly indistinguishable from certain forms of these other species.

Distribution. Only known from the type locality, Gunung Silabur, Serian, Sarawak, Malaysia.

Molecular analysis. In the ML and Bayesian analyses, all *G. pyrrhoderma* (16S: n = 28; CO1: n = 26) individuals group together in one clade with 99% BS and 100% PP. Its sister clade is *G. scalinella* + *G. kinabatanganensis*.

Discussion. In the original description, Thompson and Dance (1983) did not compare *G. pyrrhoderma* with members of their *hosei*-group (which our molecular analyses show it belongs in). Instead, they considered it allied to *G. borneensis*. Possibly this misalignment was caused by the fact that the type specimens appear to lack the series of scales that is present on most of the specimens we collected. Nonetheless, given the restricted range of collection localities at Gunung Silabur and the degree of variability in our material, we consider our and Thompson and Dance's material as conspecific. The paratypes specimen NHMUK 1984005 (Semabang entrance to Lobang Batu Cave, W. side of Gunong Selabor, 1st. Div., Sarawak, Malaysia: seen) is *G. silaburensis*.

***Georissa kinabatanganensis* sp. n.**
http://zoobank.org/A952A54F-D486-4C27-AFCA-8E7020E41ADA

Type locality. Bukit Keruak, near Kinabatangan river, Sandakan, Sabah, Malaysia (05°31.385'N, 118°17.113'E).

Type material. *Holotype*. Bukit Keruak, near Kinabatangan river, Sandakan, Sabah, Malaysia (05°31.38'N, 118°17.11'E): BOR/MOL 13921 (leg. M Schilthuizen). *Paratypes*. Bukit Keruak, near Kinabatangan river, Sandakan, Sabah (05°31.38'N, 118°17.11'E): MZU/MOL 17.26 (>50). BOR/MOL 1458, BOR/MOL 11656, BOR/MOL 11665, BOR/MOL 11711, BOR/MOL 13871. Batu Pangi, Sabah: BOR/MOL 1455. Batu Tomanggong, Sabah: BOR/MOL 1456, BOR/MOL 1457, BOR/MOL 10530.

Etymology. Named after the district of Kinabatangan, Sabah, Malaysia, where the type locality Bukit Keruak is located.

Description. *Protoconch*. Color: orange. Sculpture pattern: smooth to meshed – rounded to undefined mesh pattern. Mesh width: 14–21 μm. *Teleoconch*. Color: orange. First whorl: flat and angular at the shoulder. Subsequent whorls: angular, slightly rounded at the periphery, with number of whorls: 2–2 ¼. SH: 1.00–1.32 mm, SW: 1.13–1.37 mm, SI: 0.85–0.99. *Shell sculpture*. Radial sculpture: absent, only weak to strong growth lines are visible. Spiral sculpture: present, and strongly sculpted, with continuous to discontinuous ribbings. Scales: two series of diagonal vertical scales, widely spaced in between, both series are strongly sculpted, broad, and the scales are regularly placed. *Aperture*. Shape: oval. Basal side: rounded, angular at the columellar region. Parietal side: straight, palatal edge attached to the body whorl. AH: 0.54–0.66 mm, AW: 0.75–0.86 mm, AI: 0.65–0.80. *Holotype dimension*. SH: 1.00 mm, SW: 1.18 mm, AH: 0.54 mm, AW: 0.78 mm.

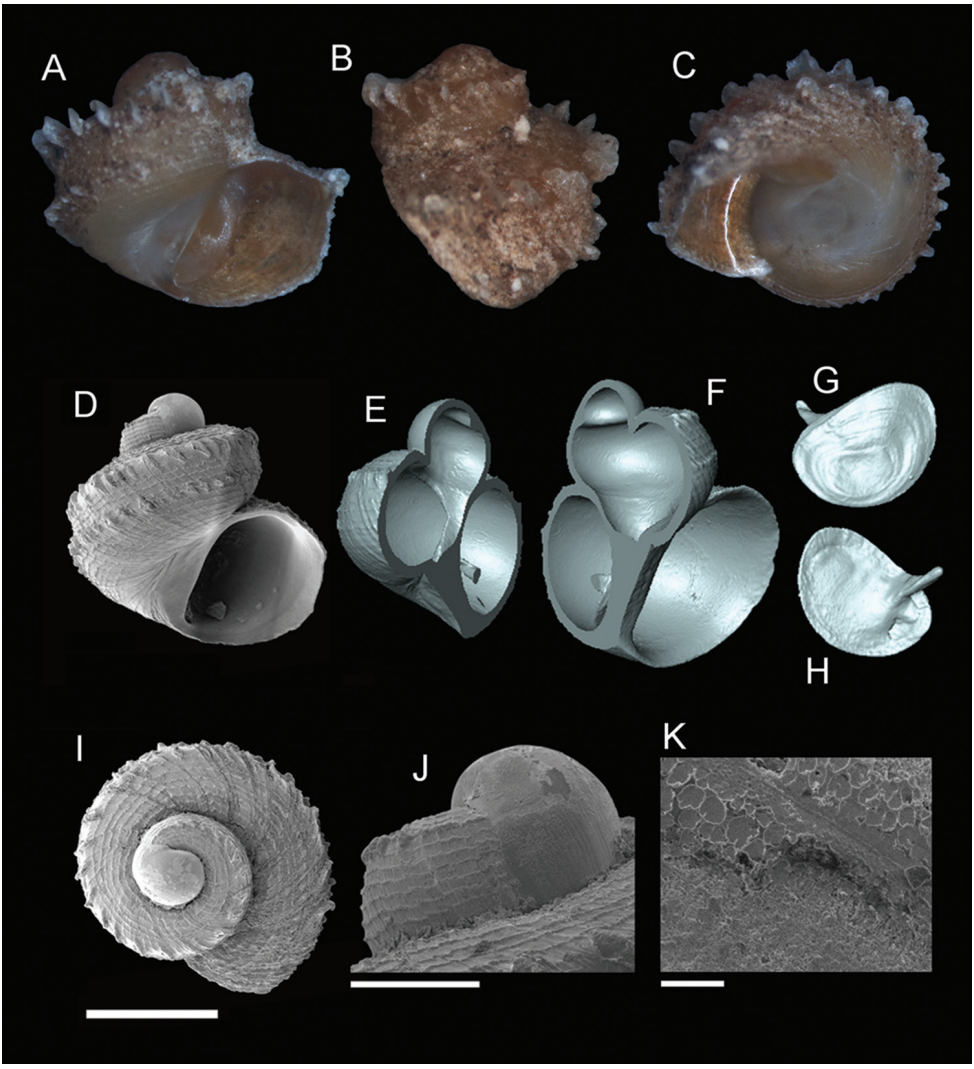


Figure 16. *Georissa kinabatanganensis* sp. n. **A–C** Holotype: BOR/MOL 13921 **D–K** Paratypes: MZU/MOL 17.26. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Cross diagnosis. *Georissa kinabatanganensis* has less variation in shell sculpture compared with *G. hosei* and *G. scalinella*. *G. kinabatanganensis* has two series of acutely projected scales on the whorls. In some cases, the second scale series is weaker than the first, and creates a series of nodular structures at the periphery. Often the shell is wider than high, which gives it a flattened appearance. In addition, *G. kinabatanganensis* has widely spaced between the scale series, similar to *G. muluensis*.

Distribution. Known from Bukit Keruak, Batu Tomanggong, and Pangi, in the region of Kinabatangan, Sabah.

Molecular analysis. RAxML and Bayesian analyses show *G. kinabatanganensis* (16S: n = 6; CO1: n = 6) forming a clade with 97% BS and 97% PP and as a sister clade to *G. sepulutensis*.

Discussion. *Georissa kinabatanganensis* is the only species in "scaly group" *Georissa* to have a flat shell habitus, all examined individuals have a shell that is broader than high.

***Georissa sepulutensis* sp. n.**
http://zoobank.org/7D2EFD37-B493-4DE4-B219-4AA94BF2BD73

Georissa scalinella van Benthem-Jutting: Schilthuizen et al. 2005: 134-135 (**non** *G. scalinella* van Benthem-Jutting, 1966).

Type locality. Sepulut valley, Gua Pungiton near Kg. Labang, Sabah, Malaysia (04°42.41'N, 116°36.04'E).

Type material. *Holotype*. Sepulut valley, Gua Pungiton near Kg. Labang, Sabah, Malaysia (04°42.41'N, 116°36.04'E); BOR/MOL 13922 (leg. M Schilthuizen). *Paratypes*. Simbaluyon limestone hill, Sabah: RMNH/MOL 333905 (18), RMNH/MOL 333982 (23), RMNH/MOL 334006 (7). Tinahas, Sabah: RMNH/MOL 333984 (>50), RMNH/MOL 334013 (>50). Sepulut valley, Gua Sanaron, Sabah (04°42.05'N, 116°36.01'E): BOR/MOL 36, BOR/MOL 39, BOR/MOL 13870 (1). Sepulut Valley, Gua Pungiton, Sabah (04°42.41'N, 116°36.04'E): BOR/MOL 12278. Sepulut valley, Batu Punggul, Sabah: RMNH/MOL 187650, BOR/MOL.40. Baturong, Sabah: BOR/MOL 37.

Etymology. Named after the town of Sepulut, Sabah, Malaysia, near which the type locality Gua Pungiton, as well as the other known localities, is located.

Description. *Protoconch*. Color: red to brown. Sculpture: smooth to meshed – semi-oval mesh to undefined mesh pattern. Mesh width: 7–17 µm. *Teleoconch*. Color: red. First whorl: flat to rounded at the shoulder. Body whorl: rounded, with number of whorls: 2–2 ¾. SH: 1.11–1.52 mm, SW: 1.11–1.37 mm, SI: 0.94–1.07. *Shell sculpture*. Radial sculpture: absent, only weak growth lines are visible. Spiral sculpture: present, and strongly sculpted. Scales: a series of pointed vertical scales, acute and highly projected, and regularly spaced. *Aperture*. Shape: oval and tilted below. Basal side: rounded, angular at the columellar region. Parietal side: straight, palatal edge attached to the body whorl. AH: 0.62–0.81 mm, AW: 0.76–0.96 mm, AI: 0.72–0.87. *Holotype dimension*. SH: 1.34 mm, SW: 1.23 mm, AH: 0.65 mm, AW: 0.82 mm.

Cross diagnosis. Unlike *Georissa kinabatanganensis*, *G. sepulutensis* has a series of scales only at the shoulder, which makes it resemble in habitus and scale characters *G. pyrrhoderma* from Gunung Silabur, Sarawak.

Distribution. Distributed in the Sepulut Valley, Sabah; known from the following limestone localities: Simbaluyon, Sanaron, Tinahas, and Pungiton.

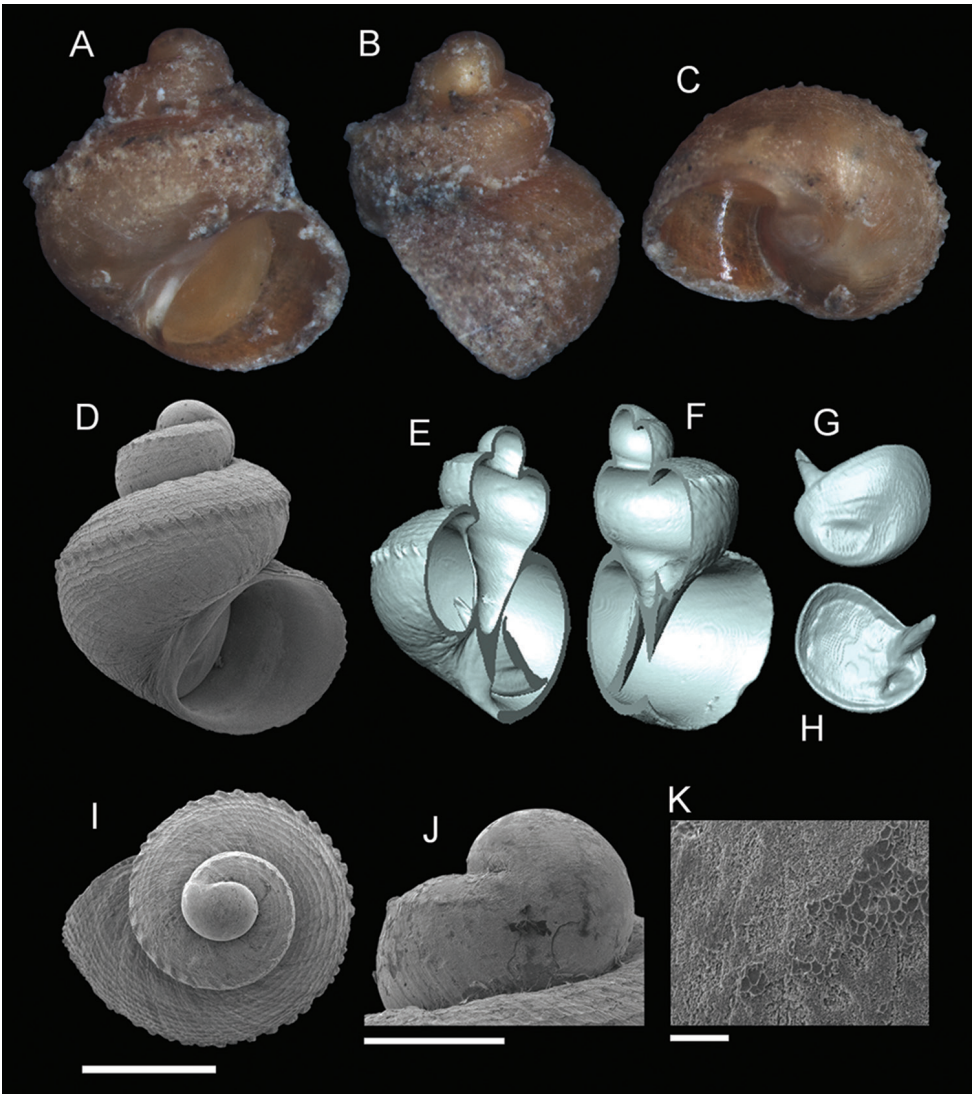


Figure 17. *Georissa sepulutensis* sp. n. **A–C** Holotype: BOR/MOL 13922 **D–K** Paratypes: BOR/MOL 12278. **A, D** Shell apertural view **B** Shell side view **C** Shell rear view **E–F** Shell cross-section from 3D model **G–H** Operculum frontal and ventral view **I** Shell top view **J** Protoconch side view **K** Close up of protoconch from top at 1000× magnification. Scale bars: 500 μm (**A–I**); 200 μm (**J**); 10 μm (**K**).

Molecular analysis. ML and Bayesian analyses show *G. sepulutensis* (16S: n = 10; CO1: n = 2) as two clades with 93% BS and 97% PP, and as the sister species to *G. kinabatanganensis* sp. n.

Discussion. *Georissa sepulutensis* and *G. kinabatanganensis* were previously included in *G. scalinella* (van Benthem-Jutting, 1966). Based on the genetic and morphological distinctness, we here consider them as separate species.

Acknowledgments

The authors thank Sarawak Forest Department (SFD) Sarawak and Economic Planning Unit (EPU), Prime Minister Office, Malaysia, and Sabah Biodiversity Centre for the fieldwork permits NCCD.907.4.4(JLD12)-155 (from SFD), UPE40/200/19/3282 (from EPU), export permit: 15982 (from SFD), and access licenses JKM/MBS.1000-2/2 JLD.5 (28), JKM/MBS.1000-2/2 JLD.3 (167), JKM/MBS.1000-2/2 (167), and JKM/MBS.1000-2/2 (121) (SaBC). Thank you to Grand Perfect Pusaka Sdn. Bhd., Siti Khadijah Reduan, Kirollina Kisun, the heads of villages and local field guides from each of the visited places who helped during the fieldworks. We also thank staff from Naturalis Biodiversity Center, Universiti Malaysia Sarawak, Universiti Malaysia Sabah, and Natural History Museum London, for all the assistance. This study was funded by KNAW Ecologie Fond (J1610/Eco/G437) and Treub Foundation. The first author thanks Ministry of Higher Education Malaysia for the PhD scholarship award at Naturalis Biodiversity Centre and University of Leiden, The Netherlands.

References

Bandel K (2008) Operculum shape and construction of some fossil Neritimorpha (Gastropoda) compared to those of modern species of the subclass. Vita Malacologica 7: 19–36. http://www.paleoliste.de/bandel/bandel_2008.pdf

Beron P (2015) Comparative study of the invertebrate cave faunas of Southeast Asia and New Guinea. Historia Naturalis Bulgarica 21: 169–210. http://www.nmnhs.com/historia-naturalis-bulgarica/pdfs/000365000212015.pdf

Berry A (1966) Population structure and fluctuations in the snail fauna of a Malayan limestone hill. Proceedings of the Zoological Society of London 150(1): 11–27. http://dx.doi.org/10.1111/j.1469-7998.1966.tb02996.x

Blanford WT (1864) XLII.-On the classification of the Cyclostomacea of Eastern Asia. Journal of Natural History 13(78): 441–465. https://doi.org/10.1080/00222936408681635

Clements R, Ng PK, Lu XX, Ambu S, Schilthuizen M, Bradshaw CJ (2008) Using biogeographical patterns of endemic land snails to improve conservation planning for limestone karsts. Biological Conservation 141(11): 2751–2764. https://doi.org/10.1016/j.biocon.2008.08.011

Clements R, Sodhi NS, Schilthuizen M, Ng PK (2006) Limestone karsts of Southeast Asia: imperiled arks of biodiversity. BioScience 56(9): 733–742. https://doi.org/10.1641/0006-3568(2006)56[733:LKOSAI]2.0.CO;2

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792–1797. http://doi.org/10.1093/nar/gkh340

Gredler PV (1902) Zur Conchylien-Fauna von Borneo and Celebes. Nachrichtsblatt der Deutschen Malakozoologischen Gesellschaft: 53–64. https://www.biodiversitylibrary.org/page/15598792#page/459/mode/1up

Godwin-Austen HH (1889) On a collection of land-shells made in Borneo by Mr. A. Everett with supposed new species. Part 1. Proceedings of the Zoological Society of London: 332–355. https://www.biodiversitylibrary.org/page/30866786#page/434/mode/1up

Haase M, Schilthuizen M (2007) A new *Georissa* (Gastropoda: Neritopsina: Hydrocenidae) from a limestone cave in Malaysian Borneo. Journal of Molluscan Studies 73(3): 215–221. https://doi.org/10.1093/mollus/eym020

Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2017) UFBoot2: Improving the Ultrafast Bootstrap Approximation. Molecular Biology and Evolution 35(2): 518–522. https://doi.org/10.1093/molbev/msx281

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods 14(6): 587–589. http://dx.doi.org/10.1038/nmeth.4285

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Liew TS, Vermeulen JJ, Marzuki ME, Schilthuizen M (2014) A cybertaxonomic revision of the micro-landsnail genus *Plectostoma* Adam (Mollusca, Caenogastropoda, Diplommatinidae), from Peninsular Malaysia, Sumatra and Indochina. ZooKeys 393: 1–107. http://doi.org/10.3897/zookeys.393.6717

Liew TS, Schilthuizen M (2016) A method for quantifying, visualising, and analysing gastropod shell form. PloS One 11(6): e0157069. https://doi.org/10.1371/journal.pone.0157069

Mayr E (1942) Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press. http://krishikosh.egranth.ac.in/bitstream/1/2050669/1/IARInew-00187.pdf

Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ (2014) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32(1): 268–274. https://doi.org/10.1093/molbev/msu300

Osikowski A, Hofman S, Georgiev D, Rysiewska A, Falniowski A (2017) Unique, ancient stygobiont clade of Hydrobiidae (Truncatelloidea) in Bulgaria: the origin of cave fauna. Folia Biologica (Kraków) 65(2): 79–93. https://doi.org/10.3409/fb65_2.79

Phung CC, Yu FTY, Liew TS (2017) A checklist of land snails from the west coast islands of Sabah, Borneo (Mollusca, Gastropoda). ZooKeys 673: 49–104. https://doi.org/10.3897/zookeys.673.12422

Puillandre N, Lambert A, Brouillet S, Achaz G (2012a) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology 21(8): 1864–1877. http://dx.doi.org/10.1111/j.1365-294X.2011.05239.x

Puillandre N, Modica MV, Zhang Y, Sirovich L, Boisselier MC, Cruaud C, Holford M, Samadi S (2012b) Large-scale species delimitation method for hyperdiverse groups. Molecular Ecology 21(11): 2671–2691. http://doi.org/10.1111/j.1365-294X.2012.05559.x

Rundell RJ (2008) Cryptic diversity, molecular phylogeny and biogeography of the rock-and leaf litter-dwelling land snails of Belau (Republic of Palau, Oceania). Philosophical Transactions

of the Royal Society B: Biological Sciences 363(1508): 3401–3412. http://doi.org/10.1098/rstb.2008.0110

Saul M (1967) Shell collecting in the limestone caves of Borneo. Sabah Society Journal 3: 105–110.

Schilthuizen M (2011) Community ecology of tropical forest snails 30 years after Solem. Contributions to Zoology 80(1): 1–15. http://www.ctoz.nl/vol80/nr01/a01

Schilthuizen M, Cabanban AS, Haase M (2005) Possible speciation with gene flow in tropical cave snails. Journal of Zoological Systematics and Evolutionary Research 43(2): 133–138. https://doi.org/10.1111/j.1439-0469.2004.00289.x

Schilthuizen M, Gittenberger E (1996) Allozyme variation in some Cretan Albinaria (Gastropoda): paraphyletic species as natural phenomena. In: Taylor JD (Ed.) Origin and Evolutionary Radiation of the Mollusca. Oxford University Press Inc., New York, 301–311.

Schilthuizen M, Liew TS, Elahan BB, Lackman-Ancrenaz I (2005) Effects of karst forest degradation on pulmonate and prosobranch land snail communities in Sabah, Malaysian Borneo. Conservation Biology 19(3): 949–954. http://dx.doi.org/10.1111/j.1523-1739.2005.00209.x

Schilthuizen M, Rutten EJM, Haase M (2012) Small-scale genetic structuring in a tropical cave snail and admixture with its above-ground sister species. Biological Journal of the Linnean Society 105(4): 727–740. https://doi.org/10.1111/j.1095-8312.2011.01835.x

Schilthuizen M, Van Til A, Salverda M, Liew TS, James SS, Elahan BB, Vermeulen JJ (2006) Microgeographic evolution of snail shell shape and predator behavior. Evolution 60(9): 1851–1858. https://doi.org/10.1554/06-114.1

Smith EA (1893) Descriptions of new species of land-shells from Borneo. Zoological Journal of the Linnean Society 24(154): 341–352. http://dx.doi.org/10.1111/j.1096–3642.1893.tb02488.x

Smith EA (1895) On a collection of land-shells from Sarawak, British North Borneo, Palawan, and other neighboring islands. In Proceedings of the Zoological Society of London 63: 97–127. https://www.biodiversitylibrary.org/item/97158#page/135/mode/1up

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Thompson FG, Dance SP (1983) Non-marine mollusks of Borneo. II Pulmonata: Pupillidae, Clausiliidae. III Prosobranchia: Hydrocenidae, Helicinidae. Bulletin of the Florida State Museum Biological Sciences 29(3): 101–152. https://www.floridamuseum.ufl.edu/files/2114/7180/1931/Vol-29-No-3.PDF

Tongkerd P, Lee T, Panha S, Burch JB, O'Foighil D (2004) Molecular phylogeny of certain Thai gastrocoptine micro land snails (Stylommatophora: Pupillidae) inferred from mitochondrial and nuclear ribosomal DNA sequences. Journal of Molluscan Studies 70(2): 139–147. https://doi.org/10.1093/mollus/70.2.139

van Benthem-Jutting WSS (1966) Two new species of Hydrocena (Neritacea) from Sabah, Borneo. Journal of Conchology 26: 39–41.

Vermeulen JJ, Junau D (2007) Bukit Sarang (Sarawak, Malaysia), an isolated limestone hill with an extraordinary snail fauna. Basteria 71(4/6): 209–220. http://natuurtijdschriften.nl/record/597351

Vermeulen JJ, Liew TS, Schilthuizen M (2015) Additions to the knowledge of the land snails of Sabah (Malaysia, Borneo), including 48 new species. ZooKeys 531: 1–139. http://doi.org/10.3897/zookeys.531.6097

Vermeulen JJ, Whitten T (1998) Fauna Malesiana guide to the land snails of Bali. Backhuys Publishers, Leiden, The Netherlands.

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29(22): 2869–2876. https://doi.org/10.1093/bioinformatics/btt499

Zilch A (1973) Die typen und typoide des Natur-Museums Senckenberg. Mollusca: Hydrocenidae. Archiv für Molluskenkunde 103(4/6): 263–272.

Supplementary material 1

An overview of scanning parameters of each examined "scaly" *Georissa*

Authors: Mohd Zacaery Khalik, Kasper Hendriks, Jaap J. Vermeulen, Menno Schilthuizen

Data type: Table in MS .docx file

Link: https://doi.org/10.3897/zookeys.773.24878.suppl1

Supplementary material 2

"Scaly" *Georissa* partitioning based on ABGD species delimitation

Authors: Mohd Zacaery Khalik, Kasper Hendriks, Jaap J. Vermeulen, Menno Schilthuizen

Data type: Images embedded in MS .docx

Link: https://doi.org/10.3897/zookeys.773.24878.suppl2

Supplementary material 3

PTP species delimitation of "scaly" *Georissa*

Authors: Mohd Zacaery Khalik, Kasper Hendriks, Jaap J. Vermeulen, Menno Schilthuizen

Data type: Image files .png and .svg, and .txt in .rar format

Link: https://doi.org/10.3897/zookeys.773.24878.suppl3

Supplementary material 4

Shell measurement of "scaly" *Georissa*

Authors: Mohd Zacaery Khalik, Kasper Hendriks, Jaap J. Vermeulen, Menno Schilthuizen

Data type: Shell measurement in MS .xlsx format

Link: https://doi.org/10.3897/zookeys.773.24878.suppl4

Supplementary material 5

Synoptic view of 13 species of "scaly" *Georissa*, and their 3D models

Authors: Mohd Zacaery Khalik, Kasper Hendriks, Jaap J. Vermeulen, Menno Schilthuizen

Data type: Video .mp4 file

Link: https://doi.org/10.3897/zookeys.773.24878.suppl5

ZooKeys 773: 57–67 (2018)
doi: 10.3897/zookeys.773.24156
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

The complete mitochondrial genome of *Xizicus* (*Haploxizicus*) *maculatus* revealed by Next-Generation Sequencing and phylogenetic implication (Orthoptera, Meconematinae)

Mao Shaoli[1], Yuan Hao[2], Lu Chao[2], Zhou Yafu[1], Shi Fuming[3], Wang Yuchao[1]

1 *Xi'an Botanical Garden of Shaanxi Province/Institute of Botany of Shaanxi Province, Xi'an 710061, China* **2** *College of Life Sciences, Shaanxi Normal University, Xi'an 710062, China* **3** *College of Life Sciences, Hebei University, Baoding, Hebei 071002, China*

Corresponding author: *Zhou Yafu* (zyf820207@126.com); *Shi Fuming* (shif_m@126.com)

Academic editor: *F. Montealegre-Z* | Received 24 February 2018 | Accepted 31 May 2018 | Published 9 July 2018

http://zoobank.org/F6956F17-2CD5-4EEC-BE8E-F9EA8F716276

Citation: Shaoli M, Hao Y, Chao L, Yafu Z, Fuming S, Yuchao W (2018) The complete mitochondrial genome of *Xizicus* (*Haploxizicus*) *maculatus* revealed by Next-Generation Sequencing and phylogenetic implication (Orthoptera, Meconematinae). ZooKeys 773: 57–67. https://doi.org/10.3897/zookeys.773.24156

Abstract

Xizicus Gorochov, 1993, the quiet-calling katydid, is a diverse genus with 68 species in world, which includes more than 45 species in China, has undergone numerous taxonomic revisions with contradicting conclusions. In this study the complete mitochondrial genome of *Xizicus* (*Haploxizicus*) *maculatus* collected from Hainan for the first time was sequenced using the Next-Generation Sequencing (NGS) technology. The length of whole mitogenome is 16,358 bp and contains the typical gene arrangement, base composition, and codon usage found in other related species. The overall base composition of the mitochondrial genome is 37.0 % A, 32.2 % T, 20.2 % C, and 10.6 % G. All 13 protein-coding genes (PCGs) began with typical ATN initiation codon. Nine of the 13 PCGs have a complete termination codon, but the remaining four genes (COI, COIII, ND5, and ND4) terminate with an incomplete T. Phylogenetic analyses are carried out based on the concatenated dataset of 13 PCGs and two rRNAs of Tettigoniidae species available in GenBank. Both Bayesian inference and Maximum Likelihood analyses recovered each subfamily as a monophyletic group. Regardless of the position of Lipotactinae, the relationships among the subfamilies of Tettigoniidae were as follows: ((((Tettigoniinae, Bradyporinae) Meconematinae) Cono-

cephalinae) Hexacentrinae). The topological structure of the phylogeny trees showed that the *Xizicus* (*Haploxizicus*) *maculatus* is closer to *Xizicus* (*Xizicus*) *fascipes* than *Xizicus* (*Eoxizicus*) *howardi*.

Keywords
mitochondrial genome, Next-Generation Sequencing, phylogenetic relationship, *Xizicus* (*Haploxizicus*) *maculatus*

Introduction

Insect mitochondrial genome (mitogenome) occurs as a small (15–20kb), circular, and double-stranded DNA molecules, including 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes (Boore 1999; Cameron 2014) and at least one large non-coding region related to the control of replication and transcription (Clayton 1992; Fernandez-Silva et al. 2003). Recently, Next-Generation Sequencing (NGS) technology, combined with bioinformatic annotation, which presently used for generating mitogenomes without using PCR, has led to a rapid increase in the number of sequenced mitogenomes (Cameron 2014). In recent years, more and more mitochondrial entire genomes of Orthoptera have been sequenced (Yang et al. 2016; Zhou et al. 2014; 2017). Up to now, 24 Tettigoniidae (Orthoptera) mitogenomes have been reported or registered in GenBank, including only five Meconematine mitogenomes.

Xizicus Gorochov, 1993 is a diverse genus in Meconematinae with 68 species in the world, currently divided into seven subgenera by traditional taxonomy based on comparative morphology: *Xizicus* s. str., *Axizicus*, *Eoxizicus*, *Furcixizicus*, *Haploxizicus*, *Paraxizicus*, *Zangxizicus* according to the OSF website (Cigliano et al. 2018). However, great controversies still exist in the defining characteristics and taxonomic status of these subgenera, and the assignment of some species changes frequently and has undergone numerous taxonomic revisions with contradicting conclusions (Gorochov 1998; Jiao et al. 2013; Wang et al. 2014). Integrative taxonomy combining multiple kinds of data and complementary perspectives (e.g. morphology, ecology and DNA sequences) has been recognized as a particularly efficient means to species delimitation and genus-level classification (Cruz-Barraza et al. 2012; Heneberg et al. 2015; Korshunova et al. 2017). The mitogenome is considered a powerful marker and is extensively applied for resolving metazoan phylogenetic relationships at both deep and shallow taxonomic levels (Cameron 2014; Zhou et al. 2017).

To date, the mitogenomes of *Xizicus* (*Xizicus*) *fascipes* and *Xizicus* (*Eoxizicus*) *howardi* have been sequenced (Yang et al. 2012; Liu 2017). *Xizicus* (*Haploxizicus*) *maculatus* (Xia & Liu, 1993) is a representative species of subgenus *Haploxizicus* distributed in Hunan of China, which was reported by Xia and Liu (1993) within the genus *Xiphidiopsis*. Later, Gorochov (1998) proposed the genus *Axizicus* and transferred *Xiphidiopsis maculatus* to this new genus. Wang et al. (2014) subsequently transferred it to the new

subgenus *Haploxizicus* mainly based on the typical characteristic of vertex disc with four longitudinal bands and the simple cercus, and this classification viewpoint was adopted by the Orthoptera Species File website (Cigliano et al. 2018). Type locality of *X.* (*H.*) *maculatus* is Cili country of Hunan Province. Specimen used in this study was collected from Hainan Province for the first time. In this study, we provided a thorough description of the complete mitochondrion genome of *X.* (*H.*) *maculatus*, compared the relative synonymous codon usage with other two subgenera species. Additionally, phylogenomic analyses were conducted based on mitochondrial genome data of Tettigoniidae available in GenBank with the purpose of investigating the phylogenetic position of *X.* (*H.*) *maculatus* and better understanding the phylogenetic relationship of Tettigoniidae.

Materials and methods

Taxon sampling and sequencing

The specimen of *X.* (*H.*) *maculatus* was collected at Jianfengling in Hainan, China in June 2017 and stored in 100 % ethanol at –4 °C. Genomic DNA was extracted from the leg muscle tissue of a single adult male species of using a DNeasy Blood & Tissue Kit (Qiagen, USA) according to the manufacturer's instruction and sent to a company for library prep and sequencing (Genesky Biotechnologies Inc., Shanghai). The library was prepared using a TruSeq DNA sample Preparation kit (Vanzyme, China) and sequenced with 150 bp pair-end reads on the Illumina Hiseq 2500 sequencing platform (Illumina, USA).

De novo assembly and annotation of the *Xizicus* (*Haploxizicus*) *maculatus* mitogenome

13,799,778 raw reads were sequenced by the Illumina Hiseq 2500 platform. The raw paired-end reads were filtered to obtain high-quality clean reads by using CLC Genomics Workbench 8 (CLC Bio, Aarhus, Denmark) with default parameters. Then the filtered reads were aligned to the mitochondrial genome of *X.* (*X.*) *fascipes* (JQ326212) as a reference using MITObim v1.8 (Hahn et al. 2013) and Mira 4.0.2 (Chevreux et al. 2004) to assembly. All of 32,608 clean mitochondrial reads yielded an average coverage of 255.4 X. The complete mitochondrial genome sequence was annotated using the software Geneious v 10.1.2 (Biomatters Ltd., Auckland, New Zealand) by comparing with the mitochondrial genome of *X.* (*X.*) *fascipes* (JQ326212). The tRNA genes were predicted using online software MITOS (Bernt et al. 2013). The nucleotide relative synonymous codon usage (RSCU) values of PCGs were analysed with MEGA v7 software (Kumar et al. 2016).

Phylogenetic analyses

Phylogenetic analyses were performed on the concatenated datasets of PCGs and rRNAs of the newly sequenced mitogenome and 24 Tettigoniidae species downloaded from GenBank, with two Phaneropteridae taxa (*Ducetia japonica* and *Phyllomimus sinicus*) selected as the outgroups. Alignment of each protein-coding gene inferred from the amino acid alignment was performed using MEGA v7.0 (Kumar et al. 2016), and the alignment results were then concatenated. Bayesian inference (BI) analysis was used for phylogenetic reconstruction with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the partitioned models chosen by PartitionFinder 2 (Lanfear et al. 2017). In the BI analysis, 10,000,000 generations were run, with four MC chains, and the trees were sampled every 1000 generations with a burn-in step. The confidence values for the BI tree were expressed as the Bayesian posterior probabilities in percentages. A maximum likelihood (ML) tree was constructed using RAxML 8.0 (Stamatakis 2014) and the optimal partitions and best models were also selected by PartitionFinder 2 and the robustness of the phylogenetic results was tested through bootstrap analysis with 1000 replicates in RAxML and the bootstrap support values were printed on the best ML tree.

Results and discussion

Genome organization

The complete mitogenome of *X.* (*H.*) *maculatus* is 16,358 bp in length and has been deposited in GenBank under accession no. MG779499. *Xizicus* (*H.*) *maculatus* mtDNA is larger than that observed in other species in Meconematinae, which typically ranged from 16,044 bp (Zhou et al. 2017) to 16,166 bp (Yang et al. 2012).

The mitochondrial genome structure is detailed in Table 1. It contained a typical gene content found in metazoan mitogenomes: 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal (rRNA) genes, and one control region (Table 1, Fig. 1). Gene order and arrangement was identical to the *X.* (*X.*) *fascipes* mitogenome. The *X. (H.) maculatus* mitochondrial genes were separated by a total of 65 bp intergenic spacer sequences, which spreaded over ten regions and range in size from one to 17 bp. There were 13 overlaps amongst all 48 bp and the longest overlaps of 8 bp were located between $tRNA^{Trp}$-$tRNA^{Cys}$ and $tRNA^{Tyr}$-COI. The overall base composition of the whole mitochondrial genome was 37.0 % A, 32.2 % T, 20.2 % C, and 10.6 % G, exhibiting obvious anti-G and AT bias (69.2 %) which was slightly lower than *X.* (*X.*) *fascipes* (70.2 %) and *X.* (*E.*) *howardi* (71.0 %).

Protein-coding genes

The total length of all 13 PCGs was 11,224 bp, and the overall A+T content of *X.* (*H.*) *maculatus* PCGs was 68.3 %. The initiation codons of all PCGs were typical with

Table 1. Organization of the *Xizicus* (*Haploxizicus*) *maculatus* mitogenome.

Gene/region	Position	Size	Direction	Initiation codon	Termination codon	anticodon
tRNA^{Ile}	1-66(-3)	66	F			GAT
tRNA^{Gln}	64-132(+8)	69	R			TTG
tRNA^{Met}	141-204	64	F			CTA
ND2	205-1233(-2)	1029	F	ATT	TAA	
tRN^{ATr}p	1232-1297(-8)	66	F			TCA
tRNA^{Cys}	1290-1353	64	R			GCA
tRNA^{Tyr}	1354-1419(-8)	66	R			GTA
COI	1412-2951	1540	F	ATT	T	
tRNA$^{Leu(UUR)}$	2452-3016(+3)	65	F			TAA
COII	3020-3703(+1)	684	F	ATG	TAA	
tRNA^{Lys}	3705-3774(-1)	70	F			CTT
tRNA^{Asp}	3774-3839	66	F			GTC
ATP8	3840-4004(-7)	165	F	ATT	TAA	
ATP6	3998-4675(-1)	678	F	ATG	TAA	
COIII	4675-5461	787	F	ATG	T	
tRN^{AGL}y	5462-5526	65	F			TCC
ND3	5527-5880(+2)	354	F	ATC	TAA	
tRNA^{Ala}	5883-5946(-1)	64	F			TGC
tR^{NAA}rg	5946-6008(+16)	63	F			TCG
tRNA^{Asn}	6025-6090(+2)	66	F			GTT
tRNA$^{Ser(AGN)}$	6093-6159	67	F			GCT
tRN^{AGL}u	6160-6226(+14)	67	F			TTC
tRNA^{Phe}	6305-6421	65	R			GAA
ND5	6306-8037	1732	R	ATT	T	
tRNA^{His}	8038-8101	64	R			GTG
ND4	8102-9440(-7)	1339	R	ATG	T	
ND4L	9434-9730(+1)	297	R	ATG	TAA	
tRNA^{Thr}	9732-9802(-1)	71	F			TGT
tRNA^{Pro}	9802-9867(+1)	66	R			TGG
ND6	9869-10396(-1)	528	F	ATA	TAA	
Cytb	10396-11532(-2)	1137	F	ATG	TAG	
tRNA$^{Ser(UCN)}$	11531-11599(+17)	69	F			TGA
ND1	11617-12570(-6)	954	R	ATA	TAG	
tRNA$^{Leu(CUN)}$	12565-12628	64	R			TAG
lrRNA	12629-13932	1304	R			
tRNA^{Val}	13933-14003	71	R			TAC
srRNA	14004-14788	785	R			
Control region	14789-16358	1570	F			

ATN (COII, ATP6, COIII, ND4, ND4L, and Cytb with ATG, ND2, COI, ATP8, ND5 with ATT; ND6, ND1 with ATA, Only ND3 with ATC). The ATN codon was prevalently regarded as initiation codons for mitogenome PCGs in insects. Seven genes (ND2, COII, ATP8, ATP6, ND3, ND4L, ND6) used TAA as the termination codons, and two genes (Cytb, ND1) were stopped with TAG. COI, COII, ND5, and

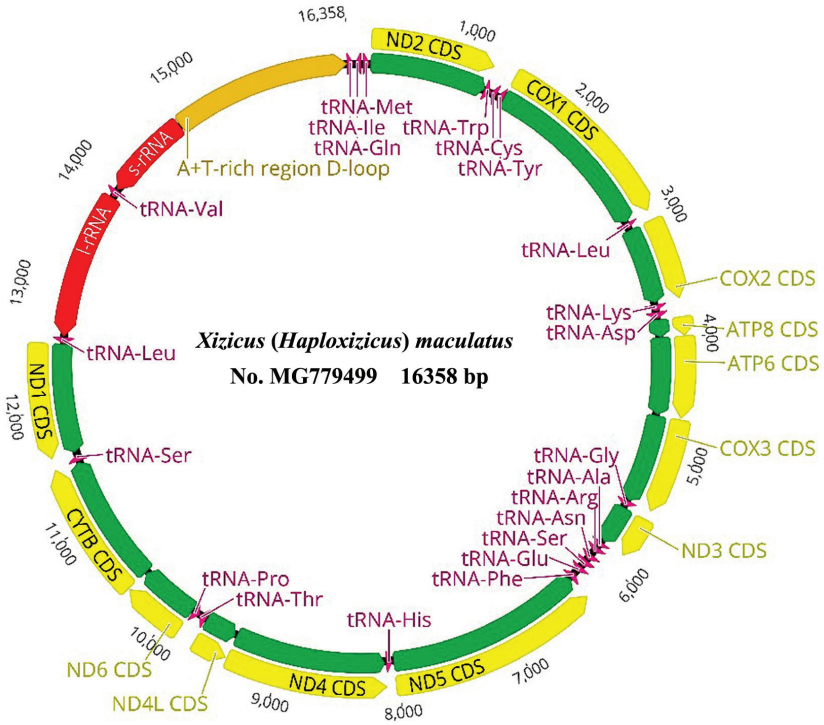


Figure 1. Circular visualization of the mitogenome of *Xizicus* (*Haploxizicus*) *maculatus.*

ND4 had an incomplete termination codon T (Table 1). The incomplete termination codon is common in metazoan mitochondrial genomes and exhibit function after post-transcription polyadenylation converts into full stop codon (Ojala et al. 1981).

The four most-used amino acids in *X.* (*H.*) *maculatus* were Leu (16.1 %), Ser (8.9 %), Phe (8.7 %), and Ile (8.4 %), whose proportions were similar to those observed in other Tettigoniidae species. All codons were present in the protein-coding genes of this mitogenome. Excluding incomplete termination codons, there were 3,735 codons in the *X.* (*H.*) *maculatus* protein-coding genes. The codon usage in *X.* (*H.*) *maculatus* appeared to be typical of other insect mitochondrial sequences. The RSCU analysis indicated that codons including A or T at the third position were always overused compared with other synonymous codons in *Xizicus* (Fig. 2). The codon usage could also reflect nucleotide bias.

Ribosomal and transfer RNA genes

The length of tRNA genes ranged from 63 to 71 bp and the relative locations for each tRNA are shown in Table 1. All tRNA genes had the typical cloverleaf secondary struc-

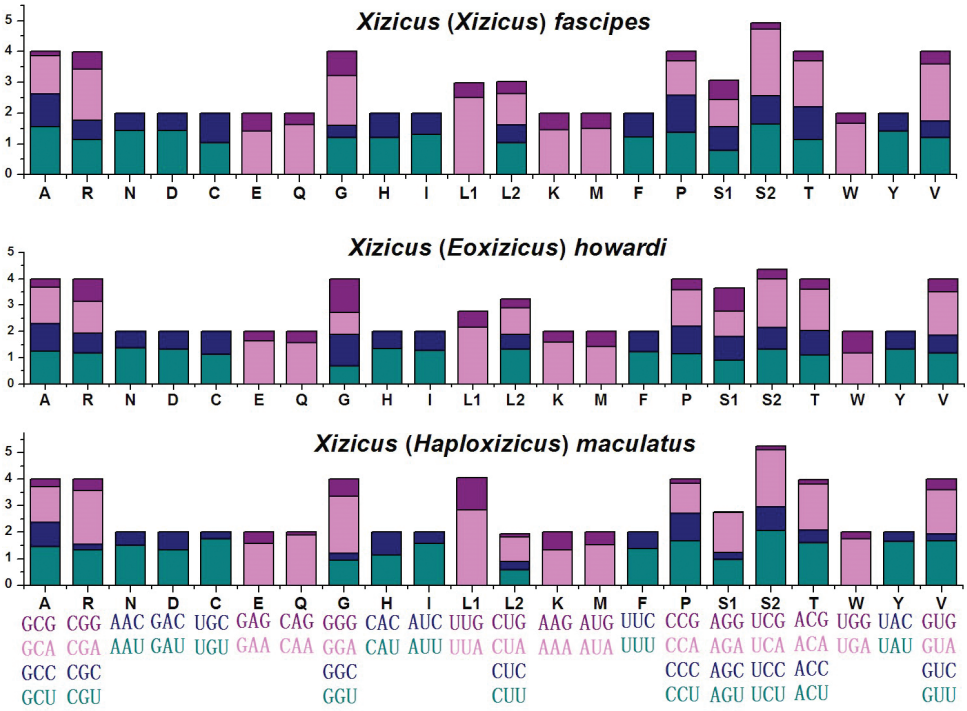


Figure 2. Relative synonymous codon usage of *X.* (*X.*) *fascipes*, *X.* (*E.*) *howardi*, *X.* (*H.*) *maculatus* mitochondrial protein-coding genes. Condon families are provided on the x-axis.

tures except for tRNA$^{Ser(AGN)}$. The secondary structures of tRNA$^{Ser(AGN)}$ was completely identical with *X. (X.) fascipes* and showed a lengthened anticodon stem (9 bp) with a bulging nucleotide in the middle, an unusual 6 bp length T-stem, a mini DHU arm (2 bp), and no connector nucleotides (Yang et al. 2012).

The lrRNA and srRNA were 1304bp and 785bp in length, respectively. They were located between tRNA$^{Leu(CUN)}$ and A+T-rich region, being separated by tRNA^{Val}.

Non-coding regions

The control region was 1570 bp in length and located between srRNA and tRNA^{Ile} in *X.* (*H.*) *maculatus* mitogenome, and was composed of 64.4 % A and T nucleotides (Fig.1; Table 1). The control region is characterized by a high AT content and is thought to be involved in the regulation of mtDNA transcription and replication (Bae et al., 2004), whose size differences are not only because of high rates of nucleotide substitution, insertion or deletion, but also due to the length of tandem repeat unit and the number of tandem repetitions (Yang et al. 2012). The sequence analysis revealed four tandem repeats size from 26 to 162 bp, contributing 738 bp to the length of the region.

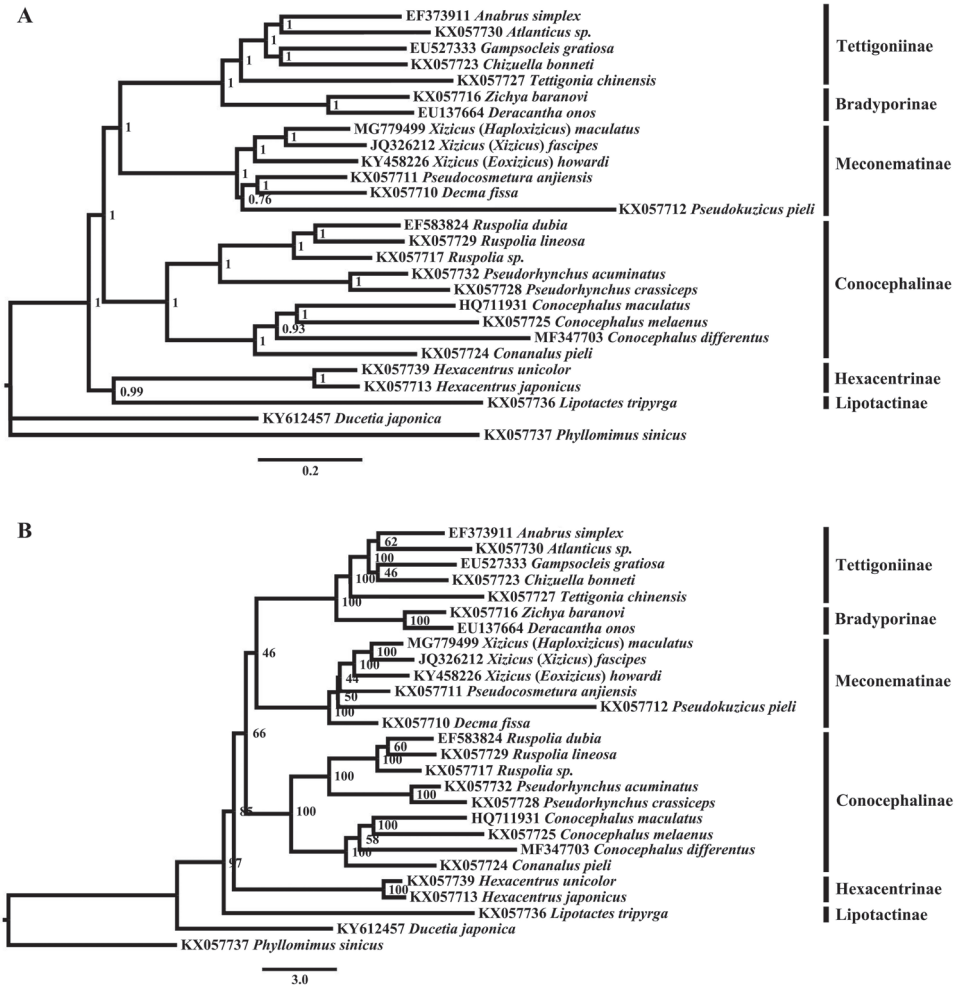


Figure 3. Phylogenetic reconstruction of Tettigoniidea using mitochondrial PCGs and rRNA concatenated dataset. **A** Bayesian result, applicable posterior probability values are shown **B** Maximum likelihood result with applicable bootstrap values shown.

Phylogenetic relationships

Bayesian analyses and maximum likelihood produced identical topologies using the best-fit partitioning scheme and site-homogeneous models, excepting the location of *Lipotactes tripyrga* (Lipotactinae) and genera relationships in Meconematinae (Fig. 3). Bayesian inference recovered each Tettigoniinae, Bradyporinae, Conocephalinae, and Meconematinae as a monophyletic group with strongly supported (PP ≥ 0.93), while the monophyly of Conocephalinae and Meconematinae (only include the tribe Meconematini) was not well supported in ML topology. The position of *L. tripyrga* was at basal in the ML tree while it formed the most basal clade together with Hexacen-

trinae in the BI analysis. The relationship of Lipotactinae with other subfamilies was unascertainable may due to only one taxa used in analyses.

In present study, regardless of the position of Lipotactinae, the relationships among the subfamilies of Tettigoniidae were as follows: ((((Tettigoniinae, Bradyporinae) Meconematinae) Conocephalinae) Hexacentrinae), which was congruent with the phylogenetic results using site-homogeneous both in ML and BI analyses (Zhou et al. 2017). The relationships among subfamilies within Tettigoniidae were sensitive to the methods used for tree reconstruction and the molecular maker used. Previous phylogenetic studies based on multi-molecular makers (28SrDNA, 18SrDNA, COII, Histone and Wingless genes) inferred a striking difference results that placed Hexacentrinae and Meconematinae as more 'advanced' groups sister to the clade consisting of Tettigoniinae and Lipotactinae, and Conocephalinae as the more 'primitive' group diverging at an earlier node (Mugleston et al. 2013). The present topologies placed Tettigoniinae as an apical node sister to Bradyporinae and then assembled with Meconematinae, which was congruent with the phylogenetic results using site-homogeneous both in ML and BI analyses (Zhou et al. 2017), while differed with the site-heterogeneous CAT-GTR model tree (Zhou et al. 2017). Recent studies suggest that analysis model used in mitochondrial phylogenetic reconstruction potentially impact phylogenies result when genomic data existing lineage compositional heterogeneity and saturation due to accelerated substitution rates causing homoplasy (Li et al. 2015; Zhou et al. 2017).

The generic relationships within the subfamily Meconematinae were not identical in the BI and ML trees. The relationships between the four genera were as follows: (((*Pseudocosmetura*, *Decma*) *Pseudokuzicus*) *Xizicus*) in the BI tree (Fig. 3A); (((*Xizicus*, *Pseudokuzicus*) *Pseudocosmetura*) *Decma*) in the ML tree (Fig. 3B). *Xizicus* (*H.*) *maculatus*, *X.* (*X.*) *fascipes* and *X.* (*E.*) *howardi* clustered into one clade in both analyses, and *X.* (*H.*) *maculatus* was closer to the nominate species *X.* (*X.*) *fascipes* than *X.* (*E.*) *howardi*. Additional taxon sampling will be needed to clarify the relationships of genera in Meconematinae.

Acknowledgements

This study is supported by the National Natural Science Foundation of China (No. 31601887, 31672259), Scientific Research Program of Shaanxi Province (No. 2015KJXX-90).

Reference

Bae JS, Kim I, Sohn HD, Jin BR (2004) The Mitochondrial Genome of The Firefly, Pyrocoelia rufa: Complete DNA Sequence, Genome Organization, and Phylogenetic Analysis with Other Insects. Molecular Phylogenetics and Evolution 204: 32978–985. https://doi.org/10.1016/j.ympev.2004.03.009

Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritzsch G, Pütz J, Middendorf M, Stadler PF (2013) MITOS: Improved de novo Metazoan Mitochondrial Genome Annotation. Molecular Phylogenetics and Evolution 69(2): 313–319. https://doi.org/10.1016/j.ympev.2012.08.023

Boore J (1999) Animal mitochondrial genomes. Nucleic Acids Research 8: 1767–1780. https://doi.org/10.1093/nar/27.8.1767

Cameron SL (2014) Insect mitochondrial genomics: implications for evolution and phylogeny. Annual Review of Entomology 50: 95–117. https://doi.org/10.1146/annurev-ento-011613-162007

Chevreux B, Pfisterer T, Drescher B, Driesel AJ, Muller WE, Wetter T, Suhai S (2004) Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. Genome Research 14: 1147–1159. http://www.genome.org/cgi/doi/10.1101/gr.1917404

Cigliano MM, Braun H, Eades DC, Otte D (2018) Orthoptera Species File. Version 5.0/5.0. http://Orthoptera.SpeciesFile.org [2018.1.7]

Clayton DA (1992) Transcription and replication of animal mitochondrial DNAs. International Review of Cytology 141: 217–232. https://doi.org/10.1016/S0074-7696(08)62067-7

Cruz-Barraza JA, Carballo JL, Rocha-Olivares A, Ehrlich H, Hog M (2012) Integrative Taxonomy and Molecular Phylogeny of Genus Aplysina (Demospongiae: Verongida) from Mexican Pacific. PLoS ONE 7(8): e42049. https://doi.org/10.1371/journal.pone.0042049

Fernandez-Silva P, Enriquez JA, Montoya J (2003) Replication and transcription of mammalian mitochondrial DNA. Experimental Physiology 88: 41–56. https://doi.org/10.1113/eph8802514

Gorochov AV (1998) New and little known Meconematinae of the tribes Meconematini and Phlugidini (Orthoptera: Tettigoniidae). Zoosystematica Rossica 7(1): 101–131.

Hahn C'Bachmann L, Chevreux B (2013) Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads-A baiting and iterative mapping approach. Nucleic Acids Research 41: e129. https://doi.org/10.1093/nar/gkt371

Heneberg P, Sitko J, Bizos J (2015) Integrative taxonomy of central European parasitic flatworms of the family Prosthogonimidae Lühe, 1909 (Trematoda: Plagiorchiida). Parasitology International 64: 264–273. https://doi.org/10.1016/j.parint.2015.02.003

Jiao J, Shi FM, Gao JG (2013) Two new species of the genus *Xizicus*, 1993 (Orthoptera: Tettigoniidae: Meconematinae) from Xizang, China. Zootaxa 3694(3): 296–300. https://doi.org/10.11646/zootaxa.3694.3.10

Korshunova T, Martynov A, Bakken T, Evertsen J, Fletcher K, Mudianta IW, Saito H, Lundin K, Schrödl M, Picton B (2017) Polyphyly of the traditional family Flabellinidae affects a major group of Nudibranchia: aeolidacean taxonomic reassessment with descriptions of several new families, genera, and species (Mollusca, Gastropoda). ZooKeys 717: 1–139. https://doi.org/10.3897/zookeys.717.21885

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(3): 1870–1874. https://doi.org/10.1093/molbev/msw054

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B (2017) PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Molecular Biology Evolutionary 34(3): 772–773. https://doi.org/10.1093/molbev/msw260

Li H, Shao R, Song N, Song F, Jiang P, Li Z, Cai W (2015) Higher-level phylogeny of paraneopteran insects inferred from mitochondrial genome sequences. Scientific Reports 5: 8527. https://doi.org/10.1038/srep08527

Mugleston DJ, Song H, Whiting MF (2013) A century of paraphyly: A molecular phylogeny of katydids (Orthoptera: Tettigoniidae) supports multiple origins of leaf-like wings. Molecular Phylogenetics and Evolution 69: 1120–1134. https://doi.org/10.1016/j.ympev.2013.07.014

Ojala D, Montoya J, Attardi G (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature 290: 470-474. https://doi.org/10.1038/290470a0

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Wang HQ, Jing J, Liu XW, Li K (2014) Revision on genus *Xizicus* Gorochov (Orthoptera, Tettigoniidae, Meconematinae, Meconematini) with description of three new species form China. Zootaxa 3861(4): 301–306. https://doi.org/10.11646/zootaxa.3861.4.1

Xia KL, Liu HL (1993[1992]). Orthoptera: Tettigonioidea and Grylloidea. In: Huang FS (Ed.) Insects of Wuling Mountains Area, southwest China. Science Press, Beijing, 87–113.

Yang J, Ye F, Huang Y (2016) Mitochondrial genomes of four katydids (Orthoptera: Phaneropteridae): New gene rearrangements and their phylogenetic implications. Gene 575: 702–711. https://doi.org/10.1016/j.gene.2015.09.052

Yang MR, Zhou ZJ, Chang YL, Zhao LH (2012) The mitochondrial genome of the quiet-calling katydids, *Xizicus fascipes* (Orthoptera: Tettigoniidae: Meconematinae). Journal of Genetics 91(2): 141–153. https://doi.org/10.1007/s12041-012-0157-3

Zhou ZJ, Zhao L, Liu N, Guo HF, Guan B, Di JX, Shi FM (2017) Towards a higher-level Ensifera phylogeny inferred from mitogenome sequences. Molecular Phylogenetics and Evolution 108: 22–33. https://doi.org/10.1016/j.ympev.2017.01.014

Zhou ZJ, Zhao L, Shi FM (2014) The First Mitochondrial Genome for the Superfamily Hagloidea and Implications for Its Systematic Status in Ensifera. PLoS ONE 9(1): e86027. https://doi.org/10.1371/journal.pone.0086027

ZooKeys 773: 69–78 (2018)
doi: 10.3897/zookeys.773.24656
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

A new species of the genus *Blaptogonia* from the Himalayas with four DNA markers (Coleoptera, Tenebrionidae, Blaptini)

Xiu-Min Li[1], Xing-Long Bai[1], Guo-Dong Ren[1]

1 *The Key Laboratory of Zoological Systematics and Application, College of Life Sciences, Hebei University, Baoding, Hebei 071002, P.R. China*

Corresponding author: *Guo-Dong Ren* (gdren@hbu.edu.cn)

Academic editor: *Y. Bousquet* | Received 24 February 2018 | Accepted 22 May 2018 | Published 9 July 2018

http://zoobank.org/18009C82-84F1-4B24-AA45-323024AF1CAE

Citation: Li X-M, Bai X-L, Ren G-D (2018) A new species of the genus *Blaptogonia* from the Himalayas with four DNA markers (Coleoptera, Tenebrionidae, Blaptini). ZooKeys 773: 69–78. https://doi.org/10.3897/zookeys.773.24656

Abstract

A new species of the genus *Blaptogonia* Medvedev, 1998, *B. zhentanga* **sp. n.**, is described from the southern Himalayas of China. Two fragments of mitochondrial protein-coding genes (COI, Cytb), one fragment of mitochondrial ribosomal RNA gene (16S), and one fragment of nuclear rRNA gene (28SD2) of the new species were obtained. A key to the known species of the genus is presented.

Keywords

biology, darkling beetles, DNA sequence, taxonomy, Tenebrioninae

Introduction

The tenebrionid genus *Blaptogonia* Medvedev, 1998 belongs to the subtribe Gnaptorinina Medvedev, 2001 of tribe Blaptini Leach, 1815 within the subfamily Tenebrioninae Latreille, 1802. To date, only four species have been described worldwide and are known to occur only in the southern Himalayas at 3000–4000 meters (Medvedev 2004).

The first species, *Trigonoides costulata* Fairmaire, 1901, was described from Sikkim, India. It is evident from the text that *Trigonoides* used by Fairmaire (1901) is an incorrect spelling for *Tagonoides* Fairmaire, 1886. The second species, *Blaps subcarinata* Blair, 1927, was described based on the specimens of the third expedition to Mt. Everest

from Tibet, China. Subsequently, a new genus, *Blaptyscelis,* was established by Koch (in Pierre, 1961), along with the combinations *Blaptyscelis costulata* Fairmaire, 1901 and *B. subcarinata* Blair, 1927. Koch (1965) redefined the diagnostic characters of this genus, which were accepted by Medvedev (1998, 2004). The third species, *Blaptyscelis zurstrasseni* Kaszab, 1977 was described from Nepal. A contribution to the knowledge of the tribe Blaptini was subsequently made by Medvedev (1998) who established the replacement name *Blaptogonia* since *Blaptyscelis* Koch was originally proposed without a type species designation. Thus, the combinations *Blaptogonia costulata* Fairmaire, 1901, *B. subcarinata* Blair, 1927, and *B. zurstrasseni* Kaszab, 1977 were established, and *Tagonoides costulata* Fairmaire, 1901 was designated as the type species.

At the beginning of the 21st century, *Blaptogonia yini* Ren, Wang & Yu, 2000 was described from Tibet and placed in *Blaptogonia* because of the distinct elytral carinae, one of the typical characters of the genus, but was moved recently to the genus *Blaps* Fabricius, 1775 (Ren et al. 2016) on the basis of additional materials and structures of male genitalia. The fourth species, *Blaptogonia tshernjachovskii* Medvedev, 2004, was described from Nepal based on two female specimens. Medvedev (2004) also characterized the structures of the apical part of the male abdomen, as well as the ovipositor and genital tubes of the female, as diagnostic characters of the genus *Blaptogonia*.

In the present study, a new species of the tribe Blaptini, collected from southern Qomolangma Nature Reserve of Tibet, is described. In addition, two fragments of mitochondrial protein-coding genes (COI, Cytb), one fragment of mitochondrial ribosomal RNA gene (16S), and one fragment of nuclear rRNA gene (28SD2) of the new species were sequenced and uploaded to GenBank.

Materials and methods

Morphology

All specimens examined in this study were deposited in the Museum of Hebei University (**MHBU**), Baoding, China. Photographs of morphological structures were taken using a Leica M205A stereomicroscope with a Leica DFC550 camera and Leica application suite 4.6. The habitus photos were taken using a Canon EOS 5D Mark III camera connected to a Canon Macro lens MP-E 65 mm.

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from leg muscle tissue of a single adult specimen using EZNA® Insect DNA Kit (Omega Bio-tek, USA). Total DNA extract was stored at -20 °C. Two fragments of mitochondrial protein-coding genes (COI, Cytb), one fragment of mitochondrial ribosomal RNA gene (16S), and one fragment of nuclear rRNA gene

Table 1. Primer sequences for PCR.

Locus	Primer (Forward / Reverse/ Internal)	Sequence (forward and reverse) 5'→3'	References
COI	F 2183	CAACATTTATTTTGATTTTTTGG	Folmer et al. 1994
	R 3014	TCCAATGCACTAATCTGCCATATTA	
Cytb	F revcb2h	TGAGGACAAATATCATTTTGAGGW	Simmons and Weller 2001
	R rebcbj	TCAGGTCGAGCTCCAATTCATGT	
16S	F 13398	CGCCTGTTTATCAAAAACAT	Simon et al. 1994
	R 12887	CCGGTCTGAACTCAGATCAT	
28SD2	F 3665	AGAGAGAGTTCAAGAGTACGTG	Belshaw and Quicke 1997
	R 4068	TTGGTCCGTGTTTCAAGACGGG	

(28SD2) were amplified using the primers of Table 1. The profile of the PCR amplification consisted of an initial denaturation step at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–58 °C for 1 min, and extension at 72 °C for 1 min, and a final 8 min extension step at 72 °C. The PCR products were subsequently checked by 1% agarose gel electrophoresis and sequencing was performed at GENEWIZ Biotech Co., Ltd. (Suzhou, China) using the same primers as in the PCR.

Results

Key to known species of the genus *Blaptogonia*

1 Surface of elytra between carinae without subcarinae (i.e. secondary carinae) ... **2**

– Surface of elytra between carinae with subcarinae ... **3**

2 Both carinae on each elytron evanescent in the basal half and 2nd carina not reaching humeral carina at the apex in male and female ... ***B. zurstrasseni*** **Kaszab, 1977**

– Both carinae on each elytron reaching the elytral base and fused with humeral carina at the apex in female ... ***B. tshernjachovskii*** **Medvedev, 2004**

3 Elytral surface between carinae and subcarinae with fine granules; carinae higher than subcarinae; paramere arcuately concave, narrowing from basal 1/5 to apex ... ***B. zhentanga*** **sp. n.**

– Elytral surface between carinae and subcarinae without granules; carinae as high as subcarinae; paramere almost straight, Parameres narrowing from base to apex ... **4**

4 Body and legs black; male elytra more oval, with a row of small punctures between 4th and 5th carinae, and between 5th and humeral carinae ... ***B. costulata*** **Fairmaire, 1901**

– Head and elytra dark brown, pronotum and legs reddish brown; male elytra narrow, more parallel sided, without rows of punctures between carinae and subcarinae ... ***B. subcarinata*** **Blair, 1927**

Genus *Blaptogonia* Medvedev, 1998

Blaptogonia Medvedev, 1998: 186; 2001: 95; 2004: 89; Löbl et al. 2008: 231; Ren et al. 2016: 332.

Type species. *Tagonoides costulata* Fairmaire, 1901.

***Blaptogonia costulata* (Fairmaire, 1901)**

Trigonoides costulata Fairmaire, 1901: 267.
Blaptyscelis costulata: Koch in Pierre 1961: 213.
Blaptogonia costulata: Medvedev 1998: 186; 2001: 95; 2004: 89; Löbl et al. 2008: 231.

Distribution. India: Sikkim.

***Blaptogonia subcarinata* (Blair, 1927)**

Blaps subcarinata Blair, 1927: 243.
Blaptyscelis subcarinata: Koch in Pierre 1961: 213.
Blaptogonia subcarinata: Medvedev 1998: 186; 2001: 95; 2004: 89; Löbl et al. 2008: 231; Ren et al. 2016: 332.

Distribution. China: Tibet (Yadong, also called Chomo).

***Blaptogonia tshernjachovskii* Medvedev, 2004**

Blaptogonia tshernjachovskii Medvedev, 2004: 177; Löbl et al. 2008: 231.

Distribution. Nepal.

***Blaptogonia zhentanga* sp. n.**
http://zoobank.org/4B416851-9F94-4EEB-B369-424908D08D54
Figs 1–4

Type material. Holotype: male (MHBU) (Fig. 3), **CHINA:** Xizang, Dinggyê County, Zhêntang Town, Power Station, 27°55.069'N, 87°28.171'E, Alt. 3418 m, 4.VIII.2014, Guo-Dong Ren, Xing-Long Bai & Jun-Sheng Shan leg. **Paratypes:** 54 males, 28 females (MHBU), same data as the holotype.

Diagnosis. This new species is closely related to *Blaptogonia subcarinata* Blair, 1927 but can be distinguished from the latter by the following character states: (1) elytral

Figure 1. Habitat and population of *Blaptogonia zhentanga* sp. n.

surface with fine granules and irregular and shallow fine punctures, whereas *subcarinata* has no granules and rows of punctures between carinae and subcarinae; (2) elytral carinae more elevated than the subcarinae, whereas the subcarinae are as high as the carinae in *subcarinata*; (3) parameres arcuately concave and narrowing from basal 1/5 to apex, whereas the parameres are nearly straight in *subcarinata* and narrowing from base to apex.

Etymology. Named after the type locality, Zhêntang.

Description. Head, palps, antennae, carinae, subcarinae, humeral carina, abdomen, tibiae, and tarsus black. Pronotum, elytra, femora, apical spurs, and claws reddish brown to brown. Shiny dorsally and ventrally, with sparse and short pubescence at apex on elytra.

Male (Figs 2a–g, 3). Labrum with sparse punctures and pale yellow setae. Anterior margin of clypeus straight, tilted at sides, surface with sparse fine punctures; frontoclypeal suture shallow. Anterior gena slightly extended before eyes, outer margins straight and converging toward base of clypeus, surface with moderately dense fine punctures; emargination of outer margins of head above antennal base widely obtuse-angular. Eyes transverse, weakly projecting and clearly wider than anterior gena. Posterior gena densely hairy, outer margins arcuately converging to neck. Surface of head with moderately dense punctures. Antennae (Fig. 1b) moderately long, with apical segment reaching beyond pronotal base, length (width) ratio of antennomeres 2 to 11 are 1.0(0.9): 1.0(0.3): 1.0(0.5): 1.0(0.5): 1.0(0.5): 1.0(0.5): 1.0(1.0): 1.0(0.8): 1.0(0.9): 1.0(0.7).

Pronotum (Fig. 2a) transverse, widest before middle, 1.3–1.5 times as wide as long, 1.6–1.8 times as wide as head. Ratio of pronotal width at anterior margin to its maximum and base 0.6: 1.0: 0.9. Anterior margin arcuate, bordered laterally by bead along entire length; posterior margin weakly arcuate, without bead. Anterior angles obtuse,

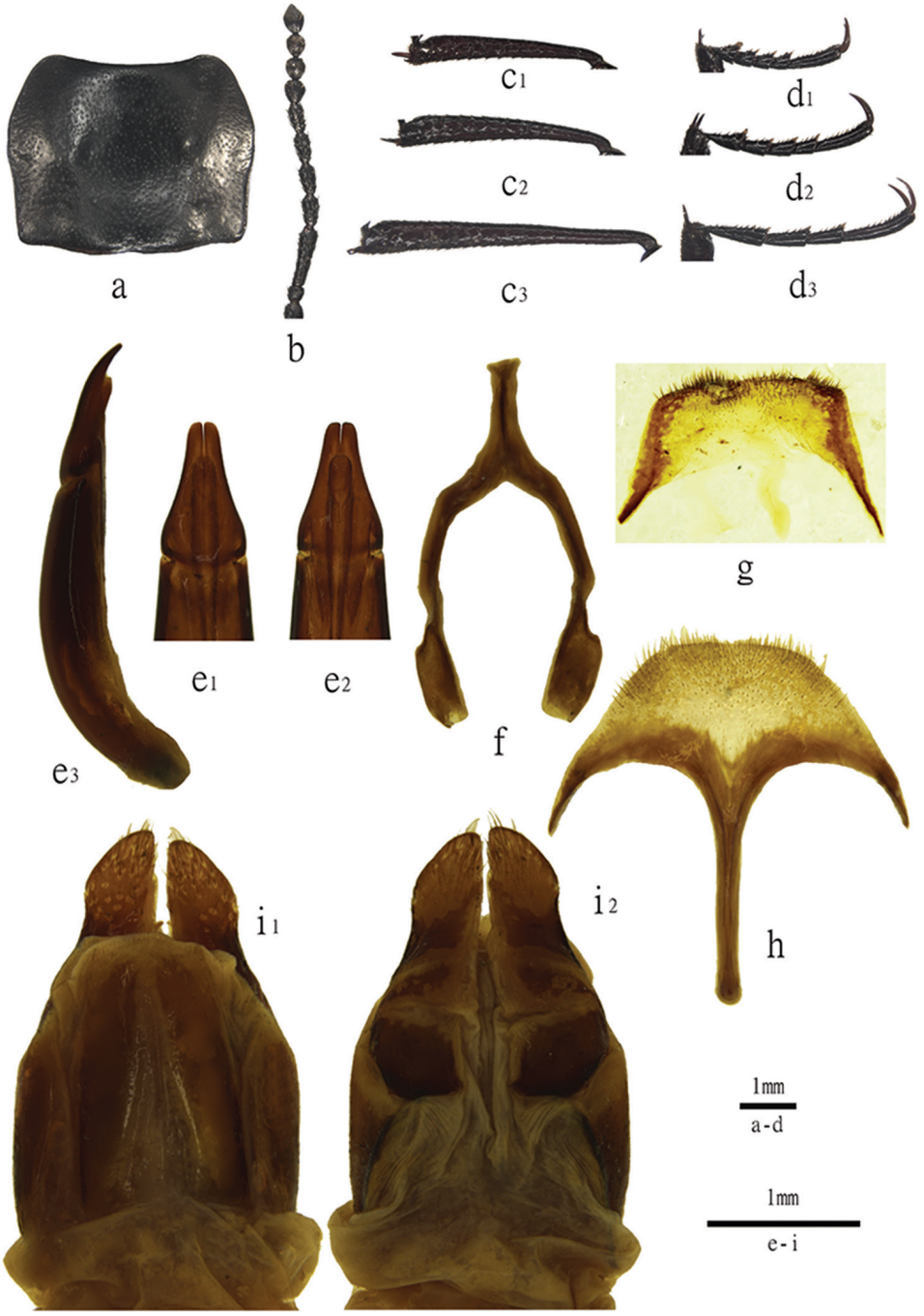


Figure 2. Characters of *Blaptogonia zhentanga* sp. n. **a–g** male: **a** pronotum **b** antenna **c_1–c_3** pro-, meso, metatibia **d_1–d_3** pro-, meso-, metatarsus **e_1–e_3** aedeagus in dorsal, ventral, and lateral view **f** *spiculum astrale* **g**. abdominal sternite VIII **h–i** female: **h** *Speculum ventrale* in ventral view **i_1–i_2** ovipositor in dorsal and ventral view.

rounded apically; posterior angles obtuse. Pronotal surface between lateral margins weakly convex, with moderately dense and shallow fine punctures on disc. Propleura with fine shallow wrinkles. Prosternal process with dense pale hairs.

Elytra elongate-oval, 1.4–1.6 times as long as wide, and 1.2–1.4 times as wide as pronotum. Each elytron between suture and humeral carina with two distinct carinae. In addition, surface of elytra between suture and 1st carina, 1st and 2nd carina, 2nd and humeral carina with lower subcarinae; subcarinae between 2nd and humeral carina indistinct. The humeral carina, subcarinae and carinae reaching base of elytra; 1st carina fused with humeral carina at apex, 2nd carina and subcarinae indistinct at apex. Surface of suture, subcarinae, carinae, humeral carina, between suture and subcarinae, between subcarinae and carinae, between subcarinae and humeral carina with irregular sparse and shallow fine punctures, sparse fine granules, and shallow wrinkles; punctures and wrinkles indistinct at apex. Epipleura not reaching suture of elytral angle, outer margin visible in dorsal view only at humeri, sometimes at apices. Visible abdominal sternites covered with short pale recumbent setae, sparse and fine shallow punctures, and fine granules; 1st to 3rd ventrites with fine longitudinal wrinkles, 2nd ventrite flattened in the middle, 4th ventrite shallowly depressed at sides.

Legs (Fig. 2c, d) rather slender. Protibia (Fig. $2c_1$) straight, inner apical spur longer than outer one; mesotibia (Fig. $2c_2$) nearly straight; metatibia (Fig. $2c_3$) straight, inner apical spur equal to or slightly longer than outer one. Ventral surface of protarsomere I (Fig. $2d_1$) with hairy brush, mesotarsomere I (Fig. $2d_2$) with hairy tuft. Length (width) ratio of pro-, meso- and metafemora 7.4(1.0): 2.5(1.0): 3.0(1.0), that of corresponding tibiae 3.4(1.0): 3.5(1.0): 4.3(1.0); that of metatarsomeres (Fig. $1d_3$) 12.7(1.0): 7.3(1.0): 6.5(1.0): 13.8(1.0). Claws slender, longer than half the length of apical tarsomere.

Aedeagus (Fig. 2e–g): length 2.9 mm, width 0.8 mm. Parameres 0.9 mm long and 0.6 mm wide, arcuately concave, narrowing from basal 1/5 to apex (Fig. $2e_1$, e_2), and curved to ventral side in lateral view (Fig. $2e_3$).

Female (Figs 2h–i, 4). Body wider than in male. Antennae reaching pronotal base. Pronotum 1.4–1.5 times as wide as long, 1.6–1.8 times as wide as head. Elytra 1.3–1.5 times as long as wide, and 1.4–1.6 times as wide as pronotum. Second visible sternite not flattened in middle. Apical spur slender, sharp at apex; inner apical spur of protibia slightly longer than outer one. Ventral surface of tarsus without hairy tuft. *Spiculum ventrale* as in Fig. 1h. Ovipositor as in Fig. 1i.

Measurements. Body length: male 11.9–13.8 mm, female 13.1–14.9 mm; width: male 5.3–6.0 mm, female 7.0–7.4 mm.

Distribution. China: Xizang.

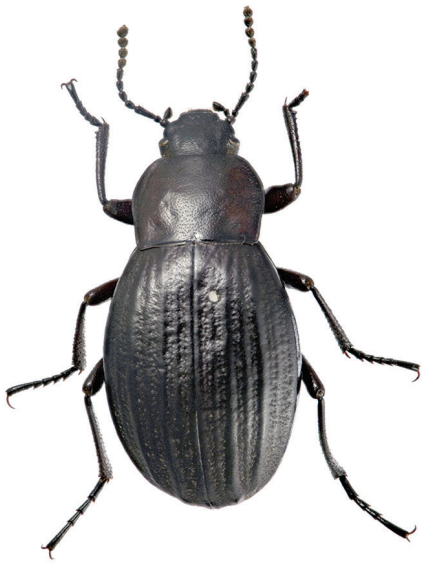
Molecular characters. Two fragments of mitochondrial protein-coding genes (COI, Cytb), one fragment of mitochondrial ribosomal RNA gene (16S), and one fragment of nuclear rRNA gene (28SD2) are deposited in GenBank with the accession numbers MG946798, MG946797, MG946800, and MG946799, respectively, based on one male, China, Xizang, Dinggyê, Zhêntang, 4 August 2014, coll. Guo-dong Ren, Xing-long Bai & Jun-sheng Shan. The sequence is presented in Table 2.

Table 2. Sequence of *Blaptogonia zhentanga* sp. n.

GenBank accession No.	Locus	Sequence
MG946798	COI	TGCCATATTAGAATGATGACAGTATAGGGAGTTCAGAATATCTGT-GTTCAGCAGGGGGAGTATTTTGTAATCATTCAATAGAGGAGGTTAT-ATTAAGCGAGGTTAAGGATTTTCGTGATGAAGAAAATCTTTCTCAT-ATAATGAAAATTAGGAATAATACTCCTACTAAAGATATTAGAGACC-CAATTGAGGAAATAATATTTCATAGGGTGTAGGCATCAGGGTAATCT-GAGTATCGTCGGGGTATTCCTCTTAATCCGAGAAAGTGTTGAGGAAA-GAAGGTAAGGTTTACGCCCACGAATATTACAAAAAATTGAATTTTA-CACAGTTTTGCCCTTAAGGATAAACCTGTGAATAAAGGGAATCAATG-TACTAATCCTCCTAGAATTGCAAATACAGCTCCTATAGATAATACG-TAATGGAAATGGGCTACTACATAATAGGTATCATGTAATATAATAT-CAATGGAAGAGTTAGCTAGAATTACTCCTGTTAATCCTCCTACTG-TAAATAAAAATACGAATCCTAATGCTCATAATATTGAGGGACTATAATT-TAGTTGTGTTCCGTGGAGAGTGGCTAATCATCTGAAAATTTTAATTC-CAGTAGGAACTGCAATAATTATTGTTGCTGAAGTGAAATATGCTC-GAGTATCTACGTCTATTCCTACTGTAAATATGTGATGGGCTCATAC-CACAAATCCTAATAATCCAATTGCTATTATAGCATAAATTATTCCCAAT-GTTCCAAAGGCCTCTTTTTTACCTCTTTCTTGTCT
MG946797	Cytb	ATCATTTTGAGGTGCTACTGTAATTACAAACTTACTTTCAGCAATTC-CATACCTAGGATCAACTATTGTACAATGATTATGAGGAGGGTTT-GCAGTAGACAATGCAACACTTACTCGATTTTTTGCATTCCATTTC-CTTCTTCCATTTATTGTAACAGCAATAGTTATAATCCATTTACT-GTTTCCCACCAAACAGGATCTAATAATCCCCTAGGATTAAACAG-TAATATTGACAAAATTCCATTTCACCCATACTTTTCCTTTAAAGA-CATTATAAGATTGATTATTACAATTATAGCTCTTGTAATACTATCTAT-TAATAGACCCTATCTACTAGGAGATCCAGACAACTTTACACCCG-CAAACCCTCTATCAACCCCAATTCATATCCAGCCAGAATGATATTTC-CTATTTGCTTATGCAATCTTACGTTCAATCCCTAATAAATTAGGAG-GAGTAATTGCACTAGCAATATCAATTGCAATCCTTTATATTT-TACCTCTATCTAATAAAAAAAAATTTGCAAGAAACTCATTTTACC-CTATAAATAAAATCCTATTCTGAATTATATTAGTTACAGTGATTCTAT-TAACATGAATTGG
MG946799	28SD2	TTCAAGACGGGTCCTGAAAGTACCCAAAGCTATAGCGTCCGCA-GATCGGCGTTTCAACGAGGTCCTGTTCGAGAACACCTCGGC-CAACAGTCGCCCGGGGACGGGACCGGCACCAGGTCCGATCAC-CGTCGCGAGAAGCGCGCTTGCCGAAGGTCGAACGCTAACT-GAATAGCGGCCCCGCGCCATCTGTATATCGTCGAGCGAGCCGAC-CGGGAAACACCGAGGGTTCGTCACGAACGCCGAAACGTCCG-GACGAACTCCACCTCGGGCCTTAGGCCGACACCCAACGAATCGC-GACGTCCTACAGGGGGAGAAGTGCACGCGTTCGACCGCAGTTCG-GAGGACGAAAGGCGCGGACGACGCGTACGCCGTCCGTCGCACGAC-CATCCAGCGCCACGATCGAGACACGCTGAATCTCCCCTTTCGAC-CTTTCGGGTTTCTCAGGTTTACCCCTGAACGGTTTCACGTACTCTT
MG946800	16S	AAAAACATGTCTTTTTGTTTTATGATTTAAAGTCTGGCCTGCCCAAT-GATTAATTTTAAATGGCTGCAGTATTTTGACTGTACAAAGGTAG-CATAATCATTAGTTTCTTAATTAGAAGCTGGAATGAATGGTTTGAT-GAAAAATTTACTGTCTCAATTCAATTGTTTTAGAATTTTATTTTTAAGT-GAAAAAGCTTAAATTTTTTAGAAAGACGAGAAGACCCTATAGAGTTTTA-TATGTTTTTTATTATTTATTATATGGTTATAATATTTTTAATTTTAAAATT-TATTTTGTTGGGGTGATGTGAAAATTTAAATAACTTTTCTTAATTT-TAACACTAATTAGTGATTAAATGATCCTTTTTAGGATTAAAAGATTAAAT-TACCTTAGGGATAACAGCGTAATTTTTTTTGAAAGTTCTTATTGA-TAAAAAAGTTTGCGACCTCGATGTTGGATTAAAATTTATTTTTGGTG-TAGAAGCTGGAAAATTTGGGTCTGTTCGACCCTTAAAATTTTACAT-GATCTG

Biology. Adults of the new species were found beneath stones in the shrubbery (Fig. 1), usually with more than ten individuals per stone. When threatened, they released quite foul smells and irritating liquids from their abdominal defensive glands. The smell persisted for a few days in the laboratory.

3



4

Figures 3–4. Habitus of *Blaptogonia zhentanga* sp. n. **3**.male, holotype, 12.2 mm **4** female, paratype, 13.5 mm.

***Blaptogonia zurstrasseni* (Kaszab, 1977)**

Blaptyscelis zurstrasseni Kaszab, 1977: 246.
Blaptogonia zurstrasseni: Medvedev,1998: 186; 2001: 95; 2004: 89; Löbl et al. 2008: 231.

Distribution. Nepal.

Acknowledgements

This study was supported financially by the National Natural Science Foundation of China (No. 31572309), the Ministry of Science and Technology of the People's Republic of China (No. 2015FY210300), the Key Laboratory of Zoological Systematics and Application in Hebei Province (No. 14967611D), and Post-graduate's Innovation Fund Project of Hebei Province (No. CXZZBS2017021).

References

Belshaw R, Quicke DLJ (1997) A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). Molecular Phylogenetics and Evolution 7(3): 281–293. https://doi.org/10.1006/mpev.1996.0400

Blair KG (1927) Heteromera of the third Mt. Everest expedition, 1924. The Annals and Magazine of Natural History (9)19: 241–255. https://doi.org/10.1080/00222932708633592

Fabricius JC (1775) Systema Entomologicae, Sistens Insectorum Classes, Ordines, Genera, Species, Adiectis Synonymis, Locis, Descriptionibus, Observationibus. Flensburgi et Lipsiae, Libraria Kortii, 832 pp.

Fairmaire L (1901) Descriptions de coléoptères des montagnes de Sikkim. Bulletin de la Société Entomologique de France 1901: 265–268.

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

Kaszab Z (1977) Tenebrionidae der Nepal-Expeditionen von Dr. J. Martens (1969–1974) (Insecta: Coleoptera). Senckenbergiana Biologica 57: 241–283.

Koch C (1965) Sur les types of Fairmaire des tribus Blaptini & Platyscelini conservés au Muséum de Paris (Coleoptera, Tenebrionidae). Annales de la Société Entomologique de France NS 1(1): 125–135.

Latreille PA (1802) Histoire naturelle, générale et particulière des crustacées et des insectes. Ouvrage faisant suite à l'histoire naturelle générale et particulière, composée par Leclerc de Buffon, et rédigée par C. S. Sonnini, membre de plusieurs sociétés savantes. Familles naturelles des genres. Tome troixième. F Dufart, Paris, 467 pp.

Leach WE (1815) Entomology. In: Brewster D (Ed.) The Edinburg Encyclopedia 9(1). Baldwin, Edinburg, 57–172.

Löbl I, Nabozhenko M, Merkl O (2008) Tribe Blaptini Leach, 1815. In: Löbl I, Smetana A (Eds) Catalogue of Palaearctic Coleoptera. Volume 5. Tenebrionoidea. Apollo Books, Stenstrup, 219–238.

Medvedev GS (1998) New species of tenebrionid beetles of the tribe Blaptini (Coleoptera, Tenebrionidae) from Hissaro-Darvaz Mountains and the Plateau of Tibet [in Russian]. Entomologicheskoe Obozrenie 77(3): 555–586.

Medvedev GS (2001) Evolution and system of darkling beetles of the tribe Blaptini (Coleoptera, Tenebrionidae). Meeting in memory of NA Cholodkovsky, ISS 53, St. Petersburg, 332 pp.

Medvedev GS (2004) New species of tenebrionids of the tribe Blaptini (Coleoptera, Tenebrionidae) from India, Nepal and China) [in Russian]. Entomologicheskoe Obozrenie 83(1): 163–189.

Pierre F (1961) Expédition francaise au Jannu (Népal Oriental). Coleoptera Tenebrionidae. Bulletin de la SociétéEntomologique de France 66: 212–214.

Ren GD, Ba YB, Liu HY, Niu YP, Zhu XC, li Z, Shi AM (2016) Fauna Sinica. Insecta. Vol. 63. Coleoptera: Tenebrionidae (I). Science Press, Beijing, 532 pp.

Ren GD, Wang XP, Yu YZ (2000) Nine new records and one new species of the tribe Blaptini from China (Coleoptera: Tenebrionidae). Journal of Hebei University (Natural Science Edition) 20: 26–33.

Simmons RB, Weller SJ (2001) Utility and evolution of cytochrome b in insects. Molecular Phylogenetics and Evolution 20: 196–210. https://doi.org/10.1006/mpev.2001.0958

Simon C, Frati F, Bekenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87(6): 651–701. https://doi.org/10.1093/aesa/87.6.651

ZooKeys 773: 79–108 (2018)
doi: 10.3897/zookeys.773.24319
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

Molecular association and morphological characterisation of *Himalopsyche* larval types (Trichoptera, Rhyacophilidae)

Anna E. Hjalmarsson[1], Wolfram Graf[2], Sonja C. Jähnig[3], Simon Vitecek[4], Steffen U. Pauls[4]

1 *Goethe-University Frankfurt, 60323 Frankfurt am Main, Germany. Senckenberg Research Institute and Natural History Museum, Senckenberganlage 25, 63025 Frankfurt am Main, Germany* **2** *Institute of Hydrobiology and Aquatic Ecology Management, University of Natural Resources and Life Sciences, Gregor-Mendel-Straße 33/DG, 1180 Vienna, Austria* **3** *Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Department of Ecosystem Research, Justus-von-Liebig-Str. 7, 12489 Berlin, Germany* **4** *Senckenberg Research Institute and Natural History Museum, Senckenberganlage 25, 63025 Frankfurt am Main, Germany*

Corresponding author: *Anna E. Hjalmarsson* (annahjalmar@gmail.com)

Academic editor: *R. Holzenthal* | Received 8 February 2018 | Accepted 23 May 2018 | Published 9 July 2018

http://zoobank.org/36751E1A-DF1A-462D-AC46-4958D0F0F899

Citation: Hjalmarsson AE, Graf W, Jähnig SC, Vitecek S, Pauls SU (2018) Molecular association and morphological characterisation of *Himalopsyche* larval types (Trichoptera, Rhyacophilidae). ZooKeys 773: 79–108. https://doi.org/10.3897/zookeys.773.24319

Abstract

Himalopsyche Banks, 1940 (Trichoptera, Rhyacophilidae) is a genus of caddisflies inhabiting mountain and alpine environments in Central and East Asia and the Nearctic. Of 53 known species, only five species have been described previously in the aquatic larval stage. We perform life stage association using three strategies (GMYC, PTP, and reciprocal monophyly) based on fragments of two molecular markers: the nuclear CAD, and the mitochondrial COI gene. A total of 525 individuals from across the range of *Himalopsyche* (Himalayas, Hengduan Shan, Tian Shan, South East Asia, Japan, and western North America) was analysed and 32 operational taxonomic units (OTUs) in our dataset delimited. Four distinct larval types of *Himalopsyche* are uncovered, and these are defined as the *phryganea* type, *japonica* type, *tibetana* type, and *gigantea* type and a comparative morphological characterisation of the larval types is presented. The larval types differ in a number of traits, most prominently in their gill configuration, as well as in other features such as setal configuration of the pronotum and presence/absence of accessory hooks of the anal prolegs.

Keywords

Caddisfly, GMYC, Hengduan Mountains, Himalaya, life stage association, PTP

Introduction

With ~15,000 described and around 50,000 presumed species, caddisflies are one of the larger insect orders and the largest primary aquatic insect order (de Moor and Ivanov 2008). Trichoptera have merolimnic life histories and the larvae have long been recognised as important ecological indicators (Resh and Unzicker 1975). They have several remarkable ecological traits, among others their diverse case-building behaviour or their ability to invade all types of aquatic habitats across the globe (except Antarctica; Holzenthal et al. 2007, Wiggins 1996). While renowned for their intricate cases, larvae of some caddis families roam freely, and only build pupal retreats. Among those, the family Rhyacophilidae Stephens, 1836 is particularly noteworthy for their diversity and ecological differentiation. This family entails the genera *Himalopsyche* Banks, 1940, *Fansipangana* Mey, 1996, *Philocrena* Lepneva, 1956, *Phoupanpsyche* Malicky, 2008, and *Rhyacophila* Pictet, 1834.

Species of the genus *Himalopsyche* are a particularly interesting group of caddisflies. They primarily inhabit mountain and alpine environments in Central and East Asia, although the genus also radiated into the Nearctic where it is represented by a single species, *H. phryganea* (Ross, 1941). *Himalopsyche* larvae mostly inhabit highly turbulent, fast-flowing streams, where they live as ferocious predators.

Including recent species descriptions, the genus *Himalopsyche* currently comprises 53 known species, adding to the status of the last major treatment of *Himalopsyche* by Schmid and Botosaneanu (1966). While our knowledge of the adult taxonomy of *Himalopsyche* is comparatively good, we know very little about the larval taxonomy and ecology of individual species. To date, five species have been described in the larval stage: *H. japonica* (Morton, 1900), *H. phryganea*, *H. gigantea* (Martynov, 1914), *H. tibetana* (Martynov, 1930), and *H. acharai* Malicky & Chantaramongkol, 1989 (Flint 1961, Graf and Sharma 1998, Lepneva 1945, 1970, Saito 1965, Tanida 1985, Thamsenanupap et al. 2005), as well as a larva corresponding to a hitherto unknown species, referred to as *H.* 'larva hoplura' (Lepneva 1945, 1970). Three distinct types of *Himalopsyche* larvae have been differentiated, most prominently based on their gill configuration, but also on differences in setal configuration of the pronotum and anal sclerites. In their comparative study, Graf and Sharma (1998) defined two of these larval types, Type A and Type B, which differ distinctly from the previously known larvae of *H. phryganea* and *H. japonica*. Type B could be assigned to *H. tibetana*. The species identity of larvae assigned to Type A could not be clarified before now, but this type shows similarities to larvae of *H. gigantea*. The larva of *H. acharai* was described by Thamsenanupap et al. (2005) and was compared to *H. japonica* and *H. phryganea*, but not with Type A and B *sensu* Graf and Sharma (1998). Thus, all *Himalopsyche* species known in the larval stage have never been compared and characterised simultaneously before.

Caddisflies are good biological indicators (e.g., Rainbow et al. 2012) and are essential elements in many standardised assessment systems, especially in North America, Europe, and Australia (e.g., Schmidt-Kloiber et al. 2008). However, the practical use of Trichoptera larvae as biological indicators is limited whenever taxonomic knowl-

edge is poor. Higher-level taxonomic resolution (e.g., genus or family level) can often mask the variability of environment/species interactions that act at the species level and make these taxa valuable indicator species (Resh and Unzicker 1975, Verdonschot 2006, Ruiter et al. 2013).

Molecular data have proven successful in facilitating the association of larvae with adults in Trichoptera (e.g., Graf et al. 2005, Ruiter et al. 2013, Zhou et al. 2007). Since the earliest studies of this kind (Graf et al. 2005, Zhou et al. 2007) the mitochondrial cytochrome c oxidase (COI) and nuclear rDNA 28S genes have been the markers of choice for life stage associations. Zhou et al. (2007) suggested a protocol for life stage association based on COI (439 bp) and 28S (~430 bp) and emphasised that it is important to use more than a single genetic marker for life stage association. There are several reasons for this. First, data from different genes provide the possibility to cross-validate the results from each gene, and to identify potential contamination issues. Second, gene trees are likely to differ from the species phylogeny among close relatives because of incomplete lineage sorting (Funk and Omland 2003). Using more than one gene therefore increases the opportunities to cross-validate results from one gene with the other gene(s).

In this study, we associate larvae with adults based on molecular data from a nuclear and a mitochondrial gene, CAD and COI, respectively, and employ three different methods for life stage association to generate a consensus result. We then present the first comparative morphological characterisation of all known larval types of *Himalopsyche*.

Materials and methods

Sampling

Larval specimens used in this study were mostly collected in Nepal and China in 2011–2013; adult material was largely obtained from research collections (Suppl. material 1). In Nepal, samples were collected in 2012 and 2013 from the Langtang, Indrawati, and Arun catchments (Tachamo Shah et al. 2015) and in China from the Lancang and Jinsha catchments in 2011 and 2013 (Figure 1). Larvae were collected from streams using hand nets, kick nets and hand-picking. Adults were collected using light traps, and occasionally with hand nets and a Malaise trap. Light traps were either set up at dusk, using a white sheet and a UV lamp (active light trapping), or left over night with the lamp placed over a small pan filled with soap water (passive light trapping; Blahnik and Holzenthal 2004, Malicky 2004). Lamps used included 12V 15W Bioform blacklights, F8W/T5/BL350 Sylvania blacklights, and 22W circline BioQuip blacklights. All specimens were stored directly in 95% ethanol in the field, and refrigerated in the lab. Museum specimens of adults were borrowed for DNA sequencing from the private collection of Hans Malicky (Lunz am See, Austria), and from the Museum für Naturkunde (Berlin, Germany).

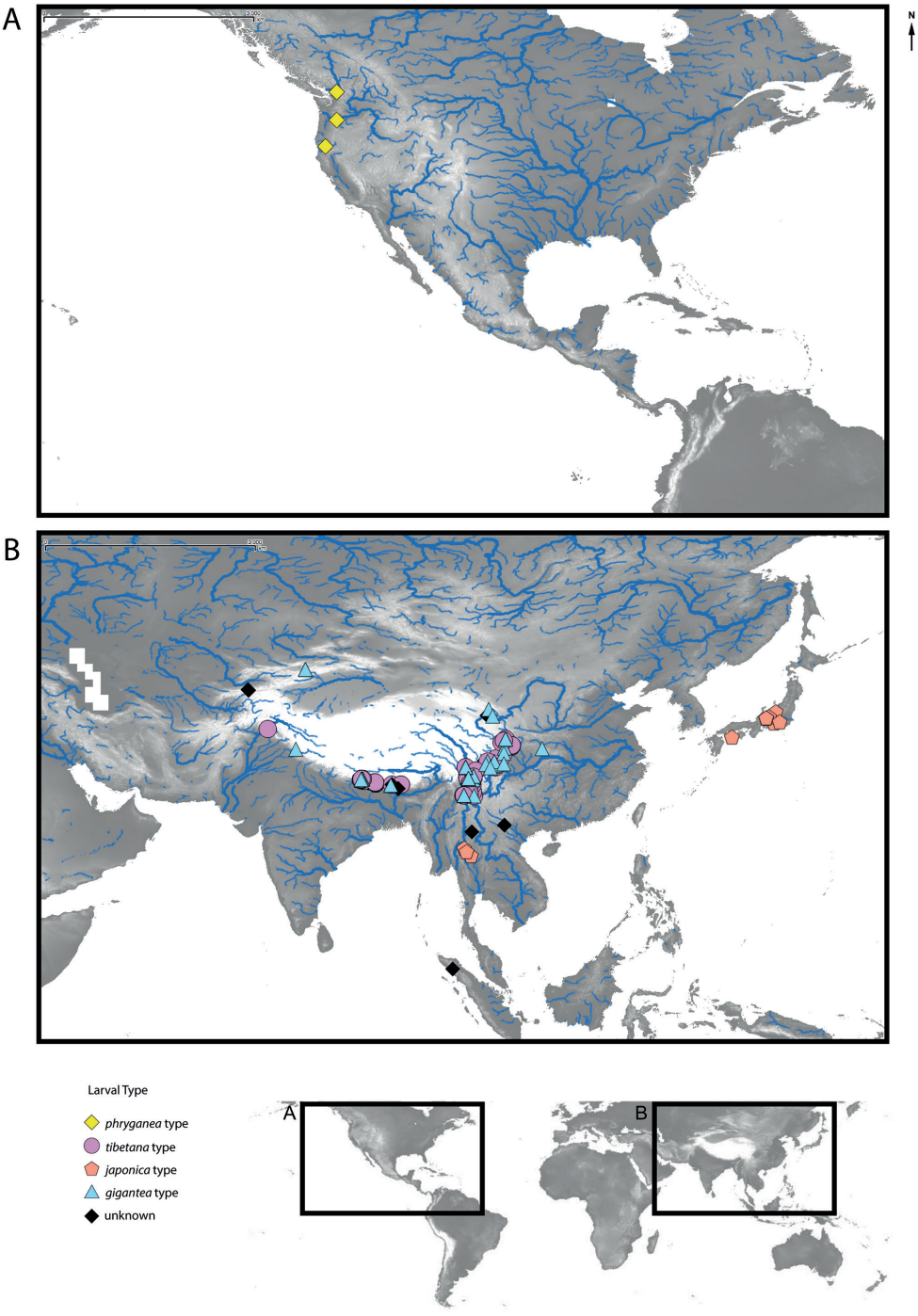


Figure 1. Map showing sampling localities in **A** North America, and **B** East Asia. Colours/symbols indicate larval types of the OTUs. Elevation data from Jarvis et al. (2008), stream data from Andreadis et al. (2013).

Molecular data

For this study, we used partial sequence data of the single copy nuclear marker CAD and the mitochondrial COI. CAD has proved useful for insect phylogenetics (Bukontaite et al. 2014, Ekrem et al. 2010, Johanson and Malm 2010, Klopfstein et al. 2013, Moulton and Wiegmann 2004, Sharanowski et al. 2011, Wild and Maddison 2008) and in species delimitation (Foster et al. 2013, Song and Ahn 2014, Vitecek et al. 2017). For COI, we targeted the 658 bp "standard barcode region" fragment of COI that has been used extensively for species identification, but also for life stage association, population genetics, and phylogeny in insects (e.g., Hebert et al. 2004, Hjalmarsson et al. 2015, Ruiter et al. 2013, Zhou et al. 2007, 2016).

DNA was extracted from legs using one of the following methods: HotShot protocol (Montero-Pau et al. 2008; mainly used for larvae), Qiagen Dneasy Blood & Tissue Kit (Qiagen, mainly used for fresh adult material), or QIAamp DNA Micro Kit (Qiagen, mainly used for museum material). We used a combination of previously published and newly developed primers, specific to *Himalopsyche*. The primers for CAD were: 743nF-ino & 1028r-ino (850 bp; Johanson and Malm 2010) and C1Fb & C7Ra (758 bp; this work, Table 1). The COI primers were: HCO1490 & LCO2198 (658 bp; Folmer et al. 1994), and B1Fa & B3Ra (367 bp; this work, Table 1). Polymerase Chain Reaction (PCR) was performed using PeqGOLD Hot Start Taq Polymerase kits (PeqLab VWR) in standard reactions, for some protocols with the addition of BSA (Table 2). Sanger sequencing of PCR products was performed on a 3730XL DNA Analyzer (Applied Biosystems) at the Senckenberg Biodiversity Climate Research Centre Laboratory Centre. Sequences were assembled and edited in Geneious 7.0.6 (Kearse et al. 2012). Ambiguities were coded using IUPAC codes. Multiple sequence alignments of CAD and COI were made using the ClustalW algorithm (Thompson et al. 1994) as implemented in Geneious, and were checked for stop codons.

Life stage association

Adult males were identified to species based on morphology. The dataset included 38 adult species based on males, including four putative new species (Hjalmarsson submitted, Kuranishi et al. unpublished data). We used and compared the results of three phylogenetic association criteria: Poisson Tree Process (PTP; Zhang et al. 2013), General Mixed Yule Coalescent method (GMYC; Fujisawa and Barraclough 2013, Pons et al. 2006), and reciprocal monophyly. In total, we had five life stage association criteria (PTP and GYMC for each gene, and reciprocal monophyly). Life stage association was considered successful if at least three criteria were fulfilled. The life stage association criteria were defined as follows. PTP: larvae are conspecific with an adult if they form a PTP cluster containing only one and the same adult species. GMYC: larvae are conspecific with adults if they form a GMYC cluster containing only one and the same adult species. Reciprocal monophyly: species are considered reciprocally

Table 1. Primers used for PCR and sequencing. Fragment lengths refer to the primer pairs 743nF-ino & 1028r-ino, C1Fb & C7Ra, HCO1490 & LCO2198, and B1Fa & B3Ra.

Gene	Primer	Sequence	Tm (°C)	Fragment length (bp)	Reference
CAD	743nF-ino	5'-GGIGTIACIACIGCITGYTTYGARCC-3'	52.4	850	Johanson and Malm 2010
CAD	1028r-ino	5'–TTRTTIGGIARYTGICCICCCAT–3'	42.1	850	Johanson and Malm 2010
$CAD_{internal}$	C1Fb	5'–TGYGTTGTRAAGATTCCGAG-3'	51.8	736	this work
$CAD_{internal}$	C7Ra	5'–TGTCCATTACAACCTCGAATG-3'	62.3	736	this work
COI	HCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	53.2	658	Folmer et al. 1994
COI	LCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	51.0	658	Folmer et al. 1994
$COI_{internal}$	B1Fa	5'-ATTGCDACWGATCAWACAAA-3'	54.9	367	this work
$COI_{internal}$	B3Ra	5'-AAYGTARTWGTWACWGCTCA-3'	47.2	367	this work

monophyletic if both genes return a monophylum containing one and the same adult species, and the same larval specimens. Reciprocal monophyly could only be tested for species with data from both genes and with >1 specimen per gene. We considered nodes as supported if they had posterior probability of at least 95%. We refer to groups of unresolved and paraphyletic species as 'species complexes'.

Gene trees of all available specimens were reconstructed in MrBayes v3.2.6 (Ronquist et al. 2012). Alignments were partitioned per codon position with independent rates among partitions. Nucleotide substitution models were determined in PartitionFinder v2.1.1. (Lanfear et al. 2016; Table 3). Runs were generated for 10 to 50 million generations and were checked for convergence in Tracer v.1.6 (Rambaut et al. 2014). Fully resolved 50% majority rule consensus trees were generated using the 'sumt' command, with 25% burn-in. PTP and test of reciprocal monophyly were performed on the gene trees from MrBayes.

The GMYC method uses a haplotype-based ultrametric gene tree to determine the transition from inter- to intraspecific branching patterns (Fujisawa and Barraclough 2013; Pons et al. 2006). For this, we reconstructed chronograms in BEAST (see below). GMYC was performed separately on chronograms from each gene in R 3.2.3 (R Core Team 2015), using the *splits* package (Ezard et al. 2014). The single-threshold option of GMYC was used. It sets a single limit between inter- and intraspecific divergence patterns and has been shown to outperform the multiple-threshold option (Fujisawa and Barraclough 2013).

Haplotype-based chronograms require that identical sequences be removed from the alignment, leaving an alignment consisting only of unique sequences. Identical haplotypes were removed from the original alignments using collapsetypes_v4.6 (Chesters 2013), which outputs a reduced fasta-alignment and haplotype assignations of each sequence. Ultrametric gene trees based on haplotype alignments were reconstructed in BEAST2 v. 2.3.1 (Bouckaert et al. 2014). Model selection was done

Table 2. Protocols for 10 µL PCR reactions, using VWR peqGOLD Hot Taq DNA Polymerase kits. BSA = Bovine serum albumin. All numbers are given in µL.

Gene	Primer pair	Buffer S	Buffer Y	dNTPS 2mM	BSA 20 mg/ml	Forward primer 10µM	Reverse primer 10µM	Taq	DNA	H_2O	Cycler
CAD	1028r-ino, 743nF-ino	1	0	1	0	0.25	0.25	0.1	1	6.4	5' 95°C, 35x (45" 95°C, 45" 55°C, 60" 72°C) 5' 72°C
CAD	C1Fb, C7Ra	1	0	1	0	0.25	0.25	0.1	1	6.4	5' 95°C, 35x (30" 95°C, 30" 50°C, 45" 72°C) 5' 72°C
COI	HCO1490, LCO2198	1	0	1	0	0.25	0.25	0.1	1	6.4	5' 95°C, 5 x (30" 95°C, 1' 44°C, 1' 72°C), 15x (30" 95°C, 30" 48°C, 1' 72°C), 20 x (30" 95°C, 30" 50°C, 1' + (10" * n) 72°C), 5' 72°C
COI	B1Fa, B3Ra	1	0	1	0.4	0.25	0.25	0.1	1	6.0	5' 95°C, 35x (30" 95°C, 30" 45°C, 45" 72°C) 5' 72°C

Table 3. Specifications of alignments used for gene tree reconstruction with BEAST and MrBayes.

Alignment	Number of sequences	Length (bp)	Variable sites	Parsimony informative sites	Missing data	Analysis	Substitution model
CAD	353	736	37,6%	28,0%	1.5%	MrBayes	1: GTR+I 2: F81 3: HKY+G
CAD haplotypes	136	736	31.8%	26.4%	1.6%	BEAST	1: BMod 2: BMod 3: BMod
COI	451	658	40,0%	37,7%	18.5%	MrBayes	1: SYM+I+G 2: F81+I 3: GTR+I+G
COI haplotypes	183	658	39.2%	35.0%	15.9%	BEAST	1: BMod 2: BMod 3: BMod

using bModeltest (Bouckaert and Drummond 2015), which estimates the best fitting model of sequence evolution simultaneously with the Bayesian tree search. The transition/transversion split option was chosen, which searches among 31 models of sequence evolution. The 'empirical' option was used for base frequencies. Alignments were partitioned per codon position with independent rates among partitions; trees and clocks were linked among partitions. Trees were reconstructed under a relaxed lognormal clock, and with a coalescent constant population tree prior. Priors were set to default settings, with infinity values replaced with hard bounds at 1000, to avoid improper priors. Independent analyses were executed with a run time of 0.5 to 1 billion generations each and runs were checked in Tracer. Maximum clade credibility trees were generated in TreeAnnotator, with 10–50% burn-in. PTP and GMYC analyses were performed on two independent trees, to check stability of the results.

Rhyacophila polonica McLachlan, 1879 was included as outgroup for MrBayes analyses but was not included in BEAST analyses.

For this paper, we define the use of the terms 'species', 'putative new species', 'cluster' and 'OTU' (operational taxonomic unit) as follows: 'Species' refers to formally described morphological taxa, following established taxonomy. With 'putative new species' we mean morphologically distinct taxa that are still unknown in the literature. The term 'cluster' refers to specific results from one of the two analyses, outputting delimited GMYC and PTP 'clusters', respectively. For our consensus result from morphology, PTP, and GMYC based on CAD and COI we use the term OTU, which can represent single species or groups of species referred to as species complexes.

Comparative morphological studies

Comparative morphological analysis of larvae followed a standard procedure. We screened all larvae of each OTU for consistent morphological characters. Instar differentiation and thus assignment of most larvae to different instars is not possible with the currently available material. Therefore, general features commonly represented by all OTUs within one phylogenetic clade were considered as synapomorphies. Some characters were found present across all size classes of single OTUs and phylogenetic clades, e.g., distolateral accessory hooks on lateral plates of anal prolegs were consistently present in even the smallest instars.

Results

Datasets

The dataset comprised 525 *Himalopsyche* individuals (205 adults, 313 larvae and 8 pupae), and *R. polonica* as outgroup for MrBayes. We generated 352 *Himalopsyche* sequences of CAD (736 bp) and 450 *Himalopsyche* sequences of COI (658 bp). After haplotype reduction of the alignments, the CAD alignment had 136 unique haplotypes and the COI had 183 unique haplotypes. The total CAD alignment had 37.6% variable sites; the total COI haplotype alignment had slightly more with 40% (Table 3). For morphological treatment, we also included larvae collected in Japan and Thailand identified as *H. japonica* and *H. acharai*, respectively, although no molecular data for these specimens were available.

Life stage association

PTP delimited 29 clusters with CAD, and 62 with COI for the ingroup. GMYC delimited 27 clusters with CAD and 46–48 clusters with COI. The results from separate runs

were stable except for GMYC with COI. This instability did not affect larval association and we hereafter only refer to COI run 1 which delimited 48 GMYC clusters (Table 4, Suppl. material 2, 3). We defined OTUs based on adult male morphospecies to the extent that it was possible. Clades containing several paraphyletic species were grouped into 'species complexes'. In all cases but two, COI clusters were nested within CAD clusters, yielding an overall compatible result. The exceptions were the OTUs *japonica*-complex (*H. japonica* and a putative new Japanese species [*H*. sp. n. 1529]), and *triloba*-complex (*H. triloba* (Hwang, 1958), *H. efiel* Malicky, 2012, *H. hageni* Banks, 1940, *H. malenanda* Schmid, 1963, *H. maculipennis* (Ulmer, 1905a), and *H. yatrawalla* Schmid & Botosaneanu, 1966), which both were paraphyletic in COI. The remaining species complexes were: *martynovi*-complex (*H. martynovi* Banks, 1940, *H. epikur* Malicky, 2011), and *excisa*-complex (*H. excisa* Ulmer, 1905b, *H. placida* Banks, 1947 and *H. maitreya* Schmid, 1963). Three OTUs were identified for which only larval material was available: *H*. sp. 1196 (L), *H*. sp. 1338 (L), and *H*. sp. 1254 (L). A *H. platon* Malicky, 2011 male formed a clade together with samples of larvae and females in COI but we lacked CAD data from the adult male so we cannot at this stage conclude whether this monophylum constitutes one or several species, and refer to this monophylum as *platon*-complex.

We could unambiguously associate 239 larvae and eight pupae to the following nine species: *H. acharai*, *H. anomala* Banks, 1940, *H. digitata* (Martynov, 1935), *H. gregoryi* (Ulmer, 1932), *H. phryganea*, *H. sylvicola*, *H. tibetana*, *H. 677*, and *H. 685* (Table 5, Suppl. material 1). We could additionally associate 65 larvae to OTU-level for seven OTUS: *excisa*-complex, *martynovi*-complex, *platon*-complex, *H*. sp. 1196 (L), *H*. sp. 1254 (L), *H*. sp. 1338 (L), and *triloba*-complex.

Morphology of *Himalopsyche* larvae

Synapomorphic larval characters of *Himalopsyche* according to Lepneva (1970), Ross (1956), Flint (1961), Ulmer (1957), and the present study are:

- Mandibles with prominent lateral protuberances (Figure 14, arrow)
- 2nd and 3rd leg with anterodorsal single coxal gills (e.g., Figure 18, arrow a, Figure 20, arrow a)
- Abdomen with prominent and complex gills consisting of a multitude of gill filaments positioned on several bases or a single base which can be slightly to distinctly protuberant (e.g. Figure 3).
- Anal proleg with two proximal accessory hooks fused with lateral sclerites (e.g. Figure 38, arrow d, Figure 41, arrow d).

Based on these characters, larvae of *Himalopsyche* can easily be differentiated from the closely related genera *Philocrena* and *Rhyacophila*. The larvae of the monotypic genera *Fansipangana* and *Phoupanpsyche* are unknown. Within *Himalopsyche*, the four different larval types can easily be differentiated based on distinct character states.

Table 4. Number of clusters delimited by PTP and GMYC. Number of PTP clusters refer to the ingroup only.

Gene	Method	Run	Chain length	Burn-in	ESS	Clusters
CAD	MrBayes & PTP	1	$1^* 10^7$	25%	All >200	29
CAD	MrBayes & PTP	2	$1^* 10^7$	25%	All >200	29
CAD	BEAST & GMYC	1	$1^* 10^9$	50%	All >200	27
CAD	BEAST & GMYC	2	$5 * 10^8$	10%	Most >200	27
COI	MrBayes & PTP	1	$5^* 10^7$	25%	All >200	62
COI	MrBayes & PTP	2	$1^* 10^7$	25%	Most >200	62
COI	BEAST & GMYC	1	$1^* 10^9$	10%	All >200	48
COI	BEAST & GMYC	2	$1 * 10^9$	10%	All >200	46

phryganea type

Himalopsyche phryganea is the only species known with this larval type. Larvae of *H. phryganea* were described and illustrated by Flint (1961) and Wiggins (1996). Larvae of the *phryganea* type are characterised by the following set of characters:

Thorax. Pronotum with a single row of long dark setae along the entire anterior margin; short light recumbent setae concentrated at anterolateral pronotal edges; Sa1 present as a transversal band of 4–5 setae, Sa2 absent (Figure 7); legs without dorsal fringe of setae (Figure 11). Gills. Ventral gills at meso- and metathorax absent (Figure 23); thoracic and abdominal gills arranged on a single suboval, slightly protuberant base extending obliquely from anterodorsal to mediolateral position (Figs 3, 15, 16, 25, 29, 30). Abdomen. Abdomen without ventral protuberances (Figure 30); single ventral medial sclerite on abdomen III-VII, oval, transversally elongated (Figure 30, arrow). Anal prolegs. Stout, distolateral accessory hook absent, dorsal plate with rounded central protuberance (Figs 37–38, arrow b); dorsal spine on basal anal claw dark (Figure 37, arrow c).

tibetana type

One species with larvae of the *tibetana* type has been described in the larval stage: *H. tibetana* (Graf and Sharma 1998, Type B). The following OTUs could be assigned to the *tibetana* type: *H. anomala*, *H. digitata*, *excisa*-complex, *H. gregoryi*, *martynovi*-complex, *platon*-complex, *H*. sp. 1196 (L), and *H.* sp. 1254 (L), *H. tibetana*, *H. 677*, and *H. 685*. Larvae of the *tibetana* type are characterised by the following set of characters:

Thorax. Pronotum with two rows of setae along the anterior edge, anteriormost row of setae short, light, recumbent and posterior row setae longer, black; Sa 1 present as a transversal band of 4–5 setae, Sa2 absent (Figure 8); legs without dorsal fringe of setae (Figure 11). Gills. Ventral gills at meso- and metathorax absent (Figure 23); thoracic gills arranged on anterodorsal and anterolateral bases, abdominal gills arranged on anterodorsal, anterolateral and posterolateral bases (Figs 4, 17, 18, 26, 31, 32); an-

Table 5. Overview of life stage association results for each OTU. Asterisks denote. The OTUs *martynovi*-complex, *H. 683*, and *H. 685,* formed a single GMYC cluster in CAD, here referred to as '*martynovi*-clade'. Key: † indicates larvae without DNA data, * indicates conflicts in COI and CAD gene tree topologies.

OTU	Samples	Species	Larvae	Larval type	CAD units (PTP/ GMYC)	COI units (PTP/ GMYC)	Association with CAD (PTP/GMYC)	Association with COI (PTP/GMYC)	Reciprocal monophyly (both genes)	Number of criteria fulfilled
H. acharai	4	*H. acharai*	yes	*japonica* type	1/1	1/1	yes/yes	yes/yes	yes	5
H. anomala	6	*H. anomala*	yes	*tibetana* type	1/1	4/3	yes/yes	no/yes	yes	4
H. auricularis	2	*H. auricularis*	no	unknown	1/1	2/1	–	–	not applicable	–
H. biansata	2	*H. biansata*	no	unknown	no data	1/1	–	–	not applicable	–
H. diehli	1	*H. diehli*	no	unknown	1/1	1/1	–	–	not applicable	–
H. digitata	34	*H. digitata*	yes	*tibetana* type	2/1	2/2	yes/yes	no/no	yes	3
H. eos	2	*H. eos*	no	unknown	no data	1/1	–	–	not applicable	–
excisa-complex	31	*H. excisa, H. placida, H. maitreya*	yes	*tibetana* type	1/1	5/2	no/no	no/no	no	0
H. gigantea	2	*H. gigantea*	no	*gigantea* type	no data	1/1	–	–	not applicable	–
H. gregoryi	77	*H. gregoryi*	yes	*tibetana* type	1/1	1/1	yes/yes	yes/yes	yes	5
H. gyamo	1	*H. gyamo*	no	unknown	1/1	1/1	–	–	not applicable	–
H. horai	5	*H. horai*	no	unknown	1/1	1/1	–	–	yes	–
japonica-complex*	8	*H. japonica, H.* sp. n. 1529	yes†	*japonica* type	2/1	4/3	–	–	no	–
H. kuldschensis	2	*H. kuldschensis*	no	unknown	1/1	1/1	–	–	yes	–
H. lanceolata	4	*H. lanceolata*	no	unknown	1/1	1/1	–	–	yes	–
H. lepcha	5	*H. lepcha*	no	unknown	1/1	2/2	–	–	yes	–
H. lua	5	*H. lua*	no	unknown	no data	2/1	–	–	not applicable	–
martynovi-complex	30	*H. martynovi, H. epikur*	yes	*tibetana* type	*martynovi*–clade	1/1	no/no	no/no	no	0
H. navasi	4	*H. navasi*	no	unknown	1/1	4/4	–	–	not applicable	–
H. phryganea	4	*H. phryganea*	yes	*phryganea* type	1/1	2/2	yes/yes	yes/yes	yes	5
platon-complex	27	*H. platon, H.* sp (F), *H.* sp (L)	yes	*tibetana* type	1/1	4/3	no/no	no/no	yes	1

OTU	Samples	Species	Larvae	Larval type	CAD units (PTP/ GMYC)	COI units (PTP/ GMYC)	Association with CAD (PTP/GMYC)	Association with COI (PTP/GMYC)	Reciprocal monophyly (both genes)	Number of criteria fulfilled
H. sp. 1196 (L)	17	NA	yes	*tibetana* type	1/1	2/1	–	–	yes	–
H. sp. 1254 (L)	4	NA	yes	*tibetana* type	1/1	no data	–	–	not applicable	–
H. sp. 1338 (L)	1	NA	yes	*gigantea* type	1/1	1/1	–	–	not applicable	–
H. sylvicola	33	*H. sylvicola*	yes	*gigantea* type	1/1	6/3	yes/yes	no/yes	yes	5
H. tibetana	58	*H. tibetana*	yes	*tibetana* type	1/1	2/2	yes/yes	yes/yes	yes	5
H. todma	4	*H. todma*	no	unknown	1/1	no data	–	–	not applicable	–
triloba-complex*	67	*H. triloba, H. hageni, H. macilipennis, H. malenanda, H. efiel, H. yatrawalla*	yes	*gigantea* type	2/2	5/3	no/no	no/no	no	0
H. yongma	1	*H. yongma*	no	*unknown*	1/1	1/1	–	–	not applicable	–
H. 677	41	H. *677*	yes	*tibetana* type	1/1	1/1	yes/yes	yes/yes	yes	5
H. 683	8	*H. 683*	no	*tibetana* type	*martynovi*– clade	1/1	–	–	yes	–
H. 685	34	*H. 685*	yes	*tibetana* type	*martynovi*– clade	1/1	no/no	yes/yes	yes	3

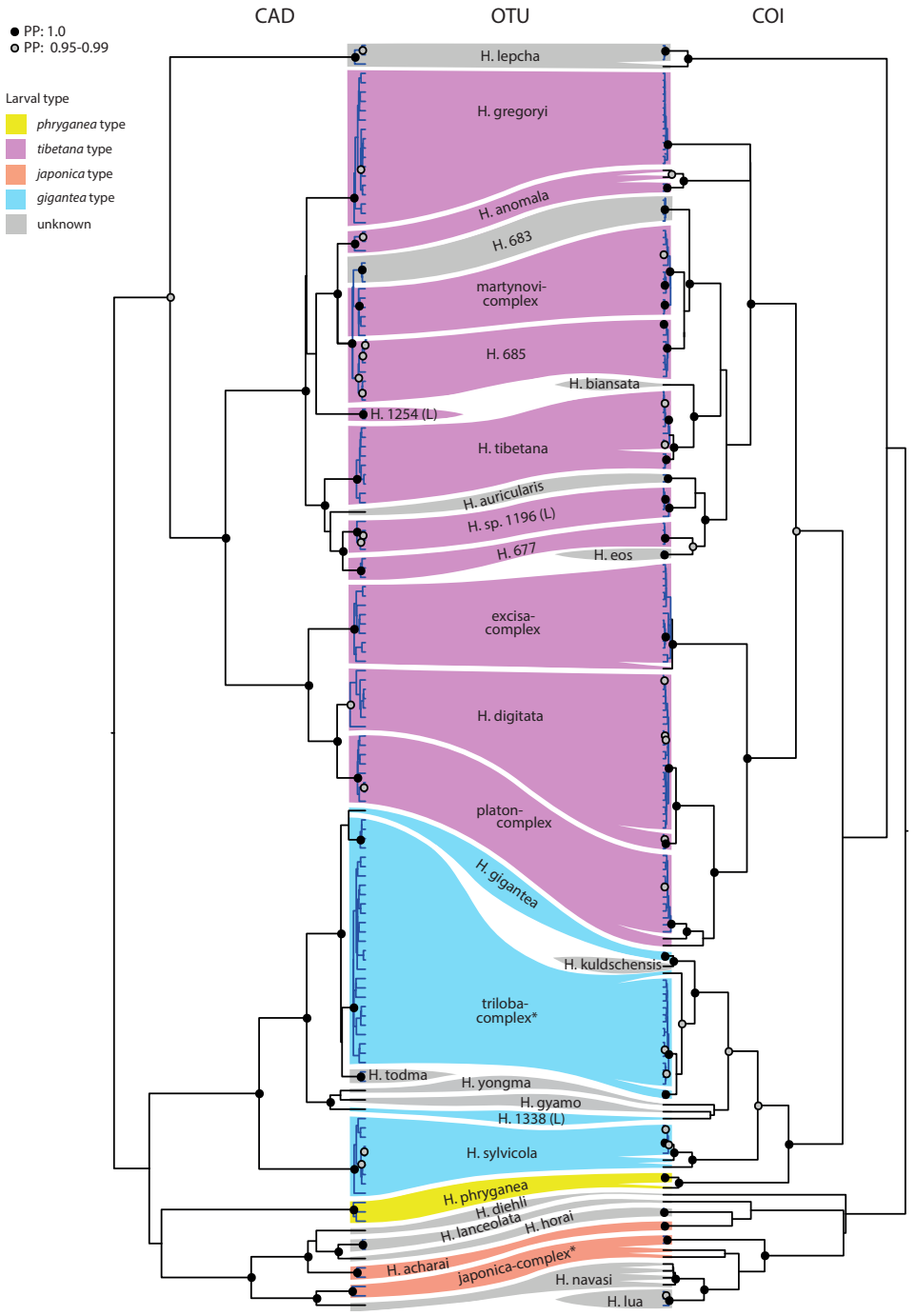
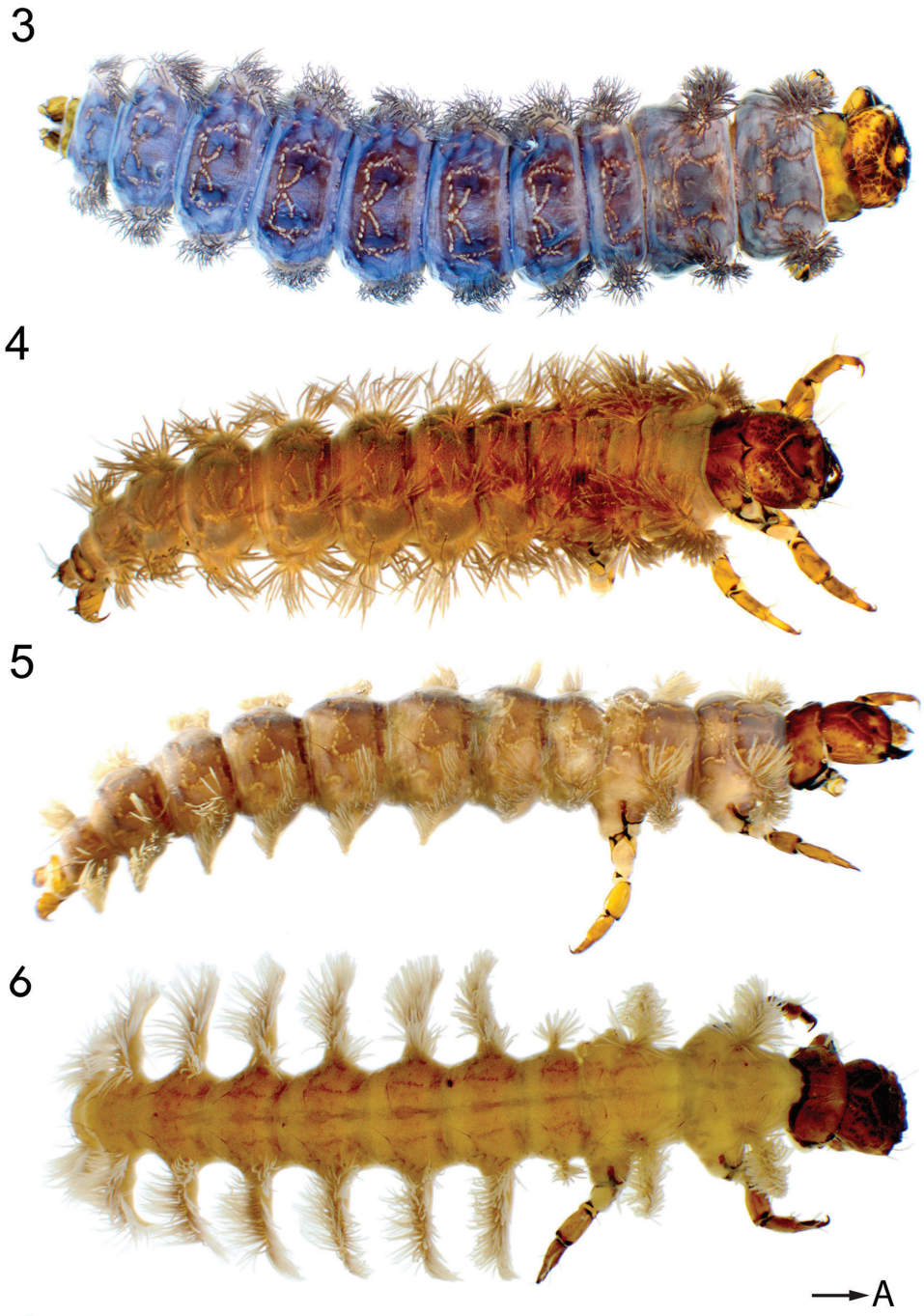


Figure 2. Summary of GMYC results from the CAD and COI, indicating the overlap in results from the two genes. Colours indicate larval types, inferred from this study and the literature.



Figures 3–6. Habitus of *Himalopsyche* larvae. **3** *H. phryganea* **4** *H. gregoryi* **5** *H. japonica* **6** *H. sylvicola*. Arrow A points to anterior.

terodorsal and anterolateral gill bases on abdomen I close, seemingly fused. Abdomen. Without ventral protuberances; single ventral medial sclerite on abdomen III-VII oval, transversally elongated (Figure 32, arrow). Anal prolegs. More elongated, with distolateral accessory hook (Figs 39–40, arrow a), dorsal plate with central hook (Figs 39–40, arrow b), dorsal spine on basal anal claw dark (Figs 39–40, arrow c).

***japonica* type**

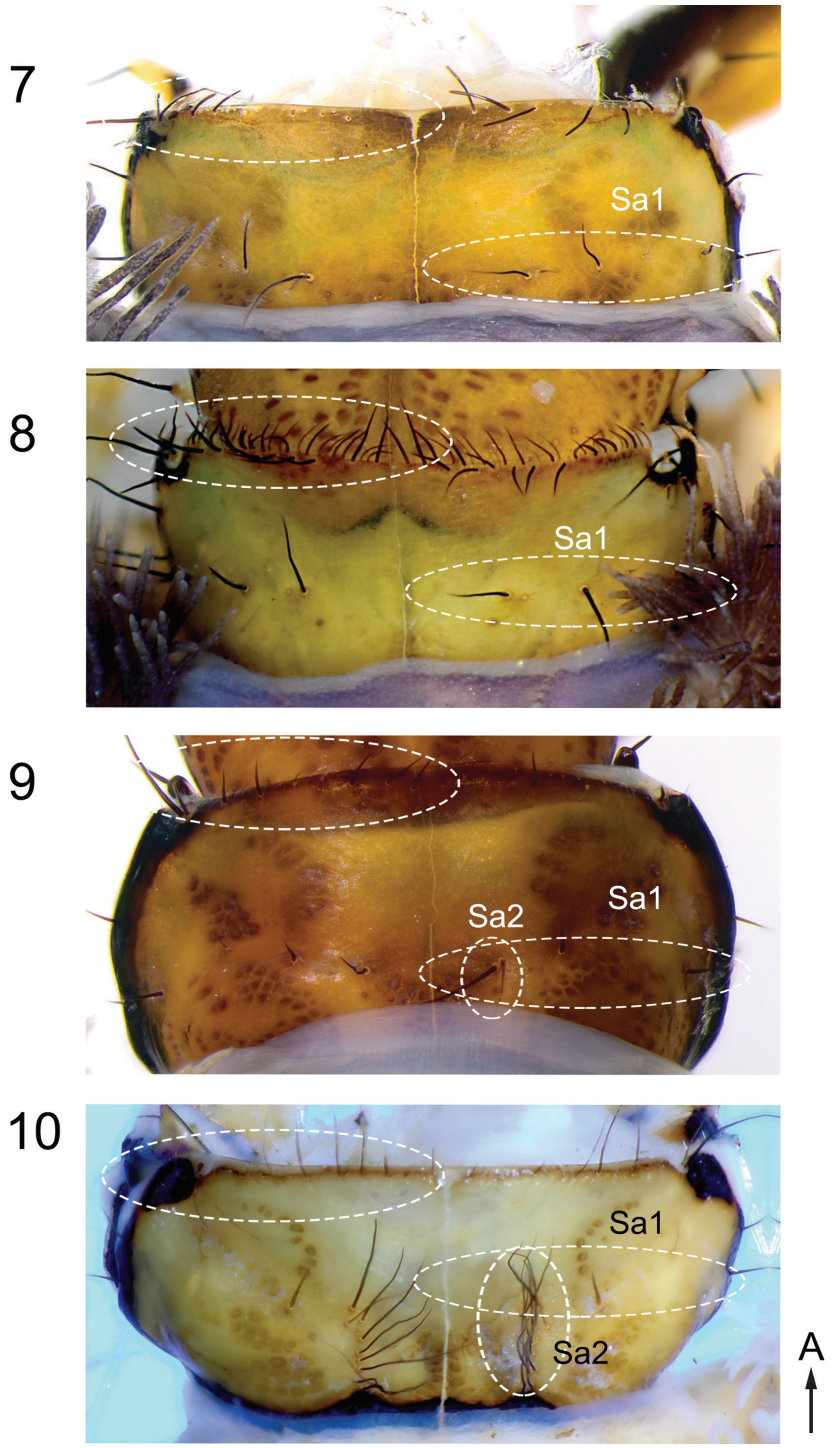
Two species of the *japonica* type have been described in the larval stage: *H. acharai* by Thamsenanupap et al. (2005), and *H. japonica* by Saito (1965) and Tanida (1985). Larvae of the *japonica* type are characterised by the following set of characters:

Thorax. Pronotum with a single row of setae along the anterior edge; Sa1 present as a transversal row of 2–4 dark setae medially, Sa2 present as a group (sometimes arranged as sagittal band) of 2–4 setae (Figure 9); legs without dorsal fringe of setae (Figure 11). Gills. Ventral gills at meso- and metathorax present (Figure 24, arrows, Figure 20, arrow b); thoracic and abdominal gills arranged on two joint bases, one anterodorsal and one on a small lateral protuberance, extending posterolaterally (Figs 5, 19, 20, 27, 33, 34); abdomen I with gills on a single base only. Abdomen. With ventral protuberances (Fig 34, arrow); ventral medial sclerites absent (Figure 34). Anal prolegs. Stout, distolateral accessory hook present (Figs 41–42, arrow a), dorsal plate flat without rounded central protuberance; dorsal spine on basal anal claw yellowish (Figure 41, arrow c).

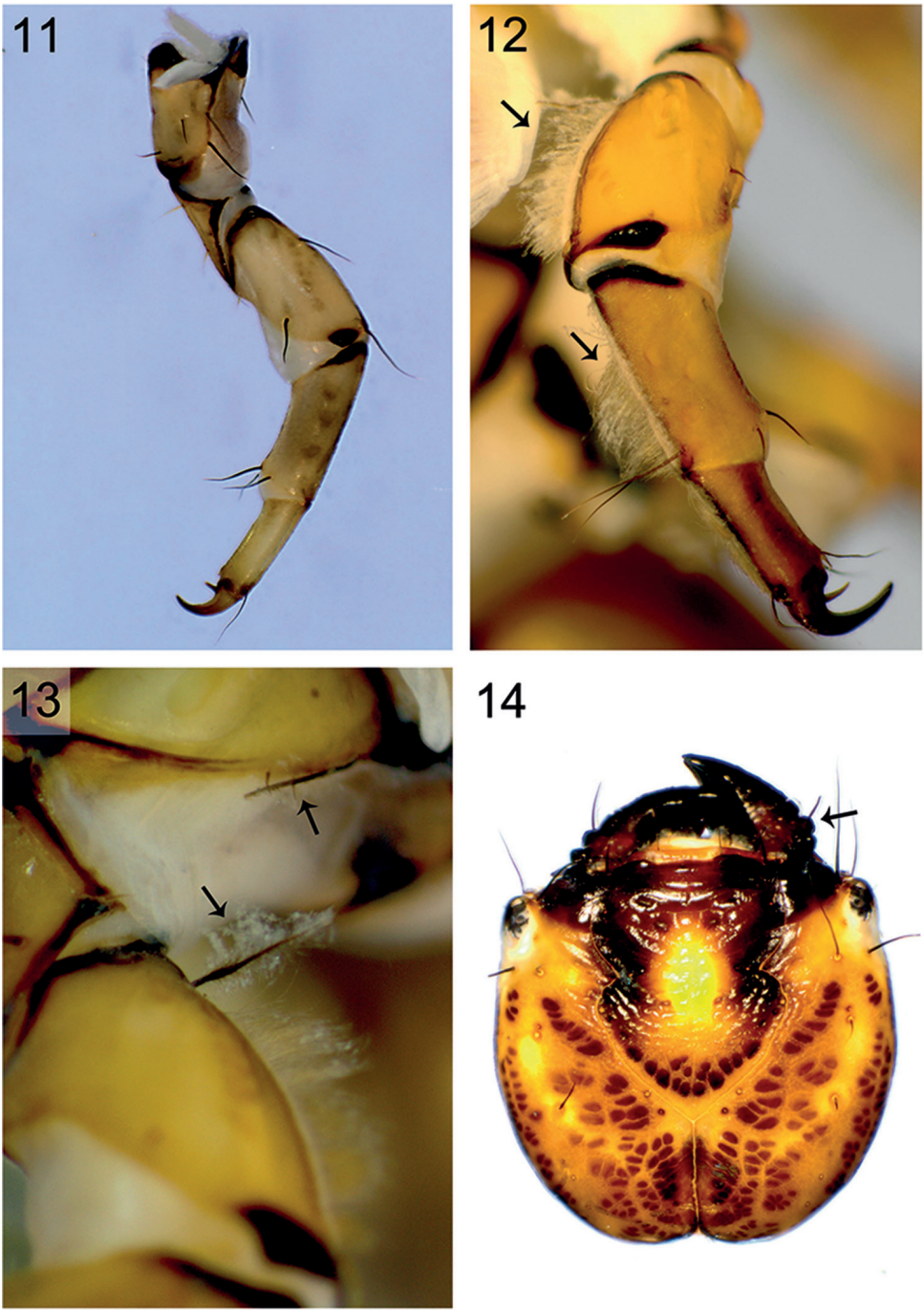
***gigantea* type**

Larvae of this type have been described by Lepneva (1945, 1970), Schmid and Botosaneanu (1966), and Graf and Sharma (1998, Type A). Both Graf and Sharma (1998) and Schmid and Botosaneanu (1966) described larvae of unknown species identity. We assigned the following OTUs to the *gigantea* type: *H. sylvicola*, *triloba*-complex, and *H.* sp. 1338 (L). Larvae of the *gigantea* type are characterised by the following set of characters:

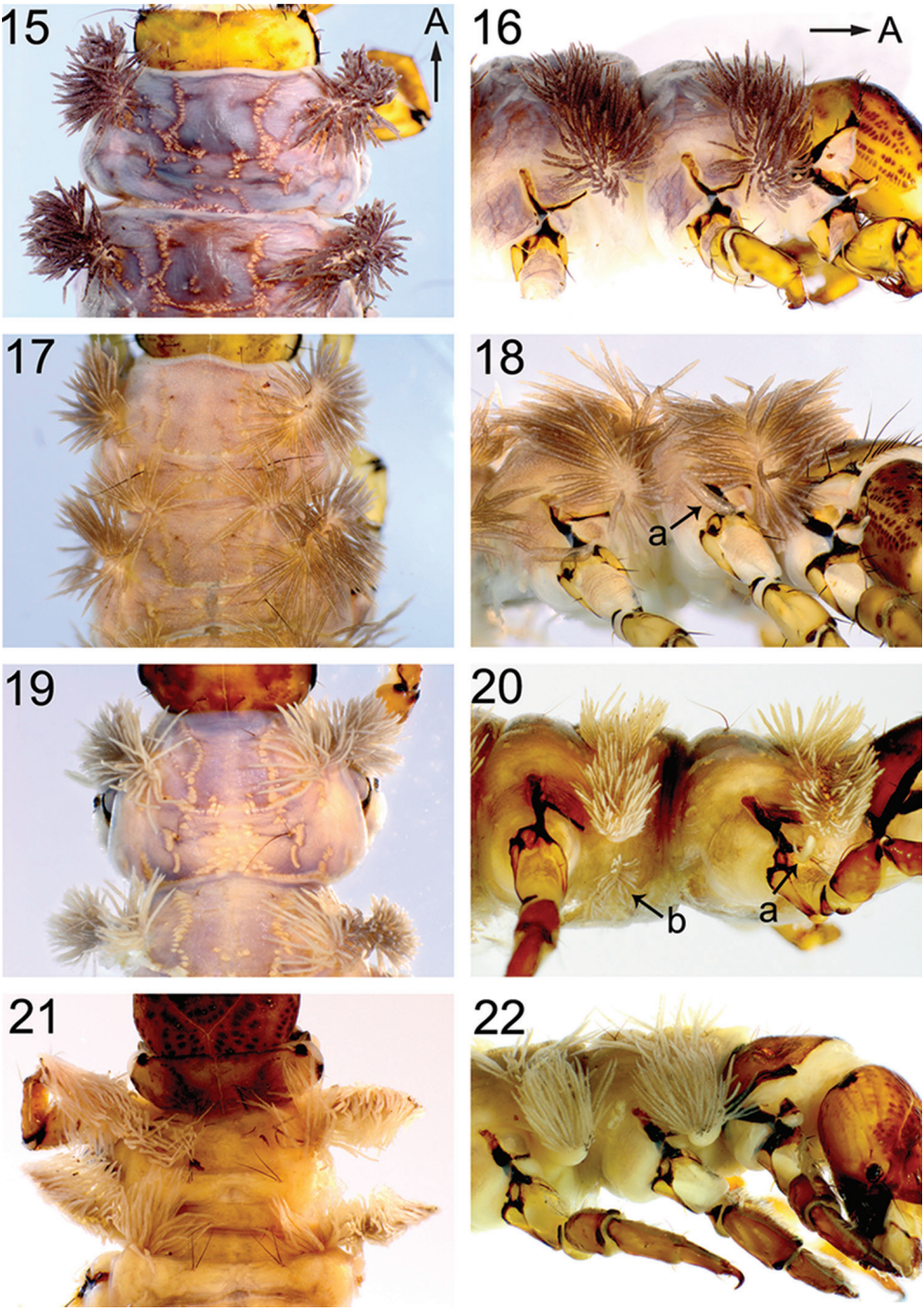
Thorax. Pronotum with a single row of setae along the anterior edge; Sa1 present as a transversal band of 2–4 setae, Sa2 present as a sagittal band of 7–9 dark setae, prominent (10); legs with dorsal fringe of setae (Figure 12), with pennate setae on coxa and femora (Figure 13). Gills. Ventral gills at meso- and metathorax absent (Figure 23); thoracic and abdominal gills arranged on large lateral processes (conical processes *sensu* Graf and Sharma 1998), lateral processes slightly smaller on meso-and metathorax and distinctly smaller on abdomen I (Figs 6, 21, 22, 28, 35, 36); lateral processes with small rounded protuberances proximoventrally (Figure 36, arrow a). Abdomen. Without ventral protuberances; single ventral medial sclerite on abdomen II-VII suboval, thin, transversally elongated (Figure 36, arrow b). Anal prolegs. Stout, without distolateral accessory hook, dorsal plate flat without protuberance or hook (Figs 43–44); dorsal spine on basal anal claw yellowish (Figs 43–44, arrow c).



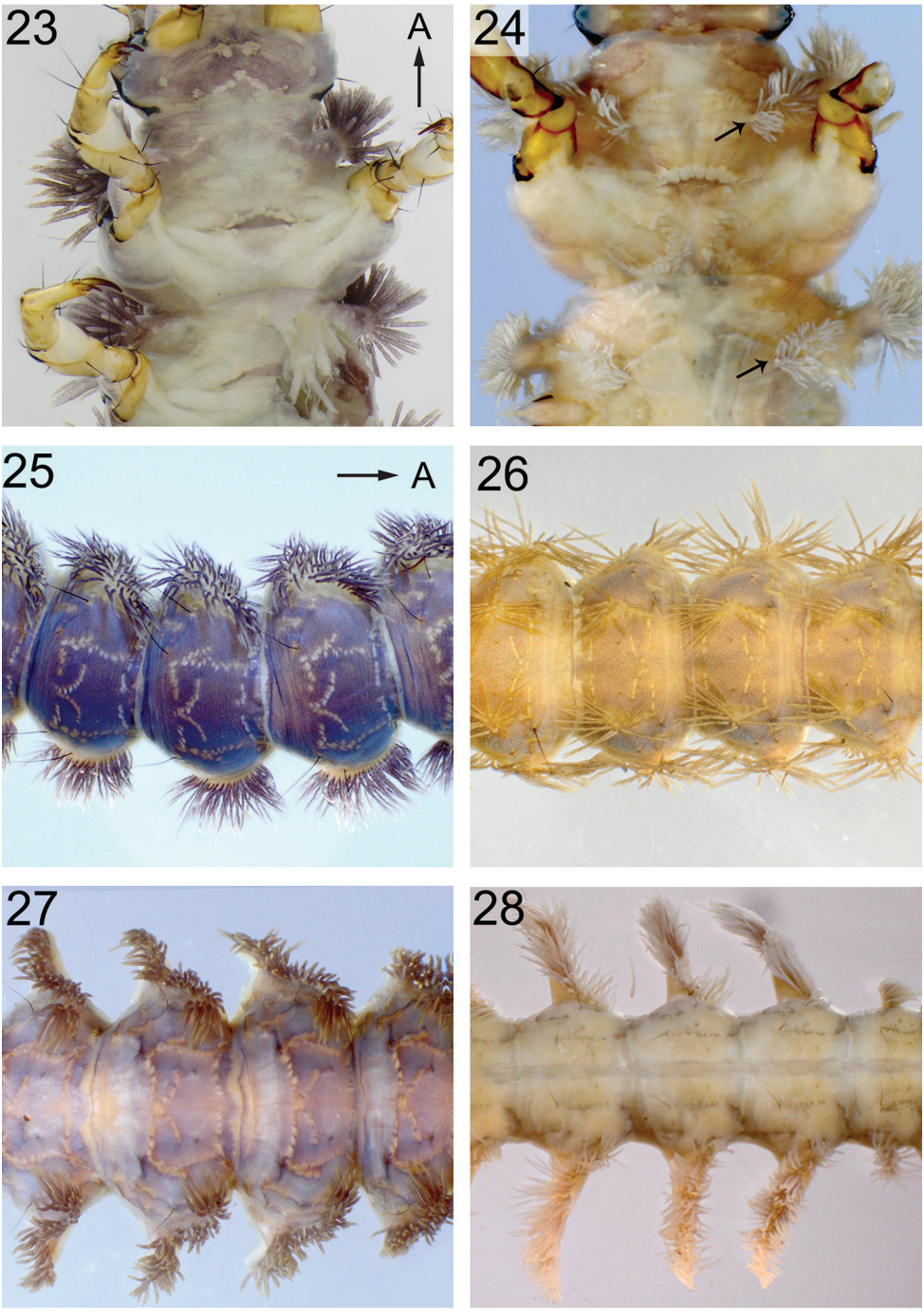
Figures 7–10. Pronotum of *Himalopsyche* larvae. **7** *H. phryganea* **8** *H. anomala* **9** *H. japonica* **10** *H. sylvicola*. Arrow A points to anterior.



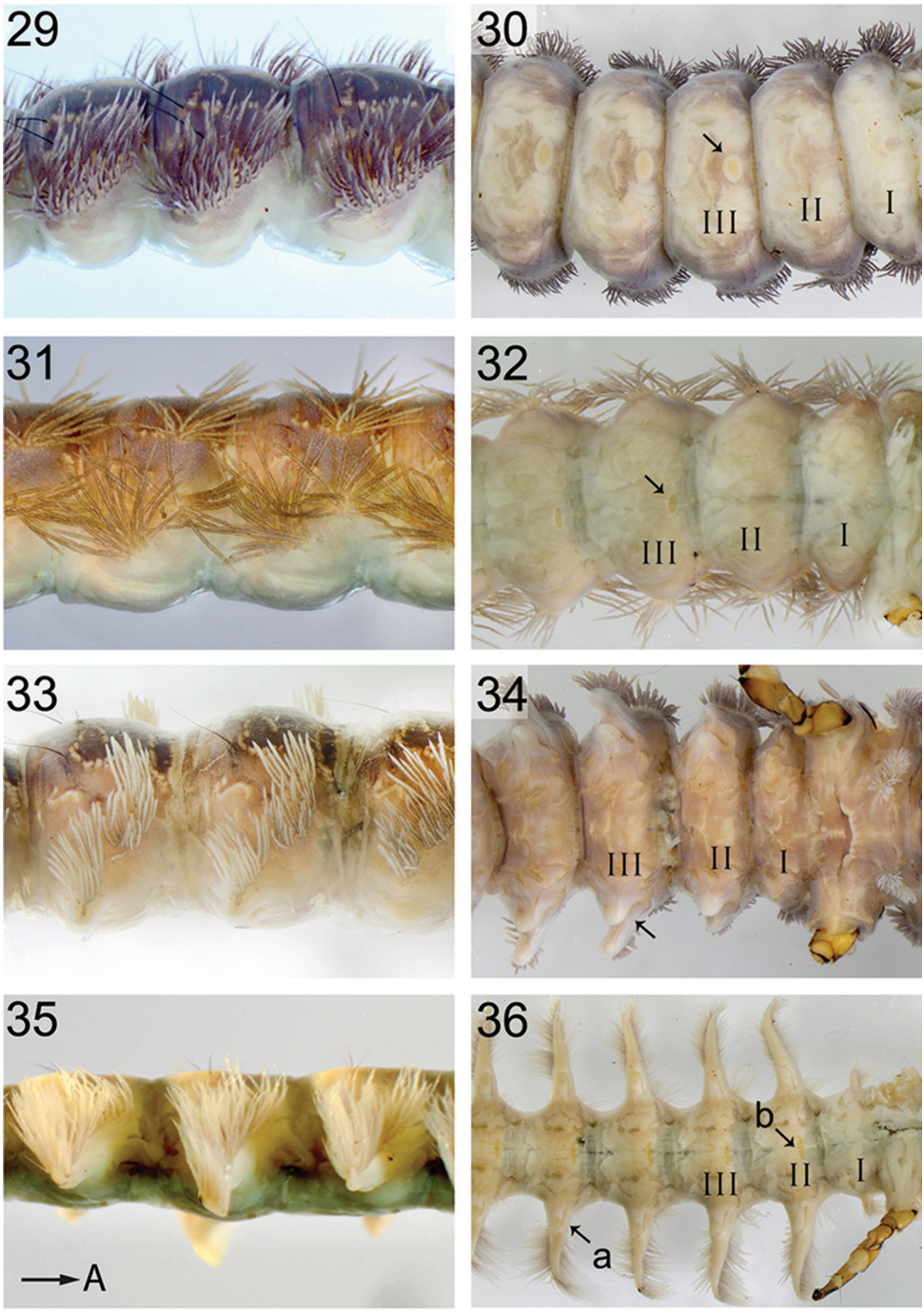
Figures 11–14. Mesolegs and head of *Himalopsyche* larvae. **11** Mesoleg of *H. gregoryi* **12** Mesoleg of *H. sylvicola,* arrows indicate dorsal fringe of setae on legs **13** Mesoleg of *H. sylvicola*. Arrows indicate pennate setae on coxa and femora **14** Head of *H. phryganea,* arrow points to lateral protuberances on mandible.



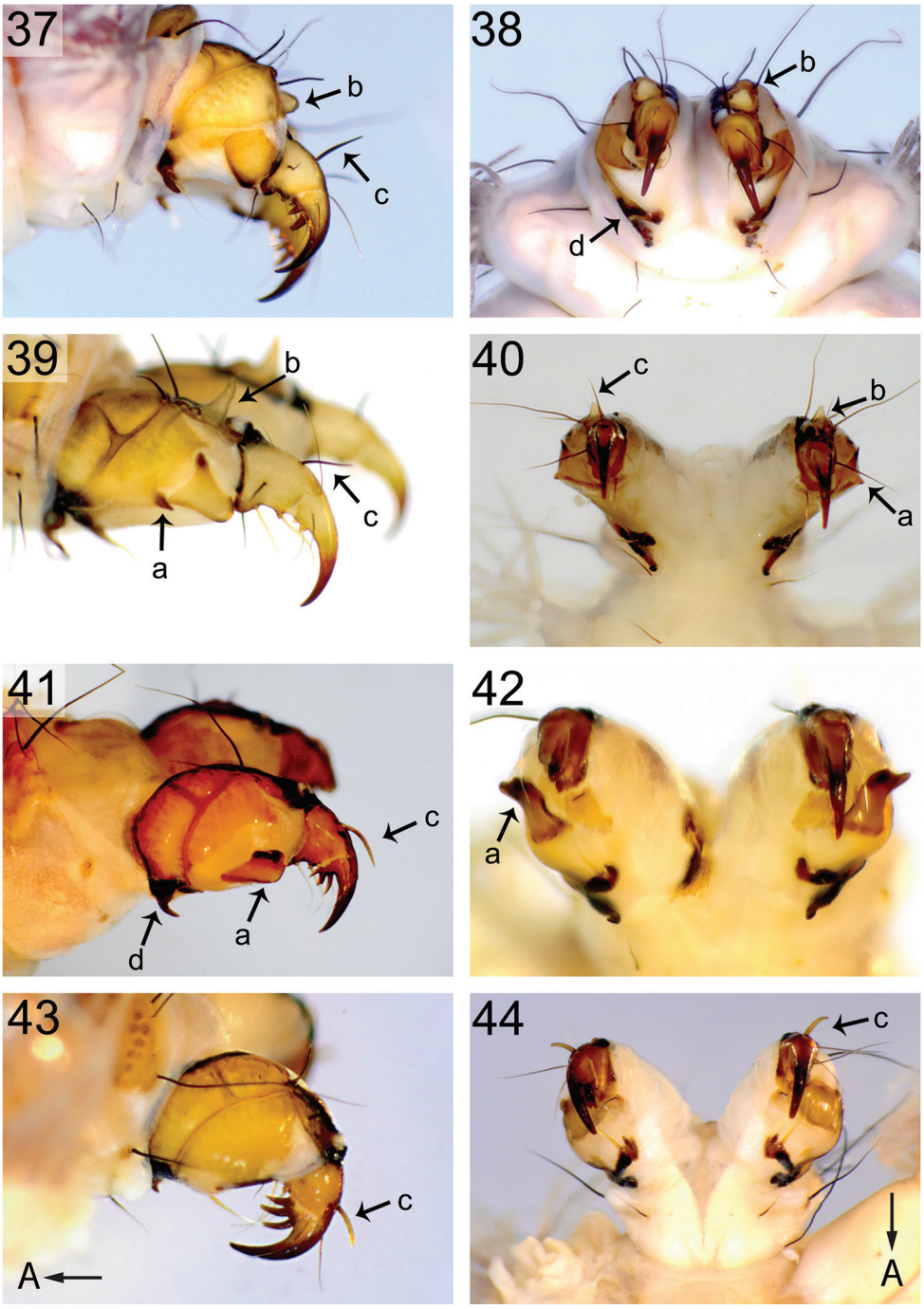
Figures 15–22. Thorax of *Himalopsyche* larvae in dorsal and lateral view. **15**, **16** *H. phryganea* **17, 18** *H. gregoryi*, arrow a points to anterodorsal single coxal gill **19**, **20** *H. japonica,* arrow a indicates anterodorsal single coxal gill; arrow b indicates the ventral gills **21**, **22** *H. sylvicola*. Arrows A points to anterior.



Figures 23–28. Thorax ventral, abdomen dorsal. **23** *H. gregoryi* **24** *H. japonica,* arrows indicate ventral gills **25** *H. phryganea* **26** *H. gregoryi*, **27** *H. acharai* **28** *H. sylvicola*. Arrows A points to anterior.



Figures 29–36. Abdomen lateral, ventral. **29**, **30** *H. phryganea,* arrow points to ventral medial sclerite **31, 32** *H. gregoryi,* arrow points to ventral medial sclerite **33**, **34** *H. japonica,* arrow points to ventral protuberance **35**, **36** *H. sylvicola,* arrow a indicates small rounded protuberance on lateral process, while arrow b indicates the ventral medial sclerite. Roman numbers indicate abdominal segments. Arrow A points to anterior.



Figures 37–44. Anal prolegs, lateral, caudal. **37**, **38** *H. phryganea* **39** *H. gregoryi* **40** *H. tibetana* **41**, **42** *H. japonica* **43**, **44** *H. sylvicola*. Key: arrows **a** distolateral accessory hook. arrows **b** protuberance/hook on dorsal plate. arrows **c** dorsal spine on basal anal claw. arrows **d** proximal accessory hooks fused with lateral sclerites. Arrow A points to anterior.

Discussion

We used several life stage association strategies based on two genes in a comparative setting: PTP, GMYC and reciprocal monophyly. All strategies acknowledge a successful association in cases of sequence identity, but differ in that PTP and GMYC use branch lengths to estimate which clades represent distinct units, and that reciprocal monophyly requires congruence of gene trees. The importance of using more than one gene (Dupuis et al. 2012) was apparent in this study, as the two genes yielded somewhat different, albeit overall compatible, results. Delimitation results based on COI tended to split species into several units, especially with PTP, where CAD did not. This could be explained by the quicker coalescence time in COI, with its matrilineal inheritance leading to an effective population size 1/4 of that of nuclear genes. The PTP and GMYC methods search for the transition between inter- and intraspecific branching patterns, and if coalescence of COI lineages occurs within isolated populations, then the PTP and GMYC methods cannot distinguish between population and species signals. CAD was generally congruent with established taxonomy except in the species complexes, which were unresolvable by either gene. The major assumption, and limitation, of all association methods employed here is that they assume monophyletic gene trees. Closely related species may be difficult to separate genetically due to incomplete lineage sorting, and recent or ongoing gene flow (e.g., Kutschera et al. 2014). Therefore, recent divergences will always be problematic for PTP and GMYC analyses. GMYC has, for example, been shown to be an accurate and conservative method under conditions where effective sample sizes are low, and divergence times between species are high, since such conditions yield monophyletic species (Esselstyn et al. 2012, Fujisawa and Barraclough 2013). To resolve the species complexes more genes should be used, ideally under a multi-species coalescence approach, such as BPP (Yang 2015), or STACEY (Jones 2017). These methods combine data from several genes to estimate the species borders, accounting for incomplete lineage sorting and conflicting information among genes.

We observed clear morphological differences in morphology between the larval types. Within larval types, however, the examined material did not show any stable and reliable morphological characters to delimitate larvae at species-level. More material, particularly of last instar larvae, and ideally from numerous sites is required to better assess interspecific, intraspecific and ontological variation in the here described as well as other morphological characters. Only then will it be possible to assess if the observed morphological variation is useful for delimiting species in the larval stage.

Conclusion

In this study, we present the characteristic morphological differences of four larval types of *Himalopsyche*. Life-stage association based on molecular data enabled us to do this, as it provided an OTU assignation for over 300 larvae. We found little or no morphological differences among species within the same type. Once we are able to

better discern organisms at lower taxonomic rank (by morphology or molecular association, e.g., in high-throughput barcoding studies), aquatic insects and other benthic invertebrates can become much more valuable for biological monitoring in poorly studied regions. More generally, it is essential to be able to distinguish taxa at the lowest taxonomic resolution (i.e., to species) to understand their ecology and evolution. This is also of great relevance when developing tools to assess ecological status to ensure sustainable use and management of natural resources.

Author contributions

AEH, SCJ, SP, and WG developed and planned the paper. AEH, SCJ, and SP conducted field work. AEH generated molecular, geographic and elevational data and performed molecular association analyses. AEH, SV and WG studied morphology of larvae; AEH drafted larval type descriptions with support from SV and WG. AEH and WG photographed larvae. AEH wrote the initial manuscript and all authors contributed substantially to further versions of the manuscript.

Acknowledgements

We thank Hans Malicky (Lunz am See), Wolfram Mey (Berlin), Bob Wisseman (Oregon), R. B. Kuranishi (Chiba), and Hiroyuki Nishimoto (Komaki) for providing valuable specimens for the study. Field work and molecular work were funded by DFG Grant PA1617/2-1. WG was financially supported by the EC-funded Synthesys Access programme (DE-TAF-5887: Morphological characterisation and description of molecularly associated and differentiated clades of larvae of genus *Himalopsyche* (Trichoptera, Rhyacophilidae)). SCJ acknowledges funding from the German Federal Ministry of Education and Research (BMBF) for the "GLANCE" project (Global change effects in river ecosystems; 01LN1320A). We thank Ram Devi Tachamo Shah, Deep Narayan Shah, Fengqing Li, Subodh Sharma, Qinghua Cai, Xiaoli Tong, and Felicitas Erzinger (née Hoppeler) for invaluable contributions in the field, as well as the Department of National Parks and Wildlife Conservation (DNPWC) Nepal for providing the research permits.

References

Andreadis KM, Schumann GJ-P, Tamlin Pavelsky T (2013) A simple global river bankfull width and depth database. Water Resources Research 49: 7164–7168. https://doi.org/10.1002/wrcr.20440

Banks N (1940) Report on certain groups of neuropteroid insects from Szechwan, China. Proceedings of the United Stated National Museum 88: 173–220. https://doi.org/10.5479/si.00963801.88-3079.173

Banks N (1947) Some neuropterous insects from Szechwan, China. Fieldiana Zoology 31(12): 97–107. https://doi.org/10.5962/bhl.title.2821

Blahnik RJ, Holzenthal RW (2004) Collection and curation of Trichoptera, with emphasis on pinned material. Nectopsyche, the Neotropical Trichoptera Newsletter 1: 8–20. http://hdl.handle.net/11299/190744

Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, Suchard MA, Rambaut A, Drummond AJ (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Computational Biology 10: e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bouckaert RR, Drummond AJ (2015) bModelTest: Bayesian phylogenetic site model averaging and model comparison. BMC Evolutionary Biology 17: 42. https://doi.org/10.1186/s12862-017-0890-6

Bukontaite R, Miller KB, Bergsten J (2014) The utility of CAD in recovering Gondwanan vicariance events and the evolutionary history of Aciliini (Coleoptera: Dytiscidae). BMC Evolutionary Biology 14: 5. https://doi.org/10.1186/1471-2148-14-5

Chesters D (2013) Collapsetypes. http://sourceforge.net/projects/collapsetypes [computer software]

Dupuis JR, Roe AD, Sperling FAH (2012) Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. Molecular Ecology 21: 4422–4436. https://doi.org/10.1111/j.1365-294X.2012.05642.x

Ekrem T, Willassen E, Stur E (2010) Phylogenetic utility of five genes for dipteran phylogeny: A test case in the Chironomidae leads to generic synonymies. Molecular Phylogenetics and Evolution 57: 561–571. https://doi.org/10.1016/j.ympev.2010.06.006

Esselstyn JA, Evans BJ, Sedlock JL, Anwarali Khan FA, Heaney LR (2012) Single-locus species delimitation: a test of the mixed Yule-coalescent model, with an empirical application to Philippine round-leaf bats. Proceedings of the Royal Society B: Biological Sciences 279: 3678–3686. https://doi.org/10.1098/rspb.2012.0705

Ezard T, Fujisawa T, Barraclough TG (2014) splits: SPecies' LImits by Threshold Statistics. R package version 1.0-19/r51. https://R-Forge.R-project.org/projects/splits/

Flint OS (1961) The presumed larva of *Himalopsche phryganea* (Ross) (Trichoptera: Rhyacophilidae). The Pan-Pacific Entomologist 37: 199–202.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299. https://www.ncbi.nlm.nih.gov/pubmed/7881515

Foster P, Bergo E, Bourke B, Oliveira T, Nagaki S, Sant'Ana D, Sallum M (2013) Phylogenetic analysis and DNA-based species confirmation in *Anopheles* (Nyssorhynchus). PLoS ONE 8: e54063. https://doi.org/10.1371/journal.pone.0054063

Fujisawa T, Barraclough TG (2013) Delimiting species using single-locus data and the generalized mixed yule coalescent approach: A revised method and evaluation on simulated data sets. Systematic Biology 62: 707–724. https://doi.org/10.1093/sysbio/syt033

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annual Review of Ecology, Evolution, and Systematics 34: 397–423. https://doi.org/10.1146/annurev.ecolsys.34.011802.132421

Graf W, Sharma S (1998) The larva of *Himalopsyche tibetana* (Insecta: Trichoptera, Rhyacophilidae) with some ecological notes on the Genus *Himalopsyche* Banks, 1940 from Nepal. In: Chalise SR, Khanal NR (Eds) International Conference on Ecohydrology of High Mountain Areas. International Centre for Integrated Mountain Development, Kathmandu, 561–571.

Graf W, Lubini V, Pauls SU (2005) Larval description of *Drusus muelleri* McLachlan, 1868 (Trichoptera: Limnephilidae) with some notes on its ecology and systematic position within the genus *Drusus*. Annales de Limnologie - International Journal of Limnology 41: 93–98. https://doi.org/10.1051/limn/2005012

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Biology 2(10): e312. https://doi.org/10.1371/journal.pbio.0020312

Hjalmarsson AE (submitted) Delimitation and description of three new species of *Himalopsyche* (Trichoptera: Rhyacophilidae) from the Hengduan Mountains, China.

Hjalmarsson AE, Bergsten J, Monaghan MT (2015) Dispersal is linked to habitat use in 59 species of water beetles (Coleoptera: Adephaga) on Madagascar. Ecography 38: 732–739. https://doi.org/10.1111/ecog.01138

Holzenthal RW, Blahnik RJ, Prather AL, Kjer KM (2007) Order Trichoptera Kirby, 1813 (Insecta), Caddisflies. In: Zhang Z-Q, Shear WA (Eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. Zootaxa 1668: 639–698. http://www.mapress.com/zootaxa/2007f/zt01668p698.pdf

Hwang C (1958) Descriptions of Chinese caddisflies (Trichoptera). Acta Zoologica Sinica 10: 279–284.

Jarvis A, Reuter HI, Nelson A, Guevara E (2008) Hole-filled SRTM for the globe Version 4. Available from the CGIAR-CSI SRTM 90m Database. http://srtm.csi.cgiar.org

Johanson KA, Malm T (2010) Testing the monophyly of Calocidae (Insecta: Trichoptera) based on multiple molecular data. Molecular Phylogenetics and Evolution 54: 535–541. https://doi.org/10.1016/j.ympev.2009.09.025

Jones G (2017) Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. Journal of Mathematical Biology 74: 447–467. https://doi.org/10.1007/s00285-016-1034-0

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Klopfstein S, Vilhelmsen L, Heraty JM, Sharkey M, Ronquist F (2013) The Hymenopteran Tree of Life: Evidence from protein-coding genes and objectively aligned ribosomal data. PLoS ONE 8: e69344. https://doi.org/10.1371/journal.pone.0069344

Kutschera VE, Bidon T, Hailer F, Rodi JL, Fain SR, Janke A (2014) Bears in a forest of gene trees: Phylogenetic inference is complicated by incomplete lineage sorting and gene flow. Molecular Biology and Evolution 31: 2004–2017. https://doi.org/10.1093/molbev/msu186

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution formolecular and morphological phylogenetic analyses. Molecular biology and evolution. https://doi.org/10.1093/molbev/msw260

Lepneva SG (1945) Remarkable larvae of the genus *Rhyacophila* Pictet (Trichoptera) from streams of Central Asia. Revue Entomologique de l'URSS 28: 64–74.

Lepneva SG (1956) Novaia licinka ruceinika (Trichoptera, Rhyacophilidre) iz vysokogorii Zakav Kaziia. Revue Entomologique de l'URSS 35(4): 899–911.

Lepneva SG (1970) Fauna of the U.S.S.R, Trichoptera, Volume II No. 1, Larvae and Pupae of Annulipalpia. Smithsonian Institution and the National Science Foundation, Washington, DC, 638 pp.

Malicky H (2004) Atlas of European Trichoptera (2nd edn). Springer Netherlands, Dordrecht.

Malicky H (2008) Beschreibungen von neuen Trichopteren aus Asien. Braueria 35: 45–57.

Malicky H (2011) Neue Trichopteren aus Europa und Asien. Braueria 38: 23–43.

Malicky H (2012) Neue asiatische Köcherfliegen aus neuen Ausbeuten (Insecta, Trichoptera) Linzer biologische Beiträge 44(2): 1263–1310.

Malicky H, Chantaramongkol P (1989) Einige Rhyacophilidae aus Thailand (Rhyacophilidae). Entomologische Zeitschrift 99(3):17–24.

Martynov AV (1914) Contributions a la faune des Trichoptères des possessions Russes dans l'Asie Centrale. Annuaire du Musée Zoologique de L'Académie Impériale des sciences de Petrograd 19(3): 401–437.

Martynov AV (1930) On the Trichopterous fauna of China and Eastern Tibet. Proceedings of the Zoological Society of London 5: 65–112.

Martynov AV (1935) A collection of Trichoptera from the Indian Museum. Records of the Indian Museum 37: 93–209.

Martynov AV (1936) On a collection of Trichoptera from the Indian Museum. Records of the Indian Museum. 38: 239–306.

McLachlan R (1879) A monographic revision and synopsis of the Trichoptera of the European fauna. Part VIII, John van Vorrt, London, 702 pp.

Mey W (1996) Die Köcherfliegenfauna des Fan Si Pan-Massivs in Nord-Vietnam. Beiträge zur Entomologie 46(1): 36–65. https://doi.org/10.21248/contrib.entomol.46.1.39-65

Montero-Pau J, Gómez A, Muñoz J (2008) Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. Limnology and Oceanography: Methods 6: 218–222. https://doi.org/10.4319/lom.2008.6.218

de Moor FC, Ivanov VD (2008) Global diversity of caddisflies (Trichoptera: Insecta) in freshwater. Hydrobiologia 595: 393–407. https://doi.org/10.1007/s10750-007-9113-2

Morton KJ (1900) Descriptions of new species of Oriental *Rhyacophila*. Transactions of the Entomological Society of London 1: 1–7.

Moulton JK, Wiegmann BM (2004) Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). Molecular Phylogenetics and Evolution 31: 363–378. https://doi.org/10.1016/S1055-7903(03)00284-7

Pictet FJ (1834) Recherches pour servir à l'histoire et à l'anatomie des Phryganides. Abraham Cherbuliez, Geneve, 300 pp. https://doi.org/10.5962/bhl.title.8547

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP (2006) Sequence-based species delimitation for the DNA Taxonomy of undescribed insects. Systematic Biology 55: 595–609. https://doi.org/10.1080/10635150600852011

R Core Team (2015) R: A language and environment for statistical computing. - R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Rainbow PS, Hildrew AG, Smith BD, Geatches T, Luoma SN (2012) Caddisflies as biomonitors identifying thresholds of toxic metal bioavailability that affect the stream benthos. Environmental Pollution 166: 196–207. https://doi.org/10.1016/j.envpol.2012.03.017

Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) Tracer v1.6. http://tree.bio.ed.ac.uk/software/tracer/

Resh VH, Unzicker JD (1975) Water quality monitoring and aquatic organisms: the importance of species identification. Journal of the Water Pollution Control Federation 47: 9–19. http://www.jstor.org/stable/25038592

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Ross HH (1941) Descriptions and Records of North American Trichoptera. Transactions of the American Entomological Society 67(1): 35–126.

Ross HH (1956) Evolution and classification of the mountain caddisflies. The University of Illinois Press, Urbana, 213 pp.

Ruiter DE, Boyle EE, Zhou X (2013) DNA barcoding facilitates associations and diagnoses for Trichoptera larvae of the Churchill (Manitoba, Canada) area. BMC Ecology 13: 5. https://doi.org/10.1186/1472-6785-13-5

Saito T (1965) Larvae of two fresh water insects, *Himalopsyche japonica* and *Neohapalothrix kanii*. Report of the Japanese Society of Systematic Zoology 1: 14–16.

Schmid F (1963) Quelques *Himalopsyche* indiennes (Trichopères, Rhyacophilidae). Bonner zoologische Beiträge 14: 206–223.

Schmid F, Botosaneanu L (1966) Le genre *Himalopsyche* Banks (Trichoptera, Rhyacophilidae). Annals of the Entomological Society of Quebec 11: 123–176.

Schmidt-Kloiber A, Graf W, Moog O, Lorenz A, Hering D (2008) The Indicator Database for European Freshwater Invertebrates. Proceedings of the first conference on faunistics and zoogeography of European Trichoptera 55: 85–88.

Sharanowski BJ, Dowling APG, Sharkey MJ (2011) Molecular phylogenetics of Braconidae (Hymenoptera: Ichneumonoidea), based on multiple nuclear genes, and implications for classification. Systematic Entomology 36: 549–572. https://doi.org/10.1111/j.1365-3113.2011.00580.x

Song JH, Ahn KJ (2014) Species delimitation in the *Aleochara fucicola* species complex (Coleoptera: Staphylinidae: Aleocharinae) and its phylogenetic relationships. Zoologica Scripta 43: 629–640. https://doi.org/10.1111/zsc.12077

Stephens JF (1836) Illustrations of British entomology; or, a synopsis of indigenous insects: containing their generic and specific distinctions; with an account of their metamorphoses, times of appearance, localities, food, and economy, as far as practicable. Vol. VI. Mandibulata. Baldwin & Cradock, London, 240 pp.

Tachamo Shah RD, Sharma S, Haase P, Jähnig SC, Pauls SU (2015) The climate sensitive zone along an altitudinal gradient in central Himalayan rivers: a useful concept to monitor cli-

mate change impacts in mountain regions. Climatic Change 132(2): 265–278. https://doi.org/10.1007/s10584-015-1417-z

Tanida K (1985) Trichoptera. In: Kawai T (Ed.) An illustrated book of aquatic insects of Japan. Tokai University Press, Tokyo, 167–215.

Thamsenanupap P, Chantaramongkol P, Malicky H (2005) Description of caddis larvae (Trichoptera) from northern Thailand of the genera *Himalopsyche* (Rhyacophilidae), *Arctopsyche* (Arctopsychidae), cf. *Eoneureclipsis* (Psychomiidae) and *Inthanopsyche* (Odontoceridae). Braueria 32: 7–11.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680. https://doi.org/10.5962/bhl.title.8547

Ulmer G (1905a) Zur Kenntnis aussereuropäischer Trichopteren. Stettin Entomologische Zeitung 66: 107–110.

Ulmer G (1905b) Neue und wenig bekannte Trichopteren der Museen zu Brüssel und Paris. Annales de la Société entomologique de Belgique. 49: 17–42.

Ulmer G (1932) Aquatic Insects of China Article III. Neue Chinesiche Trichopteren, nebst Übersicht über die bisher aus China bekannten Arten. Peking Natural History Bulletin 7: 39–70.

Ulmer G (1957) Köcherfliegen (Trichopteren) von den Sund a-Inseln. Teil III. Larven und puppen der Annulipalpia. Archiv für Hydrobiologie - Supplements 23: 109–470.

Verdonschot PFM (2006) Beyond Masses and Blooms: The indicative value of Oligochaetes. Hydrobiologia 564: 127–142. https://doi.org/10.1007/s10750-005-1714-z

Vitecek S, Kučinić M, Previšić A, Živić I, Stojanović K, Keresztes L, Bálint M, Hoppeler F, Waringer J, Graf W, Pauls SU (2017) Integrative taxonomy by molecular species delimitation: multi-locus data corroborate a new species of Balkan Drusinae micro-endemics. BMC Evolutionary Biology 17: 129. https://doi.org/10.1186/s12862-017-0972-5

Wiggins GB (1996) Larvae of the North American Caddisfly Genera (Trichoptera). 2nd ed. University of Toronto Press, Toronto, 457 pp.

Wild AL, Maddison DR (2008) Evaluating nuclear protein-coding genes for phylogenetic utility in beetles. Molecular Phylogenetics and Evolution 48: 877–891. https://doi.org/10.1016/j.ympev.2008.05.023

Yang Z (2015) The BPP program for species tree estimation and species delimitation. Current Zoology 61(5): 854–865. https://doi.org/10.1093/czoolo/61.5.854

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29(22): 2869–2876 https://doi.org/10.1093/bioinformatics/btt499

Zhou X, Kjer KM, Morse JC (2007) Associating larvae and adults of Chinese Hydropsychidae caddisflies (Insecta: Trichoptera) using DNA sequences. Journal of the North American Benthological Society 26: 719–742. https://doi.org/10.1899/06-089.1

Zhou X, Frandsen PB, Holzenthal RW, Beet CR, Bennett KR, Blahnik RJ, Bonada N, Cartwright D, Chuluunbat S, Cocks GV, Collins GE, deWaard J, Dean J, Flint OS, Hausmann A, Hendrich L, Hess M, Hogg ID, Kondratieff BC, Malicky H, Milton MA, Morinière J,

Morse JC, Ngera Mwangi F, Pauls SU, Razo Gonzalez M, Rinne A, Robinson JL, Salokannel J, Shackleton M, Smith B, Stamatakis A, StClair R, Thomas JA, Zamora-Muñoz C, Ziesmann T, Kjer KM (2016) The Trichoptera barcode initiative: a strategy for generating a species-level Tree of Life. Philosophical Transactions B 371: 20160025. https://doi.org/10.1098/rstb.2016.0025

Supplementary material 1

List of specimens

Authors: Anna E. Hjalmarsson, Wolfram Graf, Sonja C. Jähnig, Simon Vitecek, Steffen U. Pauls

Data type: speciemens data

Explanation note: Table listing all specimens included in the study including their BOLD-IDs, species/OTU assignation, collection data, and haplotype numbers.

Link: https://doi.org/10.3897/zookeys.773.24319.suppl1

Supplementary material 2

Figure S1. Life stage association results based on CAD

Authors: Anna E. Hjalmarsson, Wolfram Graf, Sonja C. Jähnig, Simon Vitecek, Steffen U. Pauls

Data type: molecular data

Explanation note: The tree was generated in MrBayes (run1). Node values represent posterior probabilities.

Link: https://doi.org/10.3897/zookeys.773.24319.suppl2

Supplementary material 3

Figure S2. Life stage association results based on COI

Authors: Anna E. Hjalmarsson, Wolfram Graf, Sonja C. Jähnig, Simon Vitecek, Steffen U. Pauls

Data type: molecular data

Explanation note: The tree was generated in MrBayes (run1). Node values represent posterior probabilities.

Link: https://doi.org/10.3897/zookeys.773.24319.suppl3

ZooKeys 773: 109–141 (2018)
doi: 10.3897/zookeys.773.21690
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

Hawaiian *Philodoria* (Lepidoptera, Gracillariidae, Ornixolinae) leaf mining moths on *Myrsine* (Primulaceae): two new species and biological data

Shigeki Kobayashi[1,2], Chris A. Johns[2,3], Carlos Lopez-Vaamonde[4,5], Camiel Doorenweerd[6,7], Atsushi Kawakita[8], Issei Ohshima[9], David C. Lees[10], Sofia Hanabergh[2,3], Akito Y. Kawahara[2,3,11]

1 *Entomological laboratory, Graduate School of life & Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, 599-8531 Japan* **2** *McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL 32611 USA* **3** *Department of Biology, University of Florida, Gainesville, FL 32611 USA* **4** *INRA, UR0633 Zoologie Forestière, F-45075 Orléans, France* **5** *Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS Université de Tours, UFR Sciences et Techniques, Tours, France* **6** *Department of Plant and Environmental Protection Sciences, University of Hawaii, 3050 Maile Way, Honolulu, HI 96822 USA* **7** *Naturalis Biodiversity Center, PO Box 9517, 2300 RA Leiden, The Netherlands* **8** *Center for Ecological Research, Kyoto University, 2-509-3 Hirano, Otsu, Shiga 520-2113 Japan* **9** *Department of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522 Japan* **10** *Natural History Museum, Cromwell Road, South Kensington, SW7 5BD, UK* **11** *Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611 USA*

Corresponding author: *Shigeki Kobayashi* (crossroad1994@hotmail.co.jp)

Academic editor: *E. van Nieukerken* | Received 17 October 2017 | Accepted 26 April 2018 | Published 9 July 2018

http://zoobank.org/2A364933-6662-4B6C-8477-4AAE1E591652

Citation: Kobayashi S, Johns CA, Lopez-Vaamonde C, Doorenweerd C, Kawakita A, Ohshima I, Lees DC, Hanabergh S, Kawahara AY (2018) Hawaiian *Philodoria* (Lepidoptera, Gracillariidae, Ornixolinae) leaf mining moths on *Myrsine* (Primulaceae): two new species and biological data. ZooKeys 773: 109–141. https://doi.org/10.3897/zookeys.773.21690

Abstract

This paper provides new taxonomic and biological data on a complex of gracillariid moths in the endemic genus *Philodoria* Walsingham, 1907 that are associated with *Myrsine* (Primulaceae) in the Hawaiian Islands, United States. Two new species, *Philodoria kauaulaensis* Kobayashi, Johns & Kawahara, **sp. n.** (host: *Myrsine lanaiensis*, *M. lessertiana*, and *M. sandwicensis*) and *P. kolea* Kobayashi, Johns & Kawahara, **sp. n.** (host: *M. lessertiana*) are described. Biological data are provided for two previously described species that also feed on *Myrsine*: *P. auromagnifica* Walsingham, 1907 and *P. succedanea* Walsingham, 1907. For the first time we detail and illustrate genital structures, immature stages, biology, and host plants of

P. auromagnifica and *P. succedanea*. *Philodoria kolea*, *P. auromagnifica,* and *P. succedanea* occur in sympatry on the island of Hawaii (Big Island), but each species differs in behavioral characters: *P. kolea* utilizes leaves of seedlings and forms a serpentine mine, whereas the latter two utilize leaves of larger plants, and form linear or serpentine to blotch mines. More broadly, leaf mine forms and diagnostic characteristics of the *Myrsine*-feeding species complex of *Philodoria* (as currently known) are reviewed and illustrated.

Keywords
DNA barcoding, leaf mine form, *Myrsine knudsenii*, *Myrsine wawraea*, taxonomy

Introduction

Hawaii constitutes one of the most geographically isolated archipelagos and harbors thousands of unusual, highly threatened endemic species. Phytophagous insects that rely on endemic Hawaiian plants are of special risk as they depend on the survival of their native host plants. The Hawaiian Islands measure just 0.02% of the area of the United States, but account for nearly 70% of the United States' historically documented plant and animal extinctions (Wagner et al. 1999). In all, over 360 Hawaiian animal and plant taxa are currently listed as either threatened or endangered under the federal and state Endangered Species Acts. More than 38% of native Hawaiian plants are threatened and 94% of Hawaiian insects are endemic (Evenhuis and Eldredge 1999). Leaf miners have achieved extraordinary localized diversity and are a major component of island ecosystems throughout the Pacific.

Philodoria Walsingham, 1907 is a genus of endemic Hawaiian leaf-mining micromoths, containing approximately 30 species, for which the classification remains largely in disarray. The genus can be distinguished from other genera in the Gracillariidae subfamily Ornixolinae by a hindwing with small frenular bristles along the costa in both sexes (Zimmerman 1978, figs 432–435); by a dorsal flap extending from the posterior margin of tergum VIII in the male; and by the female lamella antevaginalis that is sclerotized and semicircular in shape. Many *Philodoria* host plants are threatened along with their native habitat. Indeed, herbarium samples provide one of the few documented cases globally of a probable moth extinction, albeit an undescribed species (Johns et al. 2014). The genus was first described with seven species by Walsingham (1907), and the type species was designated as *P. succedanea* Walsingham, 1907. Zimmerman (1978) published a monograph of Hawaiian insects following Walsingham's work and many papers by Swezey (1910–1946). Zimmerman divided *Philodoria* into two subgenera, *P.* (*Eophilodoria*) and *P. (Philodoria*), based on the size of the maxillary palpus. His classification was recently rejected by Johns et al. (2016), who constructed a preliminary molecular phylogeny of *Philodoria* based on three genes for 11 *Philodoria* species. In their analyses, the two *Philodoria* subgenera were not monophyletic and morphological characters used to classify them were inferred as homoplasious; the subgenus *Eophilodoria* Zimmerman, 1978 was established as a subjective junior synonym of the genus *Philodoria* Walsingham, 1907. In addition, Johns et al. (2016) provided new host plant and distribution data for these 11 species. While *Philodoria* was historically treated as similar to *Elachista* (Elachistidae, Gelechioidea), it unequivocally belongs in Gracillariidae (Kawahara et al. 2017) and the

genus is unrelated to Gelechioidea (Breinholt et al. 2018). Based on taxon sampling of exemplar gracillariid genera, *Philodoria* appears to be phylogenetically closely related to the ornixoline genus *Chileoptilia* Vargas & Landry, 2005 from Chile (Kawahara et al. 2017).

Larval host plants of *Philodoria* are diverse, with up to six plant orders (Asterales, Apiales, Ericales, Malvales, Myrtales and Rosales) reported as hosts, among which Asterales (Asteraceae: *Dubautia*) and Rosales (Urticaceae: *Pipturus*) appear as dominant hosts (Swezey 1954; Zimmerman 1978). Another host plant that is used by multiple *Philodoria* species is *Myrsine* (Ericales: Primulaceae). According to Zimmerman (1978) and label data from *Philodoria* specimens in the collection of the Bernice Pauahi Bishop Museum (BPBM), there appear to be numerous undescribed *Philodoria* species on *Myrsine*. In total, 19 *Myrsine* species are known to be endemic to the Hawaiian Islands (Wagner et al. 1999), and two species of *Philodoria* that feed on *Myrsine* have been described: *P. succedanea* Walsingham, 1907 (type species of the genus) and *P. auromagnifica* Walsingham, 1907, both with similar scale colors and genital characters (Walsingham 1907, Zimmerman 1978).

In late April 2016, several of the authors collected numerous blotch mines on leaves of *Myrsine* species at two sites on the island of Hawaii (Big Island). Initially, we believed that these mines were created by a single *Philodoria* species, but after studying them, we realized that they comprised diverse larval habits (e.g., forms with spiral or linear mines, larvae in fallen or *in situ* leaves, and some adults which emerged with relatively black forewings). Recent studies (Kawahara et al. 2009, Davis and Wagner 2011, Davis and De Prins 2011, Brito et al. 2013, Moreira et al. 2017) have shown that important diagnostic characters of gracillariids are present in larvae and pupae. However, insufficient early stages have been preserved until now for diagnostics and identification. In this paper, we describe two new species, *Philodoria kauaulaensis* (hosts: *Myrsine lanaiensis*, *M. lessertiana,* and *M. sandwicensis*) and *P. kolea* (host: *M. lessertiana*), and also the genitalic structures, immature stages and new host plant information for the two previously described *Myrsine*-feeding species, *P. succedanea* and *P. auromagnifica*. Four species were reared, and their mine forms and characters are here reviewed and illustrated.

Materials and methods

Taxon sampling

All adult moths were reared from leaf mining larvae and their pupal cocoons. Leaf mines and cocoons were collected between 2013–2016 in the locations listed in Table 1. Among the material examined, the final dates refer to the adult emergence and 'em.' signifies that an adult emerged and was mounted as a dry pinned specimen; 'stored' signifies a dead adult that was stored in 99 % ethanol or RNAlater solution (Thermo Fisher Scientific). Type material designated by Lord Walsingham and specimens collected by Dr K. & Mrs. E. Sattler in the Natural History Museum (NHMUK), and those collected by Mr. O. H. Swezey at the BPBM and the National Museum of Natural History,

Table 1. Study sites of *Philodoria* species and host plants.

No.	Locality	Island	Collection Longitude and latitude	Elevation (m)	Study Specimens ID	Species name	Host plant
1	Limahuli, Upper Preserve	Kauai	22.1858°N, 158.58°W	900	AYK-HI10-001, 002	*Philodoria* sp. nr. *splendida*	Unknown
2	Kokee	Kauai	22.1508°N, 159.6370°W	1230	CJ-433, 442	*P. succedanea*	*Myrsine knudsenii*
3	Kahili	Kauai	No data	400–500	CJ-148	*P. auromagnifica*	*M. wawraea*
4	Mt. Kaala	Oahu	21.4161°N, 158.0997°W	800	CJ-526	*P. succedanea*	*M. lessertiana*
5	Kamakou	Molokai	21.1184°N, 156.9049°W	1170	CJ-241	*P. auromagnifica*	*M. lessertiana*
6	Eke	Maui	20.9379°N, 156.5801°W	870	CJ-136, 531	*P. succedanea*	*M. lessertiana*
7	Kauaula*	Maui	20.8738°N, 156.6183°W	900	CJ-381	*P. kauaulaensis*	*M. lanaiensis*
8	Waikamoi	Maui	20.7826°N, 156.2304°W	1800	CJ-539	*P. succedanea*	*M. lessertiana*
9	Upper Hamakua Ditch Trail	Hawaii	20.0511°N, 155.238°W	900	CLV6239	*Philodoria* sp. nr. *floscula*	*Pipturus* sp.
10	Kohala Watershed Partnership	Hawaii	No data	700–1500	CJ-419	*P. succedanea*	*M. sandwicensis*
11	Kaumana Trail	Hawaii	19.45°N, 155.21–155.19°W	900–1000	HILO016	*P. kolea*	*Myrsine* sp.
12	Hawai'i Volcanoes National Park†	Hawaii	19.4138°N, 155.238°W	1090	SKH-5, 10, 13, 15; HILO053, 054, 059; AYK0001, 0002, CLV6240	*P. succedanea*, *P. auromagnifica*; *P. kolea*; *P. basalis*	*M. lessertiana*; *Metrosideros polymorpha*

Type locality of **P. kauaulaensis* and †*P. kolea*

Smithsonian Institution (USNM) were also examined. Immatures in leaves were reared in plastic cups (420 ml: 129 mm in diameter at top and 60 mm in depth) containing wet cotton at 20 ± 5 °C under a photoperiod condition in the laboratory of 13–16L (hours light) 8–12D (hours darkness).

Morphology and nomenclature

Descriptions focused on the adult stage and leaf mines because of limitations of other material, and because these stages provide a wealth of morphological traits useful for diagnosis. Photographs of leaf mines were taken primarily in the field using Canon EOS 60D and 5D MKIII digital cameras. Some leafmines were scanned using an

EPSON Perfection V600 Photo scanner. Observations and measurements were made under a Leica M2 16 dissection microscope at 71–115× and a Leica S6E microscope at 6.3–40× with the aid of a micrometer scale. Images of adults were captured using a Olympus E-330 camera and Moticam 580 5.0 MP. Images were taken at various depths and subsequently stacked using the Helicon Focus 6.22. All images were then edited with Adobe Photoshop Elements 9 into final figures.

For genitalic dissections, the whole abdomen was removed and boiled for 3–4 min in 10% aqueous KOH, and residual scales and soft parts were removed in 70% ethanol. Genitalia were then stained in Chlorazol Black E (1% solution in 70 % ethanol) or acetocarmine for 0.5–1h, dehydrated in a series of 70–100 % ethanol and mounted in Canada balsam on a glass slide.

Type material and additional specimens used in the present study are preserved in the collections of the BPBM, the McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History (FLMNH) and Naturalis Biodiversity Center (RMNH). Terms used for describing wing color pattern are summarized in Fig. 1, and forewing characters follow the terminology of Walsingham (1907) and Zimmerman (1978). Terms for genitalia essentially, follow Zimmerman (1978) and "gnathos" is employed to indicate the sclerotized V-shaped transverse band joining the ventral base of tegumen. Scientific names of plants follow the Plant List (www.theplantlist.org).

DNA sequencing and analysis

A total of 16 specimens were DNA barcoded. DNA extraction, PCR amplification and sequencing of the 658 base pair Cytochrome Oxidase 1 (COI) "barcode" region for two specimens were carried out at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) following a published protocol (deWaard et al. 2008). Five specimens were extracted at the Florida Museum of Natural History, McGuire Centre for Lepidoptera and Biodiversity at the University of Florida, Gainesville, FL, USA, using the OmniPrep extraction kit and sequenced at University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR), one specimen was extracted at the Department of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto, Japan (KPU) using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, California), and single-stranded PCR and sequencing for this specimen was carried out at the Operon Sequencing Center following the manufacturer's protocol (Eurofins, Tokyo, Japan). Eight specimens that were sequenced at Naturalis Biodiversity Center were extracted using a Macherey-Nagel magnetic bead DNA extraction kit on a KingFisher automated DNA extraction robot (Table 2).

We conducted an ML analysis of the COI gene using RAxML 8.2.10 (Stamatakis 2014), searching for the best tree using the GTRCAT model and GAMMA-based likelihood optimization for the final tree, and otherwise default settings. Subsequently, 1,000 parametric bootstrap analyses with automated stopping following the extended

Table 2. Sampling information of *Philodoria* species used for molecular analysis.

Species name	Collection site	Host plant species	Host plant family	Collection ID	BOLD ID	BOLD BIN	GenBank accession no.	Institution of DNA extraction and sequencing of COI
P. succedanea	Hawaii	*Myrsine lessertiana*	Primulaceae	RMNH. INS.30669	WOGRA451-17	ADF5435	MF804823	RMNH, Netherlands
P. succedanea	West Maui	*M. lessertiana*	Primulaceae	CJ-144	WOGRA489-17	ADF5435	KT982414	FLMNH, USA
P. kauaulaensis	West Maui	*M. lessertiana*	Primulaceae	CJ-064	WOGRA487-17	ADI5327	KT982404	FLMNH, USA
P. kauaulaensis	West Maui	*M. sandwicensis*	Primulaceae	CJ-072	WOGRA488-17	ADI5327	KT982407	FLMNH, USA
P. auromagnifica	Hawaii	*M. lessertiana*	Primulaceae	RMNH.5013750	WOGRA444-17	ADD6965	MF804828	RMNH, Netherlands
P. auromagnifica	Hawaii	*M. lessertiana*	Primulaceae	CLV6240	LEPPC2422-16	ADD6965	MF804824	CCDB, Canada
P. kolea	Hawaii	*M. lessertiana*	Primulaceae	IO-322	WOGRA440-17	ADF7137	MF804825	KPU & Eurofins, Japan
P. kolea	Hawaii	*M. lessertiana*	Primulaceae	RMNH. INS.30682	WOGRA449-17	ADF7137	MF804831	RMNH, Netherlands
P. kolea	Hawaii	*M. lessertiana*	Primulaceae	RMNH.5013751	WOGRA447-17	ADF7137	MF804834	RMNH, Netherlands
P. kolea	Hawaii	*M. lessertiana*	Primulaceae	RMNH.5013752	WOGRA448-17	ADF7137	MF804832	RMNH, Netherlands
P. kolea	Hawaii	*M. lessertiana*	Primulaceae	RMNH. INS.30684	WOGRA450-17	ADF7137	MF804830	RMNH, Netherlands
Philodoria sp. nr. *floscula*	Hawaii	*Pipturus* sp.	Urticaceae	CLV6239	LEPPC2421-16	ADD6964	MF804826	CCDB, Canada
Philodoria sp. nr. *splendida*	Kauai	Unknown	Unknown	AYK-HI10-002	LNOUC1237-11	AAY7555	MF804829	FLMNH, USA
Philodoria sp. nr. *splendida*	Kauai	Unknown	Unknown	AYK-HI10-001	LNOUC1236-11	AAY7555	MF804827	FLMNH, USA
P. basalis	Hawaii	*Metrosideros polymorpha*	Myrtaceae	RMNH. INS.30680	WOGRA446-17	ADF5462	MF804833	RMNH, Netherlands
P. basalis	Hawaii	*M. polymorpha*	Myrtaceae	RMNH.5013753	WOGRA445-17	ADF5462	MF804835	RMNH, Netherlands

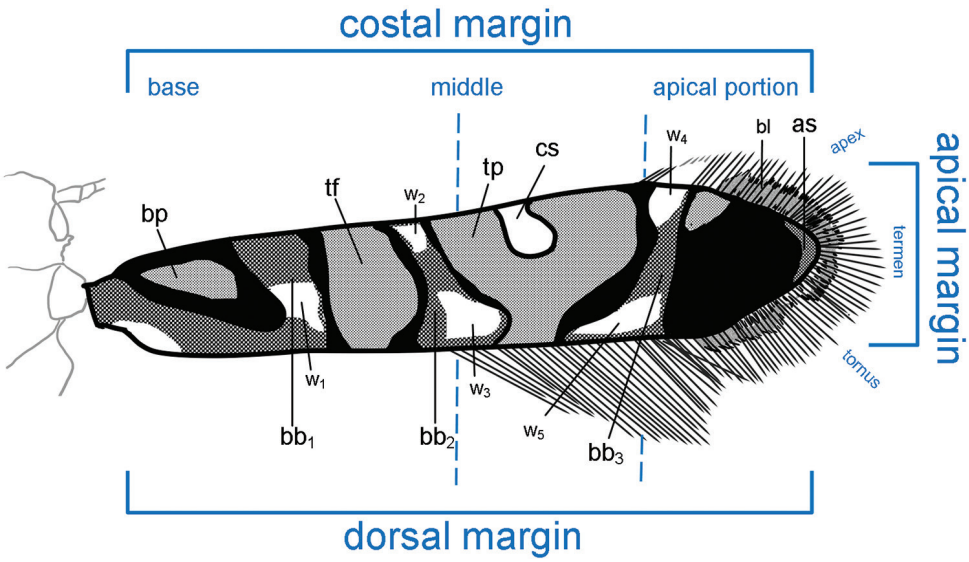


Figure 1. Nomenclature adopted in this study for the characterization of forewing pattern in *Myrsine*-feeding *Philodoria*. Abbreviations: as: apical spot; bb_1–bb_3: bronze color band; bp: basal patch; bl: basal line; cs: costal spot; tf: transverse fascia; tp: transverse patch; w_1– w_5: white color band.

Table 3. Intra- and interspecific genetic divergences in DNA barcode sequences among studied *Philodoria* species.

Species	*P. succedanea*	*P. kauaulaensis*	*P. auromagnifica*	*P. kolea*	*P. basalis*	*Philodoria* sp. nr. *splendida*
P. succedanea	[0.88]					
P. kauaulaensis	7.0	[0.17]				
P. auromagnifica	6.71	5.85	[0.31]			
P. kolea	8.91	7.38	8.43	[0.30]		
P. basalis	11.12	11.08	10.59	13.28	[1.70]	
Philodoria sp. nr. *splendida*	13.46	12.10	12.19	13.83	4.41	[1.07]
Philodoria sp. nr. *floscula*	13.46	15.07	14.78	15.90	13.84	14.93

Kimura 2-parameter (K2P) distances (%) for barcode DNA sequences of the seven analyzed species in the genus *Philodoria*; minimal pairwise distances between species are given for each species pair; values in square brackets represent maximal intraspecific distances.

majority rule criterion were performed to calculate branch support values. Phylogenetic trees were visualized in FigTree 1.4.3 (Rambaut 2009). Intra- and interspecific genetic distances were estimated using the Kimura 2-parameter model implemented within the analytical tools available in BOLDv4 (Table 3). We also used BOLD to obtain Barcode Index Numbers (BINs) (Ratnasingham and Hebert 2013).

While single-marker COI analyses can be prone to insufficient resolution and error (Rubinoff and Holland 2005), we were unable to obtain additional genetic data for these species during the time of this study. We therefore chose to use a gene-tree based approach (Hebert et al. 2003; Hajibabaei et al. 2007) as another source of evidence to complement morphology to assess species limits. Sequences, voucher data, images, and trace files are deposited in the Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007; www.barcodinglife.org). Furthermore, all sequences are deposited in GenBank, and are available as a single dataset DS-PHDRIA (http://dx.doi.org/10.5883/DS-PHDRIA)

Voucher specimen numbers

Institutional voucher numbers are given here for primary type material and museum collections. In the cases of NHMUK/BMNH numbers, for clarity and consitency they are cited without a space nor hash symbol (#) that might be read between the alpha and numeric parts of the code, since spaces and hashes create ambiguity for search, and series of institutional numbers have appeared in the past with or without such symbols

Abbreviations for collections:

BPBM Bernice P. Bishop Museum, Department of Zoology, 1355 Kalihi Street, Honolulu, Hawaii 96818, USA.

FLMNH McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA.

NHMUK Natural History Museum, Department of Zoology, Cromwell Road, London SW7 5BD, United Kingdom (formerly the British Museum [Natural History] or BMNH).

RMNH Naturalis Biodiversity Center, PO Box 9517, 2300 RA Leiden, Netherlands.

USNM National Museum of Natural History, Smithsonian Institution, 10th St. & Constitution Ave. NW, Washington, DC 20560, USA.

Results

Key to adults

1 Forewing leaden grey, externally with fuscous brown (Fig. 3) .. ***P. kolea*** **sp. n.**

– Forewing shiny, metallic bronze with bright to dark orange patches..... **2**

2 A bright orange transverse fascia at 3/4 in middle interrupted with blue patch; an orange medial transverse fascia, narrowing towards dorsum, (Figs 5I, J, 10E, F) ..... ***P. kauaulaensis*** **sp. n.**

– An orange transverse patch beyond middle to costal 3/4, narrowing towards dorsum, extending to dorsal 2/3, with white costal spot..... **3**

3 A black patch along costal fold (Figs 5C, D); a fuscous patch near apex (Figs 1A, B, 8A) ..... ***P. succedanea***

– An orange patch along costal fold, fringed with blackish scales (Figs 1E, F, 5F, H); a fuscous patch with dark orange scales at apex ..... ***P. auromagnifica***

Key to male genitalia*

1 Saccus slender, curved toward dorsum (Fig. 6B); vinculum small and inflexed on the ventral side (Fig. 6C) ..... ***P. succedanea***

– Saccus broad and straight (Fig. 6F, J); vinculum large and inflexed on the ventral side (Fig. 6G, K) **2**

2 Valva slightly narrowing in middle with terminally rounded dorsal process (Fig. 6E) ..... ***P. auromagnifica***

– Valva with short, pointed dorsal process (Fig. 7A) ..... ***P. kolea* sp. n.**

*Male of *kauaulaensis* is unknown.

Key to female genitalia

1 Signa with minute spines (Fig. 7I) ..... ***P. kolea* sp. n.**

– Signa with a pair of larger spines **2**

2 Spines long and slender (Fig. 7E, F) ..... ***P. succedanea***

– Spines on the signa small and rounded (Fig. 7H) ..... ***P. kauaulaensis* sp. n.**

– Spines on the signa blunt (Fig. 7G) ..... ***P. auromagnifica***

Key to leaf mines

1 Start of mine spiral-shape (Fig. 10B, C). Mines on *Myrsine lanaiensis*, *M. lessertiana*, *M. sandwicensis*; Maui ..... ***P. kauaulaensis* sp. n.**

– Start of mine linear or serpentine-shape **2**

2 Reddish brown long linear mine following leaf vein (Fig. 9D–F), mature larvae in fallen leaves (Fig. 9A). Mines on *M. knudsenii*, *M. lessertiana*, *M. linearifolia, M. sandwicensis*; Kauai, Oahu, Lanai, Maui, Hawaii ... ***P. succedanea***

– Brown serpentine mines, mature larvae in situ leaves **3**

3 Larvae utilize leaves on larger plants. Mines on *M. lessertiana*, *M. sandwicensis*, *M. wawraea*; Kauai, Oahu, Molokai, Hawaii ..... ***P. auromagnifica***

– Larvae utilize leaves on seedlings (Figs 12A, E, 13D). Mines on *M. lessertiana*; Hawaii (Big Island) ..... ***P. kolea* sp. n.**

***Philodoria succedanea* Walsingham, 1907**
Figs 2A–D, 5A–D, 6A–D, 7E, F, 8A, 9, 14A

Philodoria succedanea Walsingham, 1907: 717–718; pl. 25, fig. 19.
Philodoria (*Philodoria*) *succedanea* Walsingham, 1907: Zimmerman 1978: 718, figs 433, 435, 467, 472.

Type locality. Olinda, Haleakala (Maui).

Type material. Lectotype ♀, Olinda, 4000 ft., Haleakala, MAUI, Hawaiian Is. iv.1894, Perkins. 26695 [Walsingham specimen number]|PHILODORIA SUCCEDANEA Wlsm. Fn. Hawaii. I TYPE ♀ descr. fig[d].|Walsingham Collection. 1910-427.|NHMUK010305341 (here designated).

Paralectotypes 17 (2♂ 1♀ 14 unsexed; NHMUK ones are all from above Walsingham accession and 'PARATYPE' below is short for 'PHILODORIA SUCCEDANEA Wlsm. PARATYPE' as printed on large black-margined labels, with the 5-digit Walsingham specimen numbers whose first digit is '2' borne on the locality label): 1 ♂, Haleakula 4000 ft. MAUI, Hawaiian Is. V. 1896|Perkins. 28505|PHILODORIA SUCCEDANEA Wlsm. Fn. Hawaii. I TYPE ♂|BM ♂ Genitalia slide no. 2755|NHMUK010305341. 1♀ 2 unsexed: Haleakala, 5000ft, MAUI, Hawaiian Is., v.1896, Perkins. 28355|PARATYPE 3/17|NHMUK010862804|; 28230|PARATYPE 4/17♀|BPBM 34324; 28236|PARATYPE 5/17|BPBM 34321. 4 unsexed: Haleakula -4000 ft. Maui, v. 1896, Perkins. 28492|PARATYPE8/17; 28493|PARATYPE 9/17|BPBM 34320|; 28494|PARATYPE10/17|NHMUK010862806; 28495|PARATYPE 11/17|NHMUK010862807.

1 ♂ 7 unsexed, same data and locality as lectotype: 26696|NHMUK010862803; 26661|PARATYPE1/17|BPBM34325; 26667|PARATYPE2/17|BPBM 34222; 28511|PARATYPE12/17|NHMUK010862808; 28512|PARATYPE13/17|NHMUK010862809; 28513|PARATYPE14/17|NHMUK010862810; 28514|PARATYPE15/17♂|NHMUK010862811; 28552|PARATYPE 16/17|BPBM 34323.

This species was described from 19 specimens: 'type ♀ (26695); ♂ (28505)' and 17 'paratypes' from Kauai and Haleakala, Maui. This seems to indicate that Lord Walsingham considered them as holotype, allotype, and paratypes, as indicated on their specimen labels. But as a holotype was not specified in the description, the so-labelled types and paratypes are all to be considered syntypes under the present Code, Article 73.2 (ICZN 1999), and any one is thus eligible for designation as lectotype. The syntype 'type ♀ (26695)', which Walsingham listed first and figured, is here designated as lectotype (Fig. 2A). The remaining syntypes are therefore paralectotypes.

Additional material. 32 (11♂ 15♀ 6 unsexed).

Adults: Oahu Is.: 2♀, Mt. Kaala, 18.ii.1923, Swezey coll., host: "*Suttonia*" (= *Myrsine*), SK797♀, 798♀ in USNM; 3♂ 3♀, Palikea, 28.iii.2016 stored in 99 % ethanol (stored), K. Bustamente leg., host: *M. lessertiana*, 10.xi.2015, CJ-526, CJ-531, SK639♂, SK803♀, 804♀, 807♂, 808♂ in BPBM.

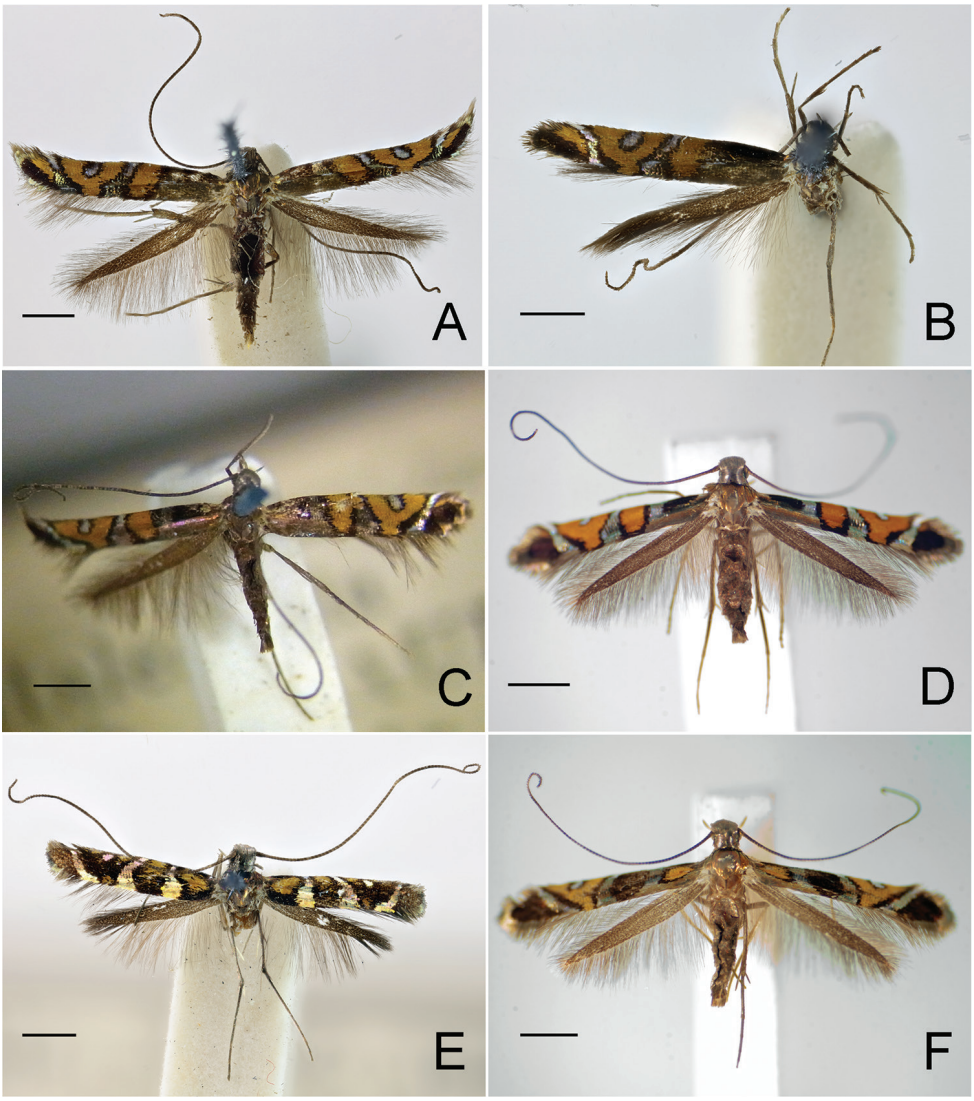


Figure 2. Adults of *Myrsine*-feeding *Philodoria* species. **A–D** *P. succedanea* Walsingham, 1907 **A** Lectotype female **B** Paralectotype male **C** Paralectotype female **D** Male Lanai **E–F** *P. auromagnifica* Walsingham, 1907 **E** Holotype male **F** Female Kauai. Scale bar: 1 mm.

Molokai Is.: 1♀, Kainalu [Kainalu Forest, South East Molokai Forest Reserve], 27?vii.1927, *Philodoria auromagnifica* Walsingham Det. by O.H. Swezey, 34145 in BPBM; 1 unsexed, 4000 ft Molokai P. 2.02 *Philodoria succedanea* Wals. 1/1 E. Meyrick det. in Meyrick coll., in NHMUK.

Lanai Is.: 2♂, 2750 ft, Munro Trail, 2.x.1976, K. & E. Sattler BM1976-605, BMNH(E)1621676 and BMNH(E)1621677, *Philodoria* sp. 8 (Lanai) Sattler coll. D.C. Lees Sep 2016.

Figure 3. Adults of *P. kolea* sp. n. **A** holotype male **B** Paratype female. Scale bar 1 mm.

Maui Is., in BPBM: 1♂ 6♀ 1 unsexed, below Eke, 17&21.v.2013 (stored), C.A. Johns leg., host: *Myrsine* sp., 24.iv.2013, CJ-136, 141, SK799♀; 1♂, Waikamoi, 24.v.2016 (stored), C.A. Johns leg., Spring.2016, CJ-539, SK641♂.

Hawaii Is., host: *M. lessertiana* in BPBM: 1♂ 1♀, Hawai'i Volcanoes National Park, Hawaii, A. Kawakita leg., "Leaf-dropper", 25.iv.2016 (larva), SK624♀, SK625♂; 2♂ 1♀ 1 unsexed, Same locality, 17&21.v.2016 em., A.Y. Kawahara leg., 29.iv.2016(Cocoon & larva), SKH-10, SK801♂, SKH-13, SK633♂. 1♀, 3800 ft, N. Kohala, Distr. Kohala Mts, Puu Laalaau area, 14–17.vii.1976, K. & E. Sattler, BM1976-605, BMNH(E)1621089, *Philodoria* sp. 9 (Hawaii) Sattler coll. D.C. Lees Sep 2016, 1621676 in NHMUK; 1♂, same data as last specimen, BMNH(E)1621090, *Philodoria* sp. 9 (Hawaii) Sattler coll. D.C. Lees Sep 2016; 1 unsexed, Kohala Watershed Partnership, 9.vi.2015 (stored), C.A. Johns leg., host: *Myrsine sandwicensis*, 18.v.2015, CJ-419 in BPBM.

Larvae: 2 unsexed, Kokee, Kauai Is., 16&26.vi.2015 (stored), C.A. Johns leg., host: *M. knudsenii* 15.vi.2015 (larva), CJ-433, 442 in FLMNH.

Diagnosis. This species is very similar to *P. auromagnifica* feeding on the same hostplant, *Myrsine*, but is recognizable by the rather bright orange patches and black triangular shaped basal patch in the forewing (Table 4; Figs 2A–D, 5A–D); in the male genitalia by the rather broad valva, slender and long saccus curving toward dorsal side (Fig. 6A–C); in the female genitalia by signa with slender and long spines (Fig. 7E, F).

Redescription. Adult (Figs 2A–D, 5A–D, 8A). Wingspan 9–10 mm in type series; forewing length 4 mm in 'TYPE ♂ (28505)' (fig. 2B), 3.6–3.8 mm in paralectotypes. Head bronze; frons white; maxillary palpus reduced; labial palpus bronze grey, with dark brown scales at apex. Antenna shiny tawny fuscous. Thorax bronze.

Forewing shiny, metallic bronze with bright orange-ochreous patches: a black triangular basal patch along the costal fold (Figs 5A, C, 8A); an oblique transverse fascia before the middle of wing, bordered with black scales; a large transverse patch after the middle to costal 3/4, distinctly narrowing in the dorsum, extending to dorsal 2/3, containing white costal spot; one white color band on the middle of the first

Table 4. Diagnostic features of four *Myrsine*-mining *Philodoria* species.

Species name	***P. succedanea***	***P. kauaulaensis***	***P. auromagnifica***	***P. kolea***
Forewing	Shiny, metallic bronze with bright orange-ochreous	Similar to *P. succedanea*	Shiny, metallic bronze with dark brownish orange	Leaden grey, externally with brownish fuscous
Basal patch	Black, triangular-shape	Absent, orange transverse fascia from costal fold to dorsal 1/4	Brownish orange with black ground color, sometimes black	Brownish fuscous
Apical orange transverse fascia	Absent	Present	Absent	Absent
Apical portion	Fuscous, sometimes orangish encroaches on the apex	Fuscous	Fusocus with dark orange scales	Leaden gray
Genitalia				
Valva	Broad	Unknown	Rather long and narrowing in the middle	having rather shorter and pointed dorsal process
Vinculum	Small, inflexed on the ventral side	Unknown	Large, inflexed on the ventral side	Small, inflexed on the ventral side
Saccus	Slender and long, curved toward dorsal side	Unknown	Broad and straight	Broad and straight
Spine on signum	Long and slender	Rather smaller and rounded	Rather blunt	Minute
Distribution[a,b]	**Kauai**, **Oahu**, **Lanai**, Maui, Hawaii	Maui	**Kauai**, Oahu, Molokai, Hawaii	Hawaii
Host plant species[a,b]	*Myrsine lessertiana*, *M. sandwicensis*, ***M. knudsenii***, ***M. linearifolia***, *Myrsine* sp.	*Myrsine lessertiana*, *M. lanaiensis*, *M. sandwicensis*	*Myrsine lessertiana*, *M. sandwicensis*, ***M. wawraea***, *Myrsine* sp.	*Myrsine lessertiana*
Larval habit type	Leaf dropper	Unknown (probably non leaf dropper)	Non leaf dropper	Non leaf dropper
Mining form	Long, linear, along leaf vein	At first spiral, later blotch	Serpentine	Serpentine
Mine color	Red	Brown	Brown	Brown

[a] As indicated by published data (Zimmerman 1978 and Johns et al. 2016) and see s also pecies description.
[b] Plant species and island name in bold indicate new records in the present study. Islands underlined denote type-locality islands.

bronze color band, others on both extremities of second and third bands; a fuscous patch extending toward the termen and apex with a black apical spot, sometimes with orange-ochreous color encroaching on the apical part; cilia tawny, with two metallic silver basal lines, one at the apical cilia, another from termen to tornus. Hindwing

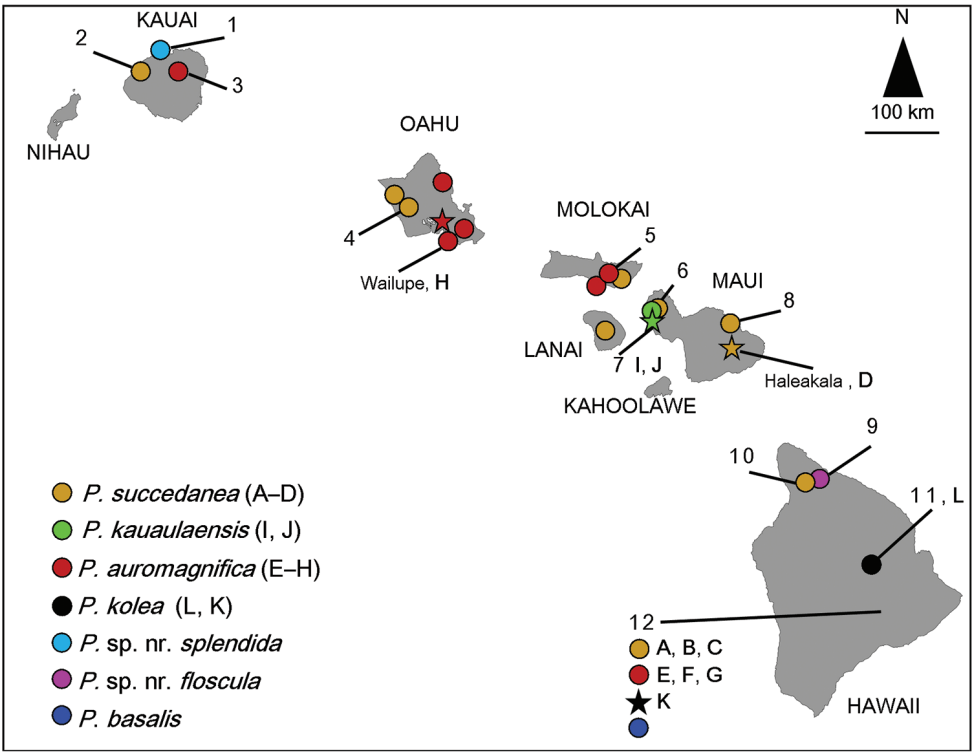


Figure 4. Distribution of *Myrsine*-feeding *Philodoria* species. The star indicates the type locality of each species. Information based on this study and label data of specimens in BPBM, USNM, and NHMUK. Symbols are numbered according to showing locality in Table 1 and alphabetical symbols (A–K) correspond to figure numbers in Figure 5.

dark tawny; cilia tawny. Abdomen tawny above, silvery beneath. Legs tawny, with silvery spurs and slightly paler tarsi.

Male genitalia (Fig. 6A–D) (n = 7). Capsule 960 µm. Uncus absent. Tegumen 570–580 µm long, 1.2–1.3× length of valva with series of long hairs at lateral side of base (Fig. 6C). Tuba analis membranous with weakly sclerotized subscaphium; gnathos V-shaped transverse band, terminal margin weakly joining subscaphium and anterior process connecting ventral base of tegumen. Valva broad, 430 µm in length covered with fine setae distally, and having a short dorsal process (Fig. 6A).Vinculum U-shaped; saccus 250 µm long, slender , curved toward dorsal side (Fig. 6B, C). Phallus 720 µm long, tubular and long about 1.2–1.3× length of valva, sinuous in lateral view with two series of minute spiniform cornuti in vesica; coecum slightly curved toward inner side (Fig. 6D).

Female genitalia (Fig. 7E, F) (n = 7). Ostium bursae rather small, opening at the middle of 7th abdominal segment; antrum cup-shaped with slender a pair of lateral lobes; ductus bursae slender, tubular, extremity connected to antrum very slender and membranous, curved inside of body, and middle part weakly sclerotized and plate-shape; end of the ductus bursae broad; inception of ductus seminalis on the posterior part of ductus bursae. Corpus bursae pyriform, anterior end weakly sclerotized; some

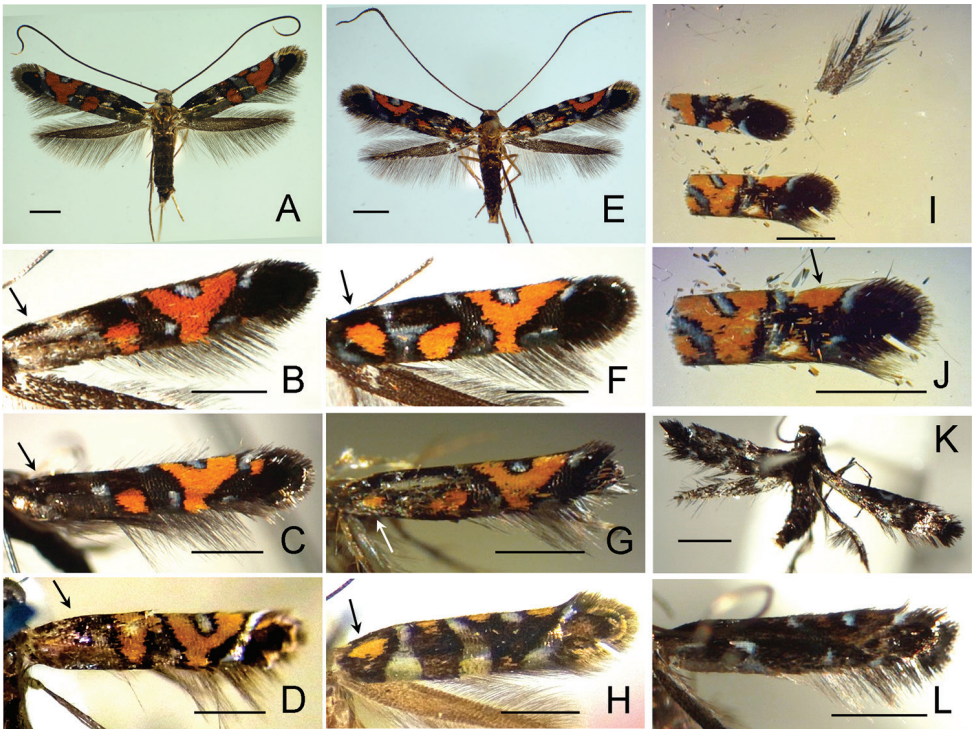


Figure 5. Forewing coloration and pattern of *Myrsine*-feeding *Philodoria* species. **A–D** *P. succedanea* **E–H** *P. auromagnifica* **I, J** *P. kauaulaensis* **K, L** *P. kolea*. **A–C, E–G, K** Hawai'i Volcanoes National Park. **A** Female SK622, leaf-dropper **B** Male SK625, leaf-dropper **C** Male SK633 **D** Paralectotype female 34320 Haleakala, Maui in BPBM **E** Female SK624, non-leaf-dropper **F** Female SK623, non-leaf-dropper **G** Male SK805 **H** Female Z-XII-20-62-6 34143 Wailupe, Oahu, in BPBM **I, J** Holotype female SK690 **K** Paratype female SK632 **L** Paratype female SK631. Scale bars: 1 mm.

lines consisting of wrinkles running longitudinally, some sclerotized; paired signa with a pair of long slender spines.

Distribution. Kauai, Oahu and Lanai: new record, Maui (Walsingham 1907), Molokai and Hawaii (Big Island) (Zimmerman 1978).

Host plants. Primulaceae: *Myrsine sandwicensis* A. DC., *M. lessertiana* A. DC. (Johns et al. 2016), *Myrsine* sp. (Zimmerman 1978). *Myrsine linearifolia* Hosaka and *M. knudsenii* (Rock) Hosaka are new host records (see Remarks).

Biology. (Figs 8A, 9, 14A). The larvae mine the adaxial side of leaves of *Myrsine* species, forming a long linear mine (Fig. 9B, G, H). The mine is at first tornus-shaped (Fig. 9C, D, I, J) and the larva broaches the mid vein towards the petiole of the leaf, forming a straight mine; the vein mine and surrounding pattern are red in coloration (Fig. 9F, H) and later instars leave the mid vein usually near the base of the leaf, gradually expanding as they feed and grow forming a full-depth mine (Fig. 9E, F). There were usually one to two mines per leaf (Fig. 9B, G, M). The pupal cocoon is situated outside of the mine, usually on the leaf surface, and also on the woody tissue of the host plant with leaf mines and larvae. At Hawai'i Volcanoes National Park, larvae were collected from leaves that had fallen

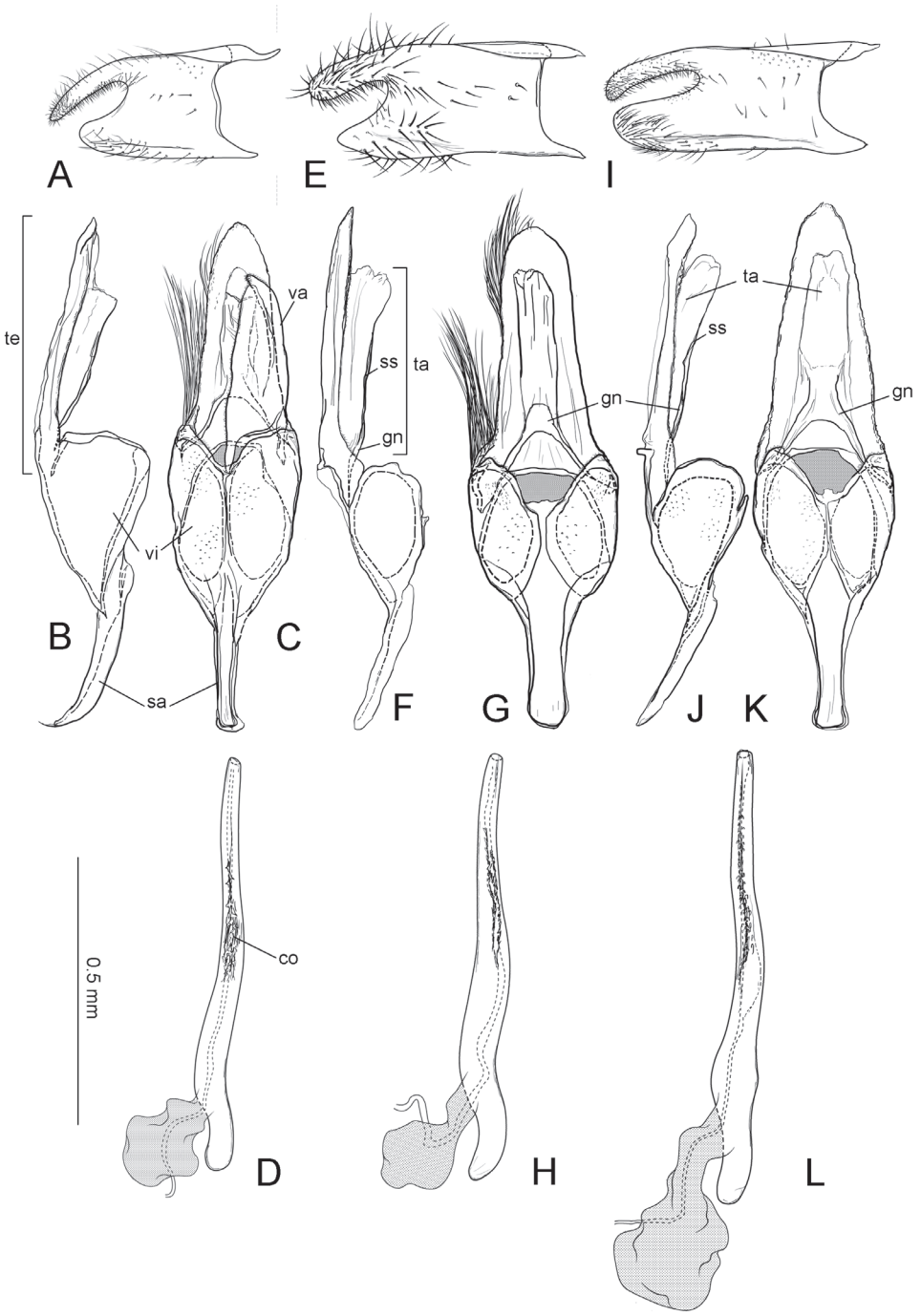


Figure 6. Male genitalia of *Philodoria*. **A–D** *P. succedanea* Maui SK641 **E–H** *P. auromagnifica* Hawaii SK800 **I–L** *P. auromagnifica* Kauai SK689 **A, E, I** Left valva **B, F, J** Genital capsule lateral view **C** Genital capsule with left valva ventral view **G, K** Genital capsule ventral view **D, H, L** Phallus lateral view. Abbreviations: co: cornuti; gn: gnathos; sa: saccus; ss: subscaphium; ta: tuba analis; te: tegumen; va: valva; vi: vinculum.

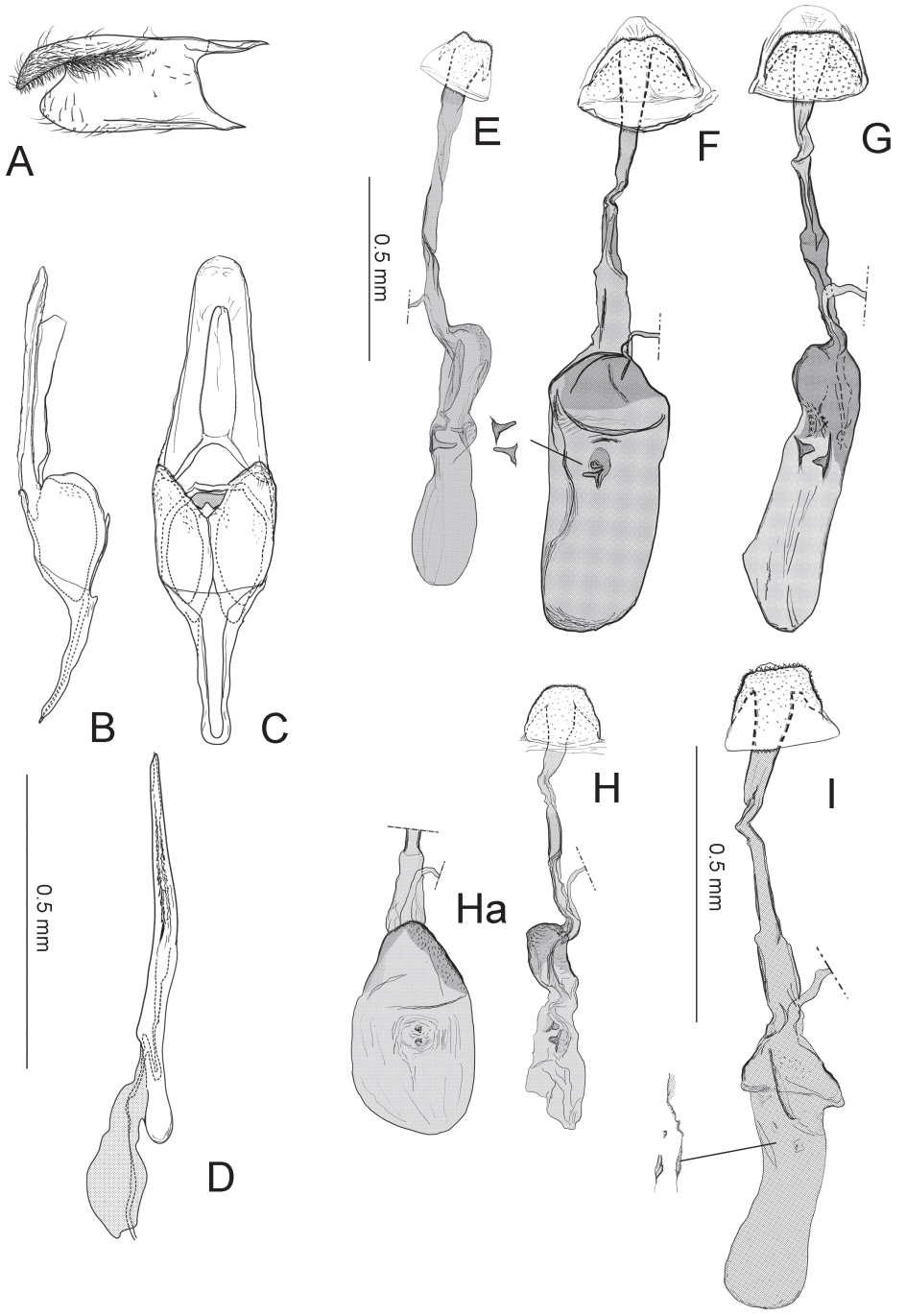


Figure 7. Genitalia of *Philodoria*. **E–I** Female. **A–D** *P. kolea* holotype male Hawaii SK851 **E** *P. succedanea* paralectotype Maui SK714 **F** *P. succedanea* leaf dropper Hawaii SK624 **G** *P. auromagnifica* non leaf dropper Hawaii SK623 **H** *P. kauaulaensis* holotype SK690 **I** *P. kolea* paratype Hawaii SK634. **A** Valva **B** Genital capsule lateral view **C** Genital capsule ventral view **D** Phallus lateral view

Figure 8. Resting posture of adult *Philodoria*. **A** *P. succedanea* Waikamoi Maui **B** *P. auromagnifica* Molokai CJ241.

to the ground and reared to adulthood (Fig. 9A). The adult has been observed during the day (Maui and Hawaii Island), resting on the upper leaf surface of the host plant (Fig. 8A).

DNA barcoding. BIN BOLD:ADF5435. The two specimens sequenced for COI, one from Maui and one from Hawaii, have identical DNA barcode sequences. The p-distance to the nearest neighbor, *P. kauaulaensis*, is 6.63%.

Remarks. We identified two adult moths (Coll ID CJ-144 / GenBank accession no. ID KT982414 and CJ-145) as *P. succedanea*, based on the presence of a basal black patch on forewing, from which whole bodies were sacrificed for molecular analysis (Johns et al. 2016; Figs 6O, 12). Zimmerman (1978) did not recognize Walsingham's (1907) Kauai record of this species because Walsingham had only one specimen at hand, which was in poor condition (specimen data: 1 ♂, Mts [which Mts not further specified], 3–4000 ft., Kauai, vi. 1894 Perkins.27297| PARATYPE 17/17 (?)|'NOT succedanea Det. by E. C. Zimmerman|NHMUK010862812). We could not find the specimen from Kauai. However, we found *Myrsine knudsenii* (Endangered, IUCN) leaves with mines with active larvae from Kokee, Kauai Is. (CJ-433, 442), which were similar in appearance to mines of *P. succedanea* on *M. lessertiana*. Judging from these data, we consider the larval mines on *M. knudsenii* were made by *P. succedanea*. We also collected active *Philodoria* leaf mines from *M. linearifolia* (Endangered, IUCN) at the same location as *M. knudsenii*, but were unable to rear adult moths. It is thus possible that *P. succedanea* also mines *M. linearifolia*, but this needs to be further examined.

***Philodoria kauaulaensis* Kobayashi, Johns & Kawahara, sp. n.**
http://zoobank.org/391CBA73-B2B0-462C-8608-0521E5B2572E
Figs 5I, J, 7H, 10, 14B

Type locality. Kauaula (Maui).

Type material. Holotype ♀, Kauaula, Maui, 18.viii.2014 (stored in 99% ethanol), C.A. Johns leg., host: *Myrsine lanaiensis*, 31.vii.2014, CJ-381, SK690 in BPBM. The

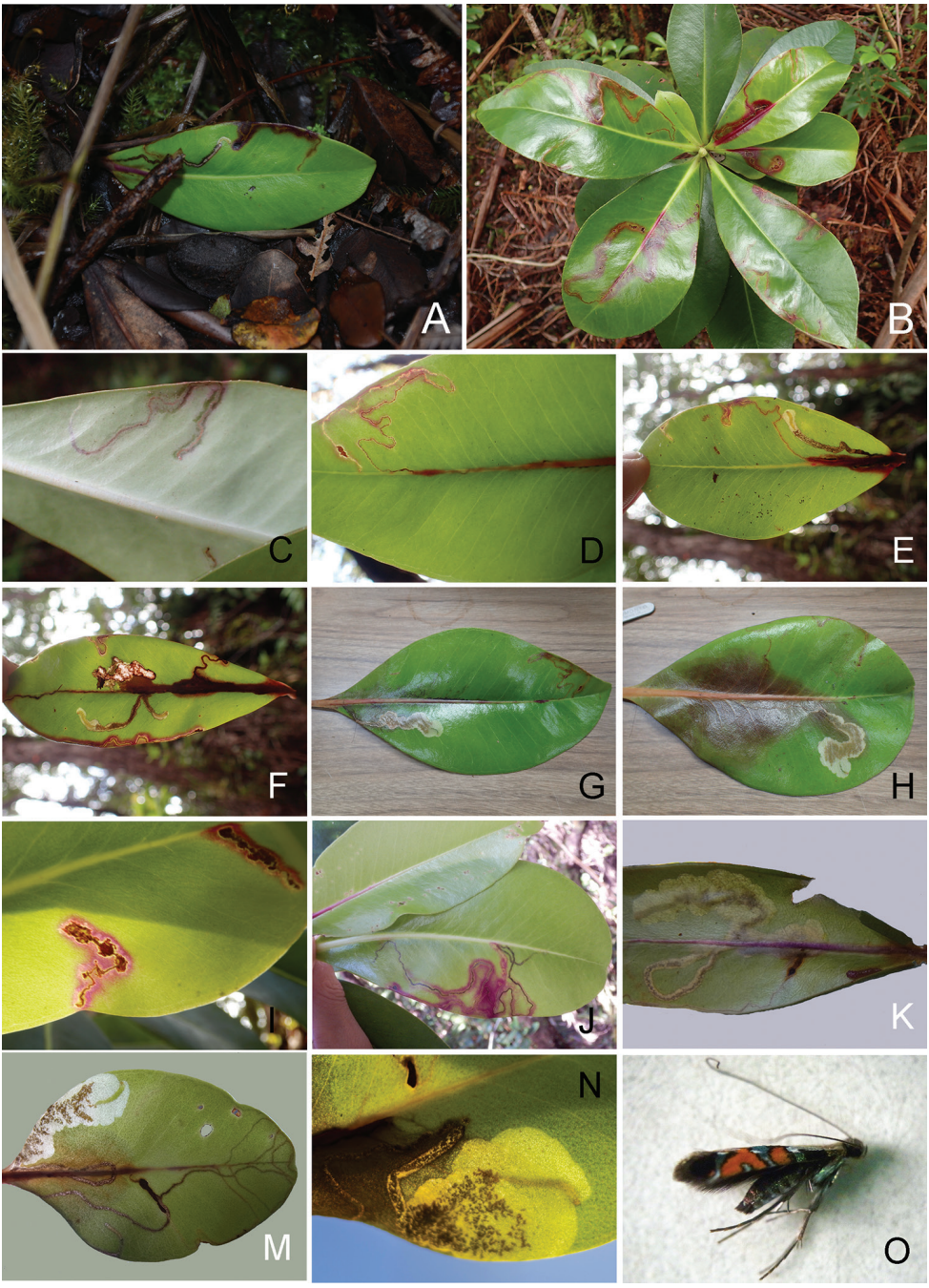


Figure 9. Biology of *Philodoria succedanea* with its hostplant, *Myrsine lessertiana*. **A–J** Hawai'i Volcanoes National Park, Hawaii (Big Island) **K–N** Maui **A** Fallen leaf and leaf mine with larva **B** Hostplants and leaf mines **C, I, J** Young mine **D** Leaf vein mine **E–G** Mine by late instar larva **H, K, M** Mature larva and mine **N** Mature larva **O** Adult, CJ-145, lateral view.

holotype is incomplete but we consider it distinctive enough to be worth describing. What remains of the holotype was mounted by placing three wings without mountant under a coverslip: two forewings (3/4 of right wing and half of left wing), and the apical portion of one hindwing (Fig. 5I). The head, antenna, thorax, and legs were sacrificed for molecular analysis.

Additional material. 2 unsexed (CJ-064, CJ-072), entirely sacrificed for molecular analysis and belonging to BIN BOLD:ADI5327 (See Remarks): 1 unsexed, Haelaau, Maui, 26.iv.2013 (stored), C.A. Johns leg., host: *M. lessertiana*, 8.iv.2013, CJ-064, KT982404; 1 unsexed, Haelaau, Maui, 29.iv.2013 (stored), C.A. Johns leg., host: *M. sandwicensis*, 8.iv.2013, CJ-072, KT982407.

Diagnosis. The forewing pattern of this species is similar to that of *P. succedanea*, but differs from the latter by having broad orange transverse fasciae (Fig. 10E, F) and a white and bronze band near the apical portion of wing, in the middle interrupted by a blue patch (Fig. 5I, J).

Description. Adult (Fig. 5I, J). Forewing length 2.4 mm, basal part of holotype forewing missing. Descriptions of the basal forewing and part of the body were based on photographs of adult moths (CJ-064, 072). Head and frons fuscous; maxillary palpus unknown; labial palpus ochreous. Antennae dark fuscous. Thorax unknown. Forewing shiny, metallic bronze with three large bright orange transverse fascia: an oblique one from costal fold to dorsal 1/4; a second at the middle of wing, narrowing greatly in the dorsum, containing a white costal spot; a third at 3/4 in the middle, interrupted by a blue patch; all fascia bordered with black scales: one white color band at middle of the first bronze color band, others on both extremities of third and fourth bands; a fuscous patch extending toward termen and apex with a black apical spot; cilia shiny, dark bronze grey. Hindwing dark tawny fuscous. Abdomen fuscous above, whitish beneath.

Male genitalia. Unknown.

Female genitalia. (Fig. 7H) (n = 1). Similar to *P. succedanea* and *P. auromagnifica*, but different in having rather smaller and rounded spines on the signa.

Distribution. Maui.

Host plants. Primulaceae: *Myrsine lanaiensis* Hillebr., *M. lessertiana* A. DC., and *M. sandwicensis* A. DC.

Biology.(Figs 10, 14B). The mine is initially spiral-shaped (Fig. 10B, C) and gradually expands as the larva feeds and the mine later gets the form of a blotch (Fig. 14B). The pupal cocoon is situated outside the mine, usually on leaf surface (Fig. 10D).

DNA barcoding. BIN BOLD:ADI5327. The two specimens sequenced for COI are from Maui and have a 0.17 p-distance between them, the p-distance to the nearest neighbor, *P. auromagnifica*, is 5.58%.

Etymology. The specific epithet is derived from the type locality, Kaua`ula (pronounced 'cow-wa-u-la') Valley, an important site for Hawaiian endemic plants and culturally and spiritually for Native Hawaiians.

Remarks. Johns et al. (2016) collected larvae from *Myrsine lessertiana* and *M. sandwicensis* in West Maui and identified the reared adult moths as *P. auromagnifica* (Coll.

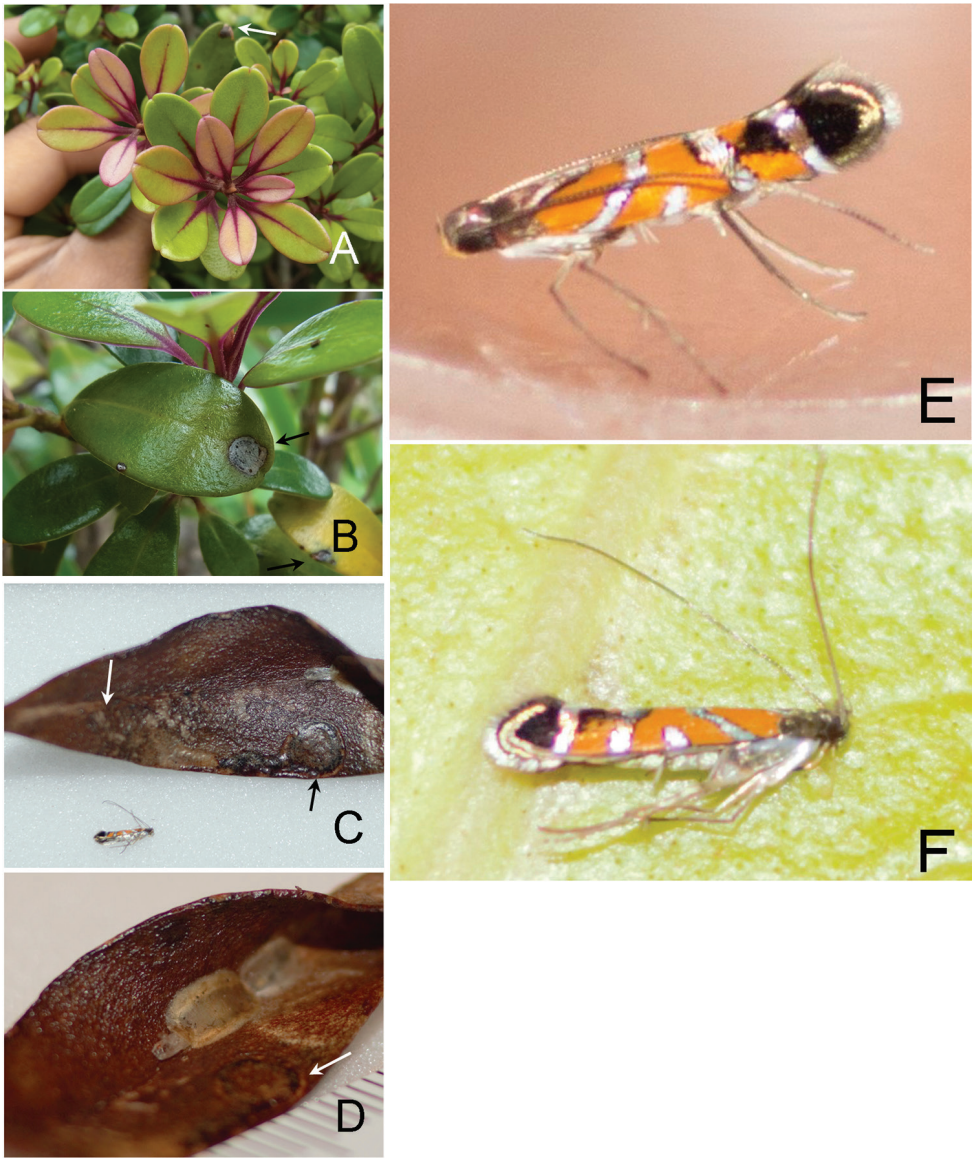


Figure 10. Biology of *Philodoria kauaulaensis* with its hostplant, *Myrsine lessertiana*, Haelaau, Maui, CJ-064. **A, B** Hostplant leaves and mines **C** Spiral mines, cocoon and adult moth **D** Cocoon with pupal exuvia **E** Resting posture of adult **F** Dead adult. Arrow showing leaf mine.

ID CJ-064 / GenBank accession no. KT982404 and CJ-072 / KT982407). Comparison of adult morphology and larval behavior with other species shows that these moths belong to *P. kauaulaensis* (Figs 10, 14). Unfortunately these specimens were sacrificed for molecular analysis, so that they cannot be added to the type series.

***Philodoria auromagnifica* Walsingham, 1907**
Figs 2E, F, 5E–H, 6E–H, 7G, 8B, 11, 14C

Philodoria auromagnifica Walsingham, 1907: 718, pl. 25, fig. 20; Swezey 1913b: 223.
Philodoria (*Philodoria*) *auromagnifica* Walsingham, 1907: Zimmerman 1978: 695, figs 461, 468, 474.

Type locality. mountains, 2000 ft near Honolulu (Oahu).

Type material. Holotype ♂, Mts. 2000 ft near Honolulu, Oahu, 25.x.1892, Perkins. 25857|BM slide no. 472|Walsingham Collection. 1910–427.|NHMUK010305330| in NHMUK. This species was described based on a single specimen from Oahu. The 'type' specimen, designated by Walsingham is here thus the holotype following article 73.1.2 (ICZN 1999).

Additional material. 22 (8♂ 11♀ 3 unsexed)

Kauai Is: 1♂, Mt. Kahili, 18.vi.2013 (stored), N. Tangalin leg., Nat Collection, host: *M. wawraea*, CJ-148, SK689♂ in BPBM; 1♀, 4000 ft, Kokee State Park, Kahuamaa Flat, 21.viii.1973|K. & E. Sattler, BM1973-498|BMNH(E)1621087|*Philodoria* sp. 5 (Kauai) Sattler coll. Colour slide 67, D.C. Lees Sep 2016 in NHMUK; 1♂, same data labels as last specimen but 28.viii.1973|67|BMNH(E)1621087; 2 unsexed, Kauai, 3600', Kokee State Park Kaumuohua Ridge (Milolii Ridge Rd) 1.vii.1982|K. & E. Sattler, BM1982-342| BMNH(E)1621081; same data, but BMNH(E)1621088; 1♂, Kauai, 3800', Kokee State Park Kumuwela Ridge Waininiua Trail 24,vi.1982|K. & E. Sattler, BM1982-342|BMNH(E)1621091.

Oahu Is: 1♂, Kahana, 1.i.1928, O.H. Swezey Collector, "*Suttonia*"(= *Myrsine*), Z-XII-20-62-5♂, BPBM no. 34142 in BPBM; 2♀, Olympus, Coll. O.H.S, ex *Myrsine*, 33, J.F.G.C. #3801♀ in USNM. 1♀, Wailupe, 11.i.1925, O.H. Swezey Collector, "*Suttonia*"(= *Myrsine*), Z-XII-20-62-6♀, 34143 in BPBM.

Molokai Is, in BPBM: 1 unsexed, Kawela, 3700ft, 23.xii.1925, O.H. Swezey Collector, "*Suttonia*"(= *Myrsine*), 34144;1♂, Kamakou Boardwalk, 24.i.2014 (stored), C.A. Johns leg., host: *M. lessertiana*, 18.xii.2013, CJ-241, SK768♂ in BPBM.

Hawaii Is., Hawai'i Volcanoes National Park, host: *M. lessertiana* in BPBM: 2♀, A. Kawakita leg., "Non-leaf-dropper", 25.iv.2016 (larva), SK622♀, SK623♀; 3♂ 4♀, 17–24.v.2016 em., A.Y. Kawahara leg., 27&29.iv.2016 (Cocoon & larva), SKH-10, SK802♀, SKH-13, SK805♂, AYK0002, SK806♂, HILO053, SK800♂, HILO054, SK811♀, HILO059, SK810♀; 1♀, Lava tube, 15.v.2016 em., C.L.-Vaamonde & C. Doorenweerd leg., 22.iv.2016, HILO020/AYK0001, SK809♀.

Diagnosis. This species is very similar to *P. succedanea*, but recognizable by the dark brownish orange patches and brownish orange basal patch in the forewing; a fuscous patch with dark orangish scales in the apical portion (Table 4; Figs 2E, F, 5E–H); in the male genitalia by the rather long valva narrowing in the middle, vinculum large, inflexed on the ventral side, broad and straight saccus (Fig. 6E–G); in the female genitalia by signa with rather blunt spines (Fig. 7G). See also diagnosis of *P. succedanea*.

Figure 11. Biology of *Philodoria auromagnifica* with its hostplant. **A** Resting posture of adult male, host: *Myrsine wawraea* Kauai CJ-148 **B, C** Later mine, host: *M. lessertiana* Hawai'i Volcanoes National Park, Hawaii.

Redescription. Adult (Fig. 2E, F). Wingspan 8 mm in holotype, 7–9 mm in other specimens; forewing length 3.5 mm in holotype, 3.2–3.9 mm in others. Head and frons dark steely fuscous; maxillary palpus reduced; labial palpus ochreous to brown. Antenna dark fuscous. Thorax: dark brownish orange, becoming fuscous posteriorly. Forewing shiny, metallic bronze with dark brownish orange patches: a large one at base bordered with black ground color (Figs 2E, F, 5E, F, H), sometimes missing orange color (Fig. 8B); an oblique transverse fascia before the middle of wing, bordered with black ground color, sometimes missing orange color (Fig. 11A); a large transverse patch after the middle to costal 3/4, narrowing greatly in the dorsum, extending to dorsal 2/3, containing a white costal spot; one white color band on the middle of the first bronze color band, others on both extremities of second and third bands; a fuscous patch mixed with dark brownish orange scales extending toward the termen and apex with a black apical spot; cilia shiny, dark bronze grey. Hindwing dark tawny fuscous. Abdomen and legs fuscous above, white beneath.

Male genitalia (Fig. 6E–L) (n = 5). Capsule 940–980 µm. Tegumen 540–580 µm long. Similar to *P. succedanea* except tegumen 1.2× length of valva; valva 460–480 µm long, broad and slightly narrowing in the middle (Fig. 6E, I); vinculum large, inflexed on the ventral side (Fig. 6G, K); saccus 300 µm long, broad and straight (Fig. 6F).

Female genitalia (Fig. 7G) (n = 7). Similar to *P. succedanea*, but different in having rather slender tapering antrum and rather blunt spines on the signa.

Distribution. Kauai: new record, Oahu (Walsingham 1907), Molokai (Swezey and Bryan 1929), and Hawaii (Big Island) (Zimmerman 1978).

Host plants. Primulaceae: *Myrsine* sp. (Swezey 1913a), *M. lessertiana* A. DC. and *M. sandwicensis* A. DC. (Johns et al 2016), *M. wawraea* (Mez) Hosaka: new record.

Biology. (Figs 8B, 11, 14C). The larvae mine the adaxial side of leaves of *Myrsine* species, forming a long serpentine mine (Fig. 11B) and gradually expanding as they feed (Figs 11C, 14C2, C3). Old mines are ocherous to brown in coloration (Fig. 14C1). There were usually one to two mines per leaf (Fig.11B). The pupal cocoon is prepared outside the mine, on either surface of the leaf, and one was found on the bark of the host.

DNA barcoding. BIN BOLD:ADD6965. The two specimens sequenced for COI are from Hawaii and diverge by 0.31%, whereas the p-distance to the nearest neighbor, *P. kauaulaensis*, is 5.58%.

Parasitoids. *Euderus metallicus* (Ashmead, 1901), Eulophidae (Zimmerman 1978).

Remarks. We collected *Philodoria* leaf mines from *Myrsine* plants on Kauai Island (See also remarks for *P. succedanea*), only one male adult identified as *P. auromagnifica* emerged from a larva that fed on *M. wawraea* (Fig. 14C3). The Kauai specimens have a black second transverse fascia (Fig. 11A), but male genital variation that we observed appears to be intraspecific (Fig. 6I–L). Some specimens have a oblong valva which narrows in the middle (Fig. 6I), while others have a long tegumen about same length of valva, and slender vinculum and saccus in ventral view (Fig. 6J, K). We notice some wing pattern variation between islands, particularly in the extent of the orange forewing markings, and detailed DNA barcoding in future may prove revealing as regard the integrity of this species as we recognize it here. Two barcoded specimens collected from Hawaii (Big) Island (RMNH.5013750, CLV6240) belong to the same BIN (BOLD:ADD6965).

Philodoria kolea Kobayashi, Johns & Kawahara, sp. n.

http://zoobank.org/36268FAD-7EAE-4761-8EC4-87E19E7BF50E
Figs 3, 5K, L, 7A–D, I, 12, 13, 14D

Type locality. Hawai'i Volcanoes National Park (Big Island).

Type material. Holotype ♂, Hawai'i Volcanoes National Park, Hawaii (Big Island), 25.iv.2016, A. Kawakita leg., host: *Myrsine lessertiana* (understory shrub), GenBank accession no. MF804825, IO-322, SK851 in BPBM. The type series was mounted from emerged adult moths.

Paratypes, in BPBM: 1♀, Kaumana Trail, Hilo, Hawaii (Big Island), 28.iv.2016, em., C.L.-Vaamonde & C. Doorenweerd leg., host: *Myrsine* sp., 20.iv.2016 (Cocoon), HILO016, SK634♀. 1♀, Thurston lava tube (Nahuku), Hawai'i Volcanoes National Park, Hawaii Is., 13.v.2016, em., S. Kobayashi leg., host: *Myrsine lessertiana*, 25.iv.2016 (larva), SKH-05-1, SK632♀; 1♀, same locality and data as holotype, IO-323, SK852; 2♀, same locality as holotype, 2&24.v.2016, em., C.L.-Vaamonde & C. Doorenweerd leg., host: *Myrsine lessertiana*, 22.iv.2016 (larva), HILO020/SKH-15, SK630♀, 631♀.

Diagnosis. Among *Philodoria* species having similar fuscous forewing coloration (i.e., *P. wilkesiella* Swezey, *P. pipturiana* Swezey, *P. epibathra* (Walsingham), and *P. nigrella* (Walsingham) (See Zimmerman 1978)), the new species is recognizable by the white and bronze color bands on the forewing (Fig. 3). The forewing pattern and the genitalia are similar to those of other *Myrsine* mining species, *P. succedanea* and *P. auromagnifica*, but *P. kolea* completely lacks the orange markings (Figs 2, 3).

Description. Adult (Figs 3, 5K, L, 12N). Wingspan 6.7 mm in holotype, 6.6, 8.5 mm in paratypes; forewing length 3.0, 3.1 mm in holotype, 2.7–4.0 mm in paratypes. Head leaden grey; frons whitish grey; maxillary palpus reduced; labial palpus greyish ochreous, terminal joint with fuscous band at middle and at apex. Antenna greyish fuscous. Thorax

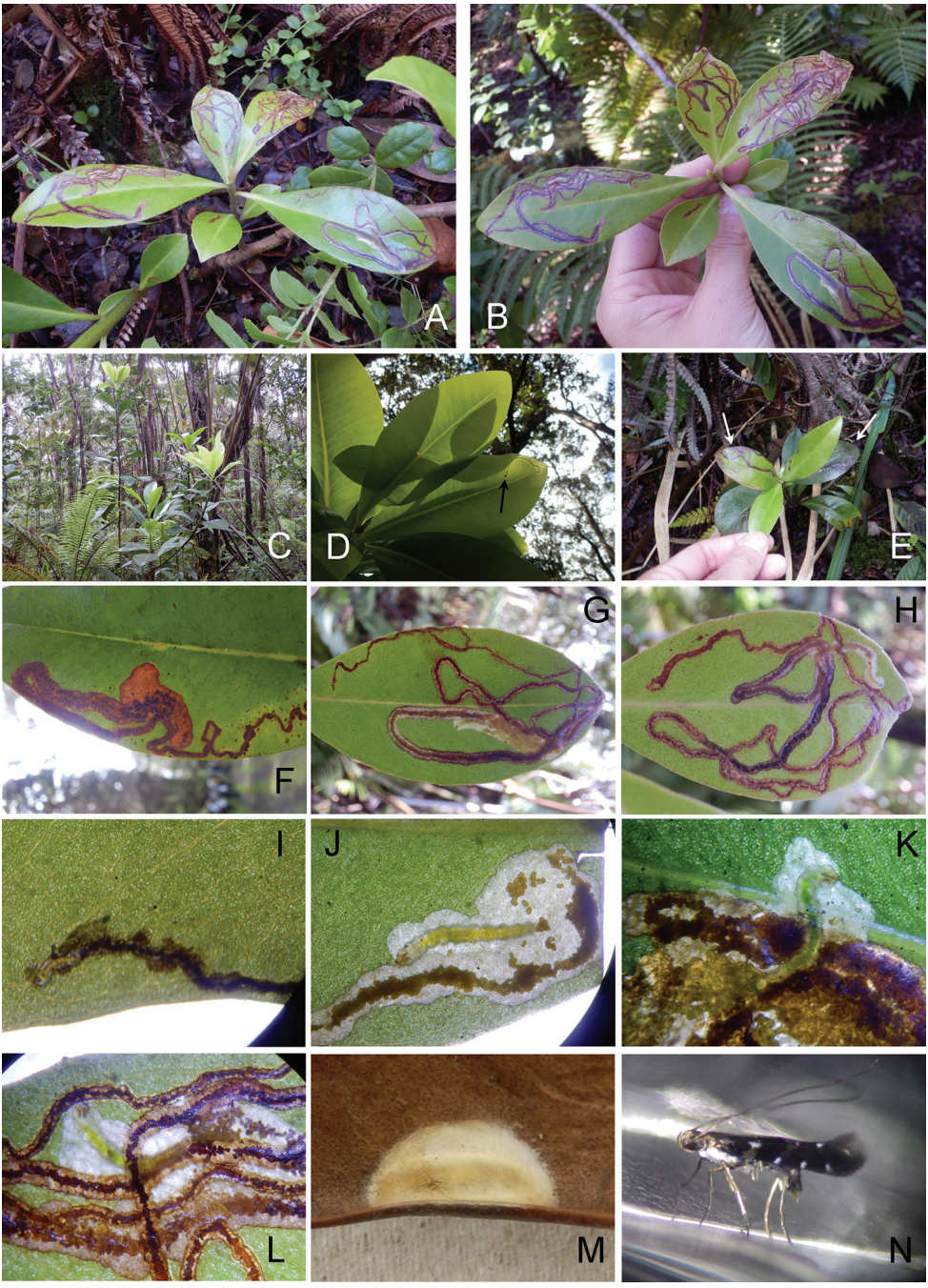


Figure 12. Biology of *Philodoria kolea* with its hostplant, *Myrsine lessertiana*. **A–M** Hawai'i Volcanoes National Park **A–B, E** Hostplant leaves and mines **C** Habitat and hostplants **D** Leaves and young mine **F–H** Later mines **I** Young larva **J–L** Mine by later instar larva **M** Cocoon **N** Resting posture of adult, paratype female lateral view.

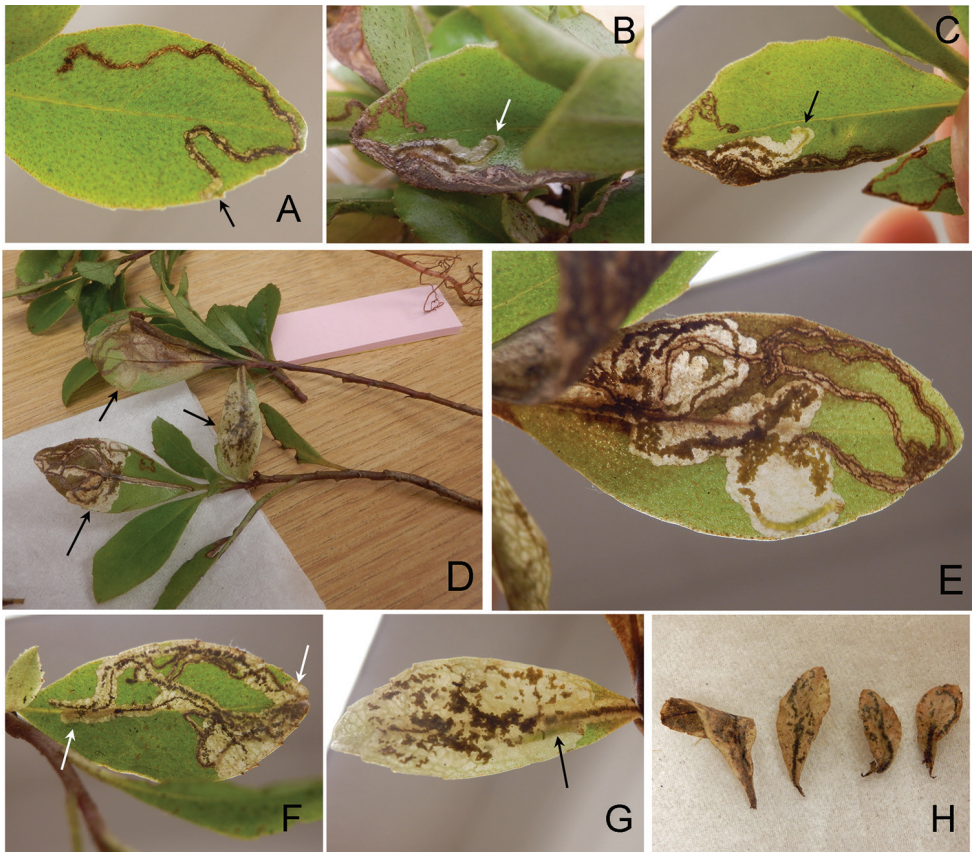


Figure 13. Biology of *Philodoria kolea* and its host, on seedlings of *Myrsine lessertiana*. **A** Young mine **B–C, E–F** Mine by later instar larva **G** Mature mine and larva **H** Full mature mine. Arrows showing larvae.

leaden grey. Forewing base leaden grey, externally suffused with brownish fuscous patches: a triangular basal patch along the costal fold; an oblique transverse fascia before the middle of wing, bordered with black scales; a large transverse patch after the middle to costal 3/4, narrowing in the dorsum, extending to dorsal 2/3, containing small white costal spot; leaden grey small median line at base with dorsal narrow patch from base to near middle; one white color band at the middle of the first bronze color band, others on both extremities of second and third bands; a leaden grey patch extending toward the termen and apex with small shiny black apical spots; cilia leaden grey with a black fringe basal line; tonal cilia with a shiny white fringe basal line. Hindwing and cilia leaden grey. Abdomen greyish fuscous above, banded with white beneath. Legs pale greyish fuscous, spurs white.

Male genitalia (Fig. 7A–D) (n = 1). Capsule 830 μm. Tegumen 600 μm long. Similar to *P. auromagnifica*, except tegumen 1.5× length of valva (Fig. 7A, C); valva 390 μm long, broad and having rather shorter and pointed dorsal process (Fig. 7A); saccus 250 μm long. Phallus 640 μm long.

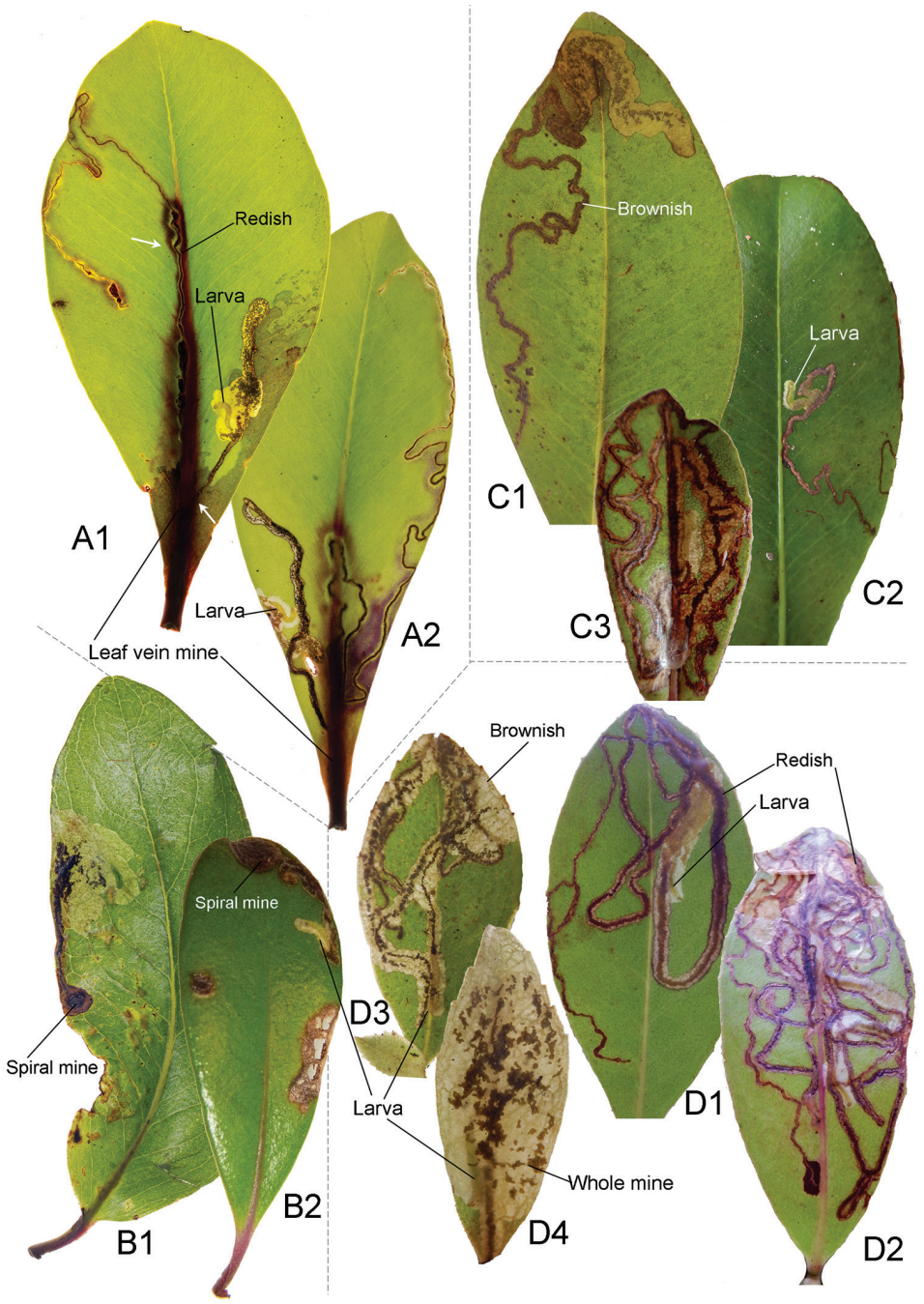


Figure 14. Mine forms and characters of *Philodoria* species and their *Myrsine* host plants. A: long linear form via mid vein ; B: Spiral to blotch form; C, D: Serpentine form. **A** *P. succedanea* **B** *P. kauaulaensis* **C** *P. auromagnifica* **D** *P. kolea* **A, C1–2, D** *M. lessertiana* **D1, 2** same collection of SKH-05-1 **B1** *M. lanaiensis*, same collection of CJ-381 **B2** *M. sandwicensis*, same collection of CJ-072 **C3** *M. wawraea*, CJ-148. **A** Molokai **B** Maui **C1–2, D** Hawaii **C3** Kauai.

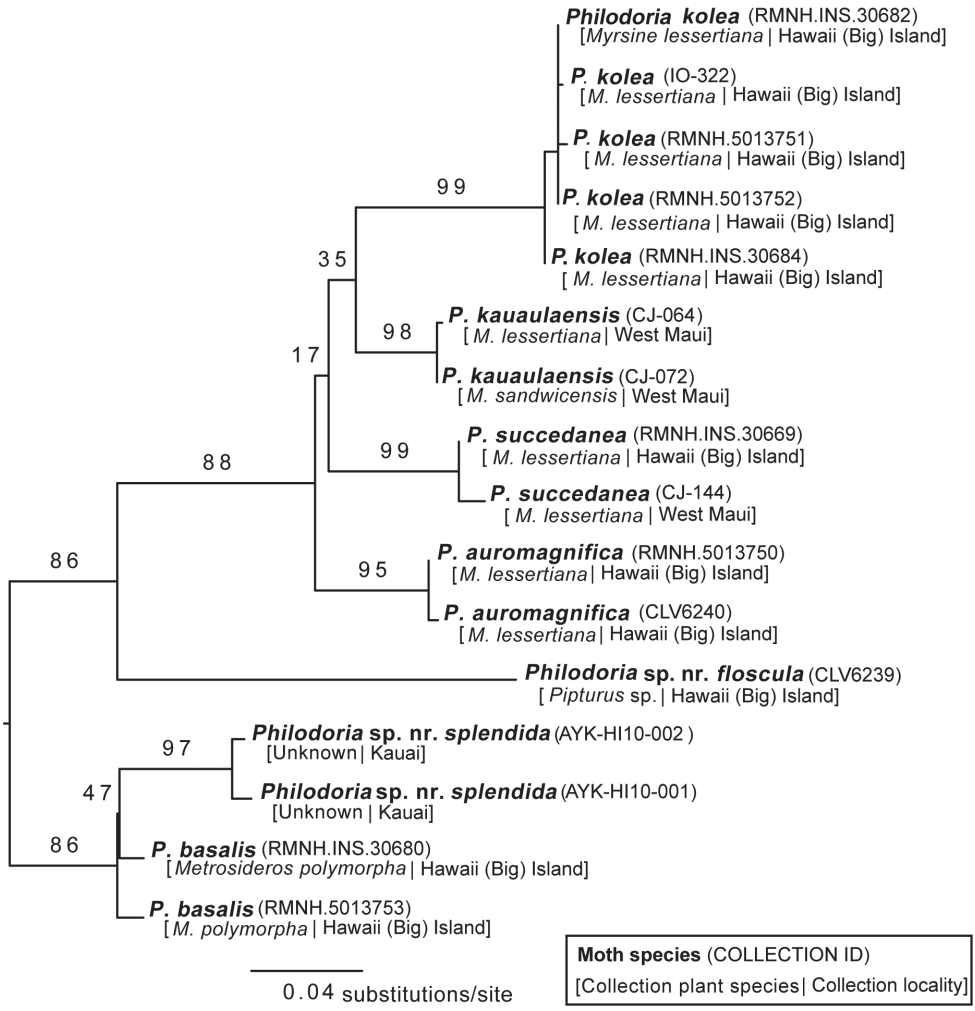


Figure 15. Maximum likelihood tree of *Philodoria* species based on the DNA barcoding region. Numbers on nodes indicate bootstrap support values. Collection ID, host plant, and collection locality, are also shown.

Female genitalia (Fig. 7I) (n = 5). Similar to *P. succedanea* and *P. auromagnifica*, but different in having two very small and narrow signa with minute spines.

Distribution. Hawaii (Big Island).

Host plants. Primulaceae: *Myrsine lessertiana* A. DC.

Biology. (Figs 12, 13, 14D). Larvae mine the adaxial side of leaves of *M. lessertiana*, forming a slender serpentine mine (Fig. 12A, B), and gradually expanding as they feed and grow forming a full-depth mine (Fig. 12G, L). Larvae consumed small amounts of leaf tissue (under 2 cm in leaf length) when feeding on seedlings (Fig. 13G, H). The young larva is about 1.5 mm long (Fig. 12I) and later instar larvae are 4–8 mm long (Fig. 12J, K). Larvae were collected from fresh leaves of seedlings. There was usually

only one mine per leaf (Fig. 12A, B, G, H). The pupal cocoon is prepared outside the mine, on either surface of the leaf; the cocoon is greyish white to ochreous and near ellipsoidal in shape (Fig. 12M); 4.0–5.0 mm in length, 1.0–3.0 mm in width.

DNA barcoding. BIN BOLD:ADF137. The five specimens sequenced for COI are from two localities on Hawaii and have maximum intraspecific p-distance of 0.17%. The p-distance to the nearest neighbor, *Philodoria kauaulaensis*, is 6.98%.

Etymology. The specific epithet, *kolea*, is a noun in apposition taken from the Hawaiian name of the host plant, *Myrsine*.

Molecular analysis

We obtained DNA barcode data for 16 individual specimens (http://dx.doi.org/10.5883/DS-PHDRIA). All species have their own unique cluster or Barcode Index Number (BIN) allowing their unequivocal identification (Table 2). The lowest interspecific distance (4.41%) was observed between *Philodoria* sp. nr. *splendida* and *P. basalis* (Table 3). Sequences of *Myrsine*-feeding *Philodoria* species, when compared pairwise, formed distinct clusters, with a maximum intraspecific divergence which varied between 0.17–0.88% and a NN distance which varied from 5.85–8.91% (Table 3). The minimum interspecific distance was smaller between *P. auromagnifica* and *P. kauaukaensis* (5.85%) than between *P. auromagnifica* and *P. succedanea* (6.71%) (Table 3). Identifying species using DNA barcodes appears to be useful for the *Myrsine*-feeding *Philodoria*. *Philodoria succedanea* belongs to BIN BOLD: ADF5435, *P. kauaulaensis* to BOLD: ADI5327, *P. auromagnifica* to BOLD: ADD6965, and *P. kolea* to BOLD: ADF7137.

Discussion

Hawaiian *Philodoria* leaf mining moths were extensively studied in the early 1910s–1940s by Otto Herman Swezey. However, little taxonomic work has been conducted since, and our investigation is revealing that several undescribed cryptic species remain to be discovered, as found in other Hawaiian micromoths (e.g., *Bedellia*, Bedelliidae: [Zimmerman 1978]; *Hyposmocoma*: Cosmopterigidae [Kawahara and Rubinoff 2012; Rubinoff 2008]). *Philodoria* is critically in need of taxonomic work considering the endemic distribution of its species on the Hawaiian islands, and the close association of the genus with native endemic and endangered host plants. Some host plants and their associated *Philodoria* have already become locally extinct (Johns et al. 2014).

Swezey collected *Myrsine*-feeding *P. succedanea* and *P. auromagnifica* from numerous localities on Oahu in the early 1900s. *Myrsine lessertiana* plants remain relatively abundant on Oahu, but *Myrsine*-mining *Philodoria* have become exceedingly difficult to find there, especially in the southeast where intense urban development has taken place over the last century. During our Oahu surveys, we were unable

to find leaf mines on *M. degeneri*, *M. fosbergii*, *M. juddii* (Critically Endangered, IUCN), *M. lanaiensis*, *M. pukooensis*, *M. punctata*, or *M. sandwicensis*, despite extensive searches for leaf mines on these host plants. It is not clear whether these absences are more due to environmental changes causing population reductions than to original host plant restriction among *Myrsine* species.

On Maui, *P. kauaulaensis* and *P. succedanea* were found in April–May 2013 at two sites separated only by 3.3 km, below the summit of Eke and on Haelaau Ridge, within the Pu'u Kukui Watershed Preserve (Fig. 2A; Johns et al 2016, fig. 1, Coll. ID CJ-064, CJ-072). In the present study, we observed *P. auromagnifica*, *P. kolea* and *P. succedanea* occurring in sympatry on April 2016 at the Hawai'i Volcanoes National Park, the island of Hawaii (Big Island) (Figs 2A, 6A–J, 8B, C, 9A–M).

We collected larvae of *P. auromagnifica* (Fig. 8B, C) on plants that were also used by *P. succedanea* (Fig. 6A). The latter species was still mining leaves from the same plants that had fallen to the ground. Larvae of *P. kolea* occurred on leaves that were intact on short (about 10–20 cm high) *Myrsine* plants at the same site (Figs 9A, B, 10D). The genetic similarity between these species could imply that perhaps competition and niche partitioning may have been the cause of speciation. Fine-scale niche partitioning has been documented in other gracillariids and their host plants, such as *Phyllocnistis* on *Persea* (Davis and Wagner 2011) and *Phyllocnistis* on *Salix* (Kobayashi et al. 2011). Our ongoing research efforts will examine the evolutionary history and colonization patterns of *Philodoria* on the Hawaiian archipelago.

In addition to providing morphological and molecular evidence to delimit species limits among the Hawaiian *Myrsine*-feeding *Philodoria*, we include a pictorial key to their leaf mines (Fig. 14). We include this information as leaf mining moths can be difficult to observe as larvae or adults to a non-specialist. Larvae of *P. succedanea* form red, long linear mines along the leaf vein (Fig. 14A), *P. kauaulaensis* produces at first spiral and later blotch mines (Fig. 14B), *P. auromagnifica* makes brown serpentine mines (Fig. 14C), and *P. kolea* creates complete serpentine mines fully occupying the adaxial side of leaf surface of *Myrsine* seedlings (Fig. 14D). We hope that local Hawaiian park rangers, naturalists, and educators can use this key as a means to identify these species, so that the collection of these much-needed data can persist.

It is likely that detailed molecular work among islands will reveal further cryptic species but native hostplants and habitats are under great threat.

Acknowledgements

We thank Cynthia King (Hawaii Department of Land and Natural Resources, Division of Forestry and Wildlife) for collecting and forest access permits, Rhonda Loa (Hawaii Volcanoes National Park) and Pat Bily (The Nature Conservancy) for land access, permitting assistance, logistical and field support, Daniel Rubinoff (University of Hawai'i at Mānoa) for conception of the project, and the participants and organizers of the leaf miner workshop conducted on Puna, HI in 2016. Klaus Sattler is thanked for extensive discussion about

material in the NHMUK. We also thank Rory Frampton (West Maui Land Company) for help with access to Kauaula and Natalia Tangalin (National Tropical Botanical Garden), Steve Perlman (Hawaii Plant Extinction Prevention Program), Lono Dunn, Daniel Tanaka, Joe Ward, Pomaikai Kanaiaupio-Crozier (Puu Kukui Watershed Preserve), Hank Oppenheimer (Hawaii Plant Extinction Prevention Program), Chris Brosius (West Maui Mountain Watershed Partnership), and Keahi M. Bustamente (Leeward Haleakala Watershed Restoration Project) for their help coordinating fieldwork and collection. Chris Hamilton (Florida Museum of Natural History) provided useful feedback on the text. The project was funded by the National Science Foundation (Graduate Research Fellowship to CAJ; DEB #1354585 to AYK), the National Geographic Society (#C283-14 to CAJ; #9686-15 to AYK), the Entomological Society of America (2014 SysEB Travel Award to CAJ), the University of Florida's Tropical Conservation and Development Program (2014 Field Research Grant to CAJ), the Florida Museum of Natural History (Lockhart Fellowship to CAJ), and an Society of Systematic Biologists SSB (2012 Mini-ARTS grant to AYK).

References

Breinholt JW, Earl C, Lemmon AR, Lemmon EM, Xiao L, Kawahara AY (2018) Resolving relationships among the megadiverse butterflies and moths with a novel pipeline for Anchored Phylogenomics. Systematic Biology 67(1): 78–93. https://doi.org/10.1093/sysbio/syx048

Brito R, Gonçalves GL, Vargas HA, Moreira GRP (2013) A new Brazilian *Passiflora* leafminer: *Spinivalva gaucha*, gen. n., sp. n. (Lepidoptera, Gracillariidae, Gracillariinae), the first gracillariid without a sap-feeding instar. ZooKeys 291: 1–26. https://doi.org/10.3897/zookeys.291.4910

Davis DR, De Prins J (2011) Systematics and biology of the new genus *Macrosaccus* with descriptions of two new species (Lepidoptera, Gracillariidae). ZooKeys 98: 29–82. https://doi.org/10.3897/zookeys.98.925

Davis DR, Wagner DL (2011) Biology and systematics of the New World *Phyllocnistis* Zeller-leafminers of the avocado genus *Persea* (Lepidoptera, Gracillariidae). ZooKeys 97: 39–73. https://doi.org/10.3897/zookeys.97.753

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA Barcodes: Analytical Protocols. In: Cristofre M (Ed.) Methods in Molecular Biology: Environmental Genomics. Humana Press Inc., Totowa, 275–293. https://doi.org/10.1007/978-1-59745-548-0_15

Evenhuis NL, Eldredge LG [Eds] (1999) Records of the Hawaii Biological Survey for 1998. Bishop Museum, Honolulu, 55 pp.

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genetics 23: 167–172. https://doi.org/10.1016/j.tig.2007.02.001

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society of London, Series B 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

ICZN (1999) International code of zoological nomenclature, fourth edition. London: International Trust for Zoological Nomenclature, London, 306 pp.

Johns CA, Tangalin N, Bustamente K, Kawahara AY (2014) Evidence of an undescribed, extinct *Philodoria* species (Lepidoptera: Gracillariidae) from Hawaiian *Hesperomannia* herbarium specimens. Proceedings of the Hawaiian Entomological Society 46: 55–57.

Johns CA, Moore MR, Kawahara AY (2016) Molecular phylogeny, revised higher classification, and implications for conservation of endangered Hawaiian leaf-mining moths (Lepidoptera: Gracillariidae: *Philodoria*). Pacific Science 70(3): 361–372. https://doi.org/10.2984/70.3.7

Kawahara AY, Nishida K, Davis DR (2009) Systematics, host plants, and life histories of three new *Phyllocnistis* species from the central highlands of Costa Rica (Lepidoptera, Gracillariidae, Phyllocnistinae). ZooKeys 27: 7–30. https://doi.org/10.3897/zookeys.27.250

Kawahara AY, Plotkin D, Ohshima I, Lopez-Vaamonde C, Houlihan P, Breinholt JW, Kawakita A, Xiao L, Regier JC, Davis DR, Kumata T, Sohn J-C, De Prins J, Mitter C (2017) A molecular phylogeny and revised higher-level classification for the leaf-mining moth family Gracillariidae and its implications for larval host-use evolution. Systematic Entomology 42: 60–81. https://doi.org/10.1111/syen.12210

Kawahara AY, Rubinoff D (2012) Three new species of Fancy Case caterpillars from threatened forests of Hawaii (Lepidoptera: Cosmopterigidae: *Hyposmocoma*). ZooKeys 170: 1–20. https://doi.org/10.3897/zookeys.170.1428

Kobayashi S, Sakamoto Y, Nakamura A, Jinbo U, Hirowatari T (2011) A new willow leaf blotch miner of the genus *Phyllocnistis* (Lepidoptera: Gracillariidae: Phyllocnistinae) from Japan, with pupal morphology and genetic comparison of Salicaceae mining species using DNA barcodes. Lepidoptera Science 62(2): 75–93.

Lees DC, Kawahara AY, Bouteleux O, Ohshima I, Kawakita A, Rougerie R, De Prins J, Lopez-Vaamonde C (2013) DNA barcoding reveals a largely unknown fauna of Gracillariidae leaf-mining moths in the Neotropics. Molecular Ecology Resources 14(2): 286–296. https://doi.org/10.1111/1755-0998.12178

Moreira GRP, Pollo P, Brito R, Gonçalves GL, Vargas HA (2017) *Cactivalva nebularia*, gen. et sp. nov. (Lepidoptera: Gracillariidae): a new *Weinmannia* leaf miner from southern Brazil. Austral Entomology 2017: 1–15. https://doi.org/10.1111/aen.12267

Rambaut A (2009) FigTree version 1.4.3. Institute of Evolutionary Biology, University of Edinburgh. http://tree.bio.ed.ac.uk/software/figtree/

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2006.01678.x

Ratnasingham S, Hebert PDN (2013) A DNA-based registry for all animal species: the barcode index number (BIN) system. PLoS ONE 8 (8): e66213. https://doi.org/10.1371/journal.pone.0066213

Rubinoff D (2008) Phylogeography and ecology of an endemic radiation of Hawaiian aquatic case-bearing moths (*Hyposmocoma*: Cosmopterigidae). Philosophical Transactions of the Royal Society B: Biological Sciences 363(1508): 3459–3465. https://doi.org/10.1098/rstb.2008.0115

Rubinoff D, Holland BS (2005) Between two extremes: Mitochondrial DNA is neither the Panacea nor the nemesis of phylogenetic and taxonomic inference. Systematic Biology 54: 952–961. https://doi.org/10.1080/10635150500234674

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and postanalysis of large phylogenies. Bioinformatics 30: 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Swezey OH (1910) Some new species of Hawaiian Lepidoptera. Proceedings of the Hawaiian Entomological Society 2(3): 106.

Swezey OH (1913a) A day's collecting at Punaluu, Oahu. Proceedings of the Hawaiian Entomological Society 2(5): 197–199.

Swezey OH (1913b) Leaf-miners of the Hawaiian Islands. Proceedings of the Hawaiian Entomological Society 2(5): 221–227.

Swezey OH (1913c) One new genus and eighteen new species of Hawaiian moths. Proceedings of the Hawaiian Entomological Society 2(5): 269–280.

Swezey OH (1915) New species of Hawaiian moths. Proceedings of the Hawaiian Entomological Society 3(2): 94–97.

Swezey OH (1920) Some new Hawaiian Lepidoptera. Proceedings of the Hawaiian Entomological Society 4(2): 376–386.

Swezey OH (1923) The leaf-miners of *Pipturus*. Proceedings of the Hawaiian Entomological Society 5(2): 293–296.

Swezey OH (1928) Some new species of lepidopterous leaf-miners in Hawaii. Proceedings of the Hawaiian Entomological Society 7(1): 187–191.

Swezey OH, Bryan EH (1929) Further notes on the forest insects of Molokai. Proceedings of the Hawaiian Entomological Society 7(3): 293–314.

Swezey OH (1934) New species of Hawaiian Lepidoptera. Proceedings of the Hawaiian Entomological Society 8(3): 523–525.

Swezey OH (1940) New species of Hawaiian Lepidoptera. Proceeding Hawaiian Entomological Society 10(3): 461–465.

Swezey OH (1946) New species of Hawaiian Lepidoptera. Proceedings of the Hawaiian Entomological Society 12(3): 625–628.

Swezey OH (1954) Forest entomology in Hawaii. An annotated check-list of the insect faunas of the various components of the Hawaiian forests. Bishop Museum Special Publication 44, 266 pp.

Wagner WL, Herbst DR, Sohmer SH (1999) Manual of the Flowering Plants of Hawai'i, Volume 1, University of Hawai'i and Bishop Museum Press, Honolulu, 943 pp.

Walsingham Lord, Thomas de Grey (1907) Microlepidoptera. In: Sharp D (Ed.) Fauna Hawaiiensis volume 1(5), Cambridge University Press, United Kingdom, 469–759.

Zimmerman EC (1978) Gracillarioidea. Microlepidoptera, Part 1. Insects of Hawaii, vol 9, University of Hawaii Press, Honolulu, 644–718.

ZooKeys 773: 143–154 (2018)
doi: 10.3897/zookeys.773.25402
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

Parasitic wasps related to *Prays oleae* (Bernard, 1788) (Lepidoptera, Praydidae) in olive orchards in Greece

Eleftherios Alissandrakis[1], Panagiota Psirofonia[1], Nickolas G. Kavallieratos[2], Saša S. Stanković[3], Vladimir Žikić[3]

1 *Laboratory of Entomology and Pesticide Science, Department of Agriculture, Technological Educational Institute of Crete, P.O. Box 1939, 71004, Heraklion, Crete, Greece* **2** *Laboratory of Agricultural Zoology and Entomology, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos str., 11855, Athens, Attica, Greece* **3** *Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000, Niš, Serbia*

Corresponding author: *Nickolas G. Kavallieratos* (nick_kaval@aua.gr)

Academic editor: *K. van Achterberg* | Received 1 April 2018 | Accepted 8 June 2018 | Published 9 July 2018

http://zoobank.org/B39CFE4B-29D4-4EED-9D32-8EA0AC59E251

Citation: Alissandrakis E, Psirofonia P, Kavallieratos NG, Stanković SS, Žikić V (2018) Parasitic wasps related to *Prays oleae* (Bernard, 1788) (Lepidoptera: Praydidae) in olive orchards in Greece. ZooKeys 773: 143–154. https://doi.org/10.3897/zookeys.773.25402

Abstract

The olive moth, *Prays oleae* (Bernard, 1788) (Lepidoptera: Praydidae) is categorised among the most devastating insect pests of olives, whose anthophagous and carpophagous generations can cause yield loss up to 581 and 846 kg of fruit per ha, respectively. In this study, results of the captured parasitoids in olive tree (*Olea europaea* Linnaeus, 1753) orchards, or infested olive plant material in Crete, Greece, is presented. Five of the six identified species captured in trap devices are related to *P. oleae*, i.e., *Chelonus elaeaphilus* Silvestri, 1908, *Chelonus pellucens* (Nees, 1816), *Apanteles xanthostigma* (Haliday, 1834), *Diadegma armillatum* (Gravenhorst, 1829), and *Exochus lentipes* Gravenhorst, 1829. The species *Eupelmus urozonus* Dalman, 1820 and *Pnigalio mediterraneus* Ferrière & Delucchi, 1957 were reared from infested *P. oleae* leaves. *Chelonus pellucens* is reported for the first time from Greece. According to the international literature, 59 hymenopterous and dipterous parasitoid species are associated with *P. oleae* in Europe.

Keywords

Diptera, Greece, Hymenoptera, parasitoids, *Prays oleae*

Introduction

Olive trees growing has been traditionally localised in the Mediterranean Basin for thousands of years, where almost 97.9% of the cultivated areas are located (Rallo et al. 2018). The list of potentially harmful organisms includes more than 255 species and the losses due to insect pests alone are estimated to be approximately 15% of production (Haniotakis 2003). Among them, the most common species are the olive fruit fly, *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae), the olive moth, *Prays oleae* (Bernard, 1788) (Lepidoptera: Praydidae), and the Mediterranean black scale, *Saissetia oleae* (Olivier, 1791) (Hemiptera: Coccidae) (Haniotakis 2003).

Prays oleae is one of the main pests infesting olives of commercial production, since larvae of the first, second, and third generations attack flowers, fruits, and leaves, respectively (Kavallieratos et al. 2005; Nave et al. 2017). The anthophagous generation can cause yield losses up to 581 kg of fruit per ha and the corresponding carpophagous up to 846 kg per ha, an issue that justifies the imposed control measures (Bento et al. 2001). In recent years, high socioeconomic pressures have forced olive growers to develop alternative control strategies in an effort to mitigate the undesirable side effects of pesticides on trophic chains and biological balances (Nave et al. 2017). In this sense, not only the economic losses due to the pest should be evaluated, but also the possible secondary effects that such control measures can have on beneficial fauna (Ramos et al. 1998).

Previous research has revealed a wide parasitoid spectrum that is related to *P. oleae*, resulting to biological control efforts against this pest. The first parasitoid used in biocontrol program was *Trichogramma embryophagum* (Hartig, 1838) (Hymenoptera: Trichogrammatidae) in former Yugoslavia (Brnetić 1988). In Spain, three species have been released against *P. oleae* with various levels of success; i.e., *Chelonus elaeaphilus* Silvestri, 1908 (Hymenoptera: Braconidae), the specialised *Ageniaspis fuscicollis* (Dalman, 1820) var. *praysincola* Silvestri, 1907 (Hymenoptera: Encyrtidae) and *T. embryophagum* (Civantos and Caballero 1993). *Trichogramma cacaeciae* Marchal, 1927 (Hymenoptera: Trichogrammatidae) has been utilised in Portugal (Bento et al. 1998) and *Trichogramma evanescens* Westwood, 1833 in Egypt (Agamy 2010).

Although there are previous records concerning the occurrence of *P. oleae* parasitoids in Greece, there are no data available from the island of Crete, the most important olive production area with almost 200,000 ha cultivated with olive trees (i.e., nearly 25% of the total island area is covered with olive plantations; Hellenic Statistical Authority 2014). Given that the knowledge of the beneficial entomofauna of the olive crop is clearly linked with the biological control of pests infesting this crop and that indigenous strains of parasitoids occurring in olive groves can be more effective against certain olive pests than the commercially available parasitoids (Herz and Hassan 2006), the objective of this study was to further investigate the parasitoid complex that is associated with *P. oleae* in the overlooked area of Crete by using trap devices and collecting plant material.

Materials and methods

All parasitoids were collected in olive orchards from the island of Crete, Greece from June to October 2017. A part of the material was captured in five glass McPhail trap devices, installed from June to October in an olive orchard at Messara (Crete) that covers an area of approx. 0.5 ha baited with 200 ml aqueous solution of 2% hydrolysed protein (Entomela 75 SL, 25% w/w urea; BASF Hellas, Amaroussion, Greece). Each trap device was placed with its lower part at a height of 2 m from the ground. The distances among trap devices were approx. 100 m. The solution was replaced every week. Additional specimens were reared from *P. oleae* infested plant material (*O. europaea* var. *koroneiki*). Infested leaves by *P. oleae* larvae were collected from olive trees, separately transferred into plastic vials covered with mesh, and transferred to the laboratory. Vials were maintained at 25 °C and 60% relative humidity and inspected daily for emergence of parasitoids. All parasitoid individuals, either from trap devices or plant material, were preserved in 96% alcohol. Specimens were dissected and slide mounted in Berlese medium. The identification of the captured and reared specimens was conducted under a Nikon SM2 745T binocular stereomicroscope (Nikon CEE GmbH, Wien, Austria) or an Olympus SZX9 (Olympus Corporation, Tokyo, Japan) using appropriate keys (Tobias et al. 1986; Askew and Nieves Aldrey 2000; Tolkanitz 2007; Broad 2011). Part of the specimens was deposited in the insect collection of the Laboratory of Agricultural Zoology of Entomology, Agricultural University of Athens, Greece, and a part was deposited in the insect collection of the Faculty of Sciences and Mathematics, Department of Biology and Ecology, University of Niš, Serbia.

Additional to field research, we critically reviewed all recorded parasitoids of *P. oleae* in Greece and Europe indicating the pest's stage they attack. The synonymy among taxa was checked and adopted according to online databases (van Achterberg 2013; Fernandez Triana and Ward 2015; Noyes 2017; Tschorsnig 2017), and the database provided by Yu et al. (2012).

Results

In total, five out of six species captured in McPhail trap devices are related to *P. oleae*, i.e., *C. elaeaphilus*, *Chelonus pellucens* (Nees, 1816) (Hymenoptera: Braconidae), *Apanteles xanthostigma* (Haliday, 1834) (Hymenoptera: Braconidae), *Diadegma armillatum* (Gravenhorst, 1829) (Hymenoptera: Ichneumonidae), and *Exochus lentipes* Gravenhorst, 1829 (Hymenoptera: Ichneumonidae), while two species were reared from *P. oleae* infested olive leaves.

The exhaustive investigation of the international literature revealed 59 hymenopterous and dipterous parasitoid species that attack *P. oleae* in Europe; 14 Braconidae, 2 Chalcididae, 1 Encyrtidae, 20 Eulophidae, 1 Eupelmidae, 7 Icheumonidae, 1 Platygastridae, 3 Pteromalidae, 2 Tachinidae, and 8 Trichogrammatidae (Table 1). Thirty-one out of

Table 1. Parasitoids of *Prays oleae* recorded in Europe and their presence in Greece: (+) recorded, (-) not recorded.

Family	Species	Source of host record	Host stage attacked	Recorded or not in Greece
Braconidae	*Aleiodes circumscriptus* (Nees, 1834)	Beyarslan (2015)	larva	+
	Aleiodes gastritor (Thunberg, 1822)	Halperin (1986)	larva	+
	Apanteles xanthostigma (Haliday, 1834)	Nave et al. (2016)	larva	+
	Bracon hebetor Say, 1836	Falcó et al. (1993)	larva	+
	Bracon laetus (Wesmael, 1838)	Aubert (1966)	larva	+
	Bracon crassicornis Thomson, 1892	Silvestri (1908)	larva	+
	Chelonus (*Microchelonus*) *elaeaphilus* Silvestri, 1908	Nave et al. (2016)	larva	+
	Chelonus (Microchelonus) silvestrii (Papp, 1999)	Papp (1999)	larva	-
	Chelonus (*Parachelonus) pellucens* (Nees, 1816)	Texeira et al. (2000)	larva	-
	Clinocentrus testaceus (Kriechbaumer, 1894)	Texeira et al. (2000)	larva	-
	Dolichogenidea dilecta (Haliday, 1834)	Telenga (1955)	larva	-
	Dolichogenidea ultor (Reinhard, 1880)	Arambourg (1969)	larva	-
	Meteorus rubens (Nees, 1811)	Texeira et al. (2000)	larva	+
	Phanerotoma dentata (Panzer, 1805)	Texeira et al. (2000)	larva	+
Chalcididae	*Hockeria bifasciata* Walker, 1834	Madl (2008)	larva	-
	Hockeria unicolor Walker, 1834	Stavraki (1977)	larva	+
Encyrtidae	*Ageniaspis fuscicollis* (Dalman, 1820) var. *praysincola* Silvestri, 1907	Nave et al. (2016)	larva	+
Eulophidae	*Asecodes erxias* (Walker, 1848)	Silvestri (1908)	larva	+
	Baryscapus nigroviolaceus (Nees, 1834)	Noyes (2017)	larva	-
	Chrysocharis gemma (Walker, 1839)	Noyes (2017)	larva	+
	Chrysocharis nephereus (Walker, 1839)	Noyes (2017)	larva	+
	Cirrospilus elongatus Boucek, 1959	Noyes (2017)	larva	-
	Dicladocerus westwoodii Westwood, 1832	Ramos and Panis (1975)	larva	+
	Elasmus arcuatus Ferrière, 1947	Ferrière (1947)	larva	-
	Elasmus flabellatus (Fonscolombe, 1832)	Nave et al. (2016)	larva	+
	Elasmus masii Ferrière, 1929	Anonymous (2006)	larva	-
	Elasmus nudus (Nees, 1834)	Ramos and Panis (1975)	larva	-
	Elasmus steffani Viggiani, 1967	Redolfi and Campos (2010)	larva	+
	Elasmus westwoodi Giraud, 1856	Noyes (2017)	larva	+
	Euderus albitarsis (Zetterstedt, 1838)	Nave et al. (2016)	larva	-
	Hemiptarsenus unguicellus (Zetterstedt, 1838)	Noyes (2017)	larva	-
	Pediobius bruchicida (Rondani, 1872)	Bouček (1974)	larva/ pupa	+
	Pnigalio agraules (Walker, 1839)	Nave et al. (2016)	larva/ pupa	+
	Pnigalio epilobii Bouchek, 1966	Stavraki (1970)	larva/ pupa	+
	Pnigalio longulus (Zetterstedt, 1838)	Stavraki (1970)	larva/ pupa	+
	Pnigalio mediterraneus Ferrière & Delucchi, 1957	Stavraki (1970)	larva/ pupa	+
	Pnigalio pectinicornis (Linnaeus, 1758)	Ramos and Panis (1975)	larva/ pupa	+
Eupelmidae	*Eupelmus urozonus* Dalman, 1820	Noyes (2017)	larva	+
Ichneumonidae	*Diadegma armillatum* (Gravenhorst, 1829)	Bento et al. (1998)	larva/ pupa	+

Family	Species	Source of host record	Host stage attacked	Recorded or not in Greece
Ichneumonidae	*Diadegma semiclausum* (Hellén, 1949)	Torres (2010)	larva/ pupa	+
	Exochus lentipes Gravenhorst, 1829	Texeira et al. (2000)	larva	-
	Himertosoma superbum Schmiedeknecht, 1900	Vidal (1997)	larva/ pupa	-
	Itoplectis alternans (Gravenhorst, 1829)	Silvestri (1908)	larva/ pupa	-
	Lissonota superbator Aubert, 1967	Aubert (1969)	larva	+
	Scambus elegans (Woldstedt, 1877)	Nave et al. (2017)	larva	-
Platygastridae	*Platygaster apicalis* Thomson, 1859	Stavraki (1970)	larva	+
Pteromalidae	*Mesopolobus mediterraneus* (Mayr, 1903)	Bozbuğa and Elekçioğlu (2008)	pupa	-
	Pteromalus chrysos Walker, 1836	Noyes (2017)	pupa	-
	Pteromalus semotus Walker, 1834	Noyes (2017)	pupa	-
Tachinidae	*Phytomyptera nigrina* (Meigen, 1824)	Kara and Tschorsnig (2003)	larva	-
	Phytomyptera vaccinii Sintenis, 1897	Tschorsnig (2017)	larva	-
Trichogrammatidae	*Trichogramma bourarachae* Pintureau & Babault, 1988	Polaszek (2009)	egg	-
	Trichogramma brassicae Bezdenko, 1968	Polaszek (2009)	egg	-
	Trichogramma cordubensis Vargas & Cabello, 1985	Jardak (1980)	egg	-
	Trichogramma dendrolimi Matsumura, 1926	Polaszek (2009)	egg	-
	Trichogramma euproctidis (Girault, 1911)	Pereira et al. (2004)	egg	+
	Trichogramma minutum Riley, 1871	Stavraki (1985)	egg	-
	Trichogramma oleae Voegele & Pointel, 1979	Polaszek (2009)	egg	+
	Trichogramma pretiosum Riley, 1879	Polaszek (2009)	egg	-

these 59 parasitoid species have been recorded in Greece: 9 Braconidae, 1 Chalcididae, 1 Encyrtidae, 13 Eulophidae, 1 Eupelmidae, 3 Icheumonidae, 1 Platygastridae, and 2 Trichogrammatidae. All Braconidae, Chalcididae, Encyrtidae, Eupelmidae, Platygastridae, and Tachinidae which are parasitoids of *P. oleae* attack only larvae. All eulophids parasitise larvae of *P. oleae* while some of them attack both larvae and pupae. Three icheumonids parasitise larvae exclusively and four both larvae and pupae. All pteromalids are pupal parasitoids whilst all trichogrammatids are egg parasitoids.

Family Braconidae

***Apanteles xanthostigma* (Haliday, 1834)**
Material examined: 11 ♀, Messara (Crete) (35°2'20"N, 24°50'54"E), 16–23.06.2017, captured in McPhail trap device.

***Chelonus* (*Microchelonus*) *elaeaphilus* (Silvestri, 1907)**
Material examined: 4 ♀, 4 ♂, Messara (Crete) (35°2'20"N, 24°50'54"E), 09–16.06.2017, captured in McPhail trap device.

***Chelonus* (*Parachelonus*) *pellucens* (Nees, 1816)**
Material examined: 6 ♀, Messara (Crete) (35°2'20"N, 24°50'54"E), 09–16.06.2017, captured in McPhail trap device.

***Glyptapanteles vitripennis* (Curtis, 1830)**
Material examined: 2 ♀, 7 ♂, Messara (Crete) (35°2'20"N, 24°50'54"E), 23–30.06.2017, captured in McPhail trap device.

Family Eulophidae

***Pnigalio mediterraneus* Ferrière & Delucchi, 1957**
Material examined: 12 ♂, Heraklion, Voutes, (Crete) (35°15'54"N, 25°03'26"E), 15.03.2017 (date of host collection). Host: *Prays oleae* on *Olea europaea* var. *koroneiki*.

Family Eupelmidae

***Eupelmus urozonus* Dalman, 1820**
Material examined: 8 ♀, 12 ♂, Heraklion, Voutes, (Crete) (35°15'54"N, 25°03'26"E), 15.03.2017 (date of host collection). Host: *Prays oleae* on *Olea europaea* var. *koroneiki*.

Family Ichneumonidae

***Diadegma armillatum* (Gravenhorst, 1829)**
Material examined: 3 ♀, 5 ♂, Messara (Crete) (35°2'20"N, 24°50'54"E), 16–23.08.2017, captured in McPhail trap device.

***Exochus lentipes* Gravenhorst, 1829**
Material examined: 6 ♀, 8 ♂, Messara (Crete) (35°2'20"N, 24°50'54"E), 16–23.09.2017, captured in McPhail trap device.

Discussion

Microgastrinae is one of the largest subfamilies of Braconidae with about 2,000 described species worldwide (Pérez Rodríguez et al. 2013). Very recently, the hymenopteran parasitoid complex of *P. oleae* was studied in Portugal where, among the 22 recorded parasitoid taxa, *A. xanthostigma* was the major natural enemy (Nave et al. 2017). Furthermore, in Egypt *A. xanthostigma* was found to parasitise the larval stage of *P. oleae* at a rate of more than 50% (Herz et al. 2005). Apart from *P. oleae*, this parasitoid species parasitises a high number of microlepidopterous species, mainly Tortricidae, Gracillariidae, and Yponomeutidae, particularly the genera *Paraswammerdamia* Friese, 1960 and *Swammerdamia* Hübner, 1825 (Yu et al. 2012). *Glyptapanteles* Ash-

mead, 1904 is a genus with about 200 species in Palaearctic and Nearctic regions and, like all Microgastrinae, are koinobiont endoparasitoids of lepidopteran larvae (Penteado Dias et al. 2011). *Glyptapanteles vitripennis* was first reported in southern Greece in 1978 (Papp 2007) without further records since then. This parasitoid species was the second most abundant recovered from Malaise traps placed in the Artikutza forest of Pyrenees (Spain) (Pérez Rodríguez et al. 2013) while it is also known that it attacks *Yponomeuta malinellus* (Zeller, 1838) (Lepidoptera: Yponomeutidae) (Velcheva et al. 2012). Given that this species parasitises numerous other lepidopterous species belonging to Geometridae, Noctuidae, Plutellidae, and Tortricidae (Nixon 1973), it could be a good candidate for biological control purposes. Whether *G. vitripennis* parasitises *P. oleae*, it remains to be confirmed with additional field efforts.

The subfamily Cheloninae is formed by more than 1,300 species belonging to 15 genera, thus constituting a quite large part of Braconidae (Kittel and Austin 2014). They oviposit into eggs and larvae of various lepidopterous species, a fact that makes them valuable potential biocontrol agents (Inayatullah and Naeem 2004; Walker and Huddleston 1987; Edmardash et al. 2011). The subgenus *Microchelonus* Szepligeti, 1908 is even considered as a valid genus, following the standpoints of Papp (2014a, b). The genus *Chelonus* Panzer, 1806 counts 601 species in the Holarctic region (Papp 2014c) with *M. elaeaphilus* being known in the Mediterranean region, either as *M. elaeaphilus* or *C. elaeaphilus* (Papp 2012; Nave et al. 2017). This species has been introduced and established in Greece from France (Yamvrias 1998). On the other hand, *C. pellucens* has a wider European distribution than *M. elaeaphilus* (van Achterberg 2013). *Chelonus pellucens* is reported for the first time from Greece and although *C. elaeaphilus* parasitises *P. oleae* (Bento et al. 1998), there are no relevant records for *C. pellucens*, an issue that merits further investigation.

Although Eupelmidae is a relatively small family with approximately 1000 species, the genus *Eupelmus* Dalman, 1820 is a large taxon containing more than 300 species (Gibson and Fusu 2016) whilst Eulophidae is one of the largest families within chalcidoid wasps, with almost 5,000 species (Aguiar et al. 2013). The genus *Pnigalio* Schrank, 1802 is comprised by 61 valid species (Li et al. 2017). Several hosts of *Pnigalio mediterraneus* Ferrière & Delucchi, 1957 (Hymenoptera: Eulophidae) are major pests of plants of ornamental and agricultural importance belonging to different orders, such as *B. oleae*, *Phyllocnistis citrella* Stainton, 1856 (Lepidoptera: Gracillariidae), and *Cameraria ohridella* Deschka & Dimić, 1986 (Lepidoptera: Gracillariidae) (Gebiola et al. 2009). Both *Eupelmus urozonus* Dalman, 1820 (Hymenoptera: Eupelmidae) and *P. mediterraneus* were found in the Greek island of Corfu as primary parasitoids of *B. oleae* (Kapatos and Fletcher 1986). Based on our results, these species are also parasitoids of *P. oleae* that occur in Greece since they were recorded from infested olive leaves.

The genus *Diadegma* Förster, 1869 constitutes a large group of Ichneumonid wasps with more than 200 known species worldwide (Wagener et al. 2006). *Diadegma armillatum* is a known parasitoid of various lepidopterous species (Velcheva et al. 2012; Fernandez Triana et al. 2014) that has been recently recorded attacking *P. oleae* larvae (Nave et al. 2017). The genus *Exochus* Gravenhorst, 1829 is the largest group of

Metopiinae including the widely distributed in Europe, *E. lentipes* that attacks various Tortricidae and Gelechiidae larvae (Yu et al. 2012).

Our original findings on associated parasitoids of *P. oleae* and the compiled information revealed could trigger further studies that deal with the management of this noxious insect species in the target area from a biological control point of view. The identified parasitoid spectrum was broad, despite the short interval of obtaining the data, indicating a potential positive impact of natural enemies to *P. oleae*, an issue however that merits further field efforts. Last but not least, given that *C. pellucens* is identified as a new member of the entomofauna of Greece during the present first attempt to record the beneficial parasitoids in olive orchards in Crete, we may expect that additional parasitoid species may occur in this agroecosystem.

Acknowledgments

We thank Martin Schwarz (Biologiezentrum, Linz, Austria) for consultation on the identification of *Diadegma* specimens.

References

Agamy E (2010) Field evaluation of the egg parasitoid, *Trichogramma evanescens* West. against the olive moth *Prays oleae* (Bern.) in Egypt. Journal of Pest Science 83: 53–58. https://doi.org/10.1007/s10340-009-0273-x

Aguiar AP, Deans AR, Engel MS, Forshage M, Huber JT, Jennings JT, Johnson NF, Lelej AS, Longino JT, Lohrmann V, Mikó I, Ohl M, Rasmussen C, Taeger A, Yu DSK (2013) Order Hymenoptera. Zootaxa 3703: 51–62. https://doi.org/10.11646/zootaxa.3703.1.12

Anonymous (2006) *Prays* del olivo (*Prays oleae*). Boletín Phytosanitario 6: 1–16.

Arambourg Y (1969) Inventaire de la biocœnose parasitaire de *Prays oleae* dans le bassin méditerranéen. Entomophaga 14: 185–194. https://doi.org/10.1007/BF02371159

Askew RR, Nieves Aldrey JL (2000) The genus *Eupelmus* Dalman, 1820 (Hymenoptera: Chalcidoidea: Eupelmidae) in peninsular Spain and the Canary Islands, with taxonomic notes and descriptions of new species. Graellsia 56: 49–61. https://doi.org/10.3989/graellsia.2000.v56.i0.309

Aubert JF (1966) Liste d'identification No. 6 (présentée par le service d'identification des Entomophages). Entomophaga 11: 115–134. https://doi.org/10.1007/BF02371463

Aubert JF (1969) Les Ichneumonides ouest-paléarctiques et leurs hôtes, Volume I Pimplinae, Xoridinae, Acaenitinae. Éditions Quatrefeuilles, Paris, 302 pp.

Bento A, Torres LM, Lopes J (2001) Avaliação de prejuízos causados pela traça da oliveira, *Prays oleae* (Bern.) em Trás-os-Montes. Revista de Ciências Agrárias 24: 89–96.

Bento A, Ilideo J, Campos M, Torres L (1998) Parasitismo associado à traça da oliveira *Prays oleae* Bern. em Trás-os-Montes (Nordeste de Portugal). Boletín de Sanidad Vegetal Plagas 24: 949–954.

Beyarslan A (2015) Taxonomic survey on the Rogadinae Förster, 1862 (Hymenoptera: Braconidae) in the northeastern Anatolian region, Turkey. Turkish Journal of Zoology 39: 811–819. https://10.3906/zoo-1407-35

Bouček Z (1974) On the Chalcidoidea (Hymenoptera) described by C. Rondani. Redia 55: 241–285.

Bozbuğa R, Elekçioğlu Z (2008) Pests and natural enemies determined in olive orchards in Turkey. Türk Bilimsel Derlemeler Dergisi 1: 87–97.

Brnetić D (1988) Biological control of the olive pests in the Yugoslav littoral. Olea 19: 92–93.

Broad G (2011) Identification key to the subfamilies of Ichneumonidae (Hymenoptera). Natural History Museum, London, 40 pp.

Civantos M, Caballero JM (1993) Integrated pest management in olive in the Mediterranean area. EPPO Bulletin 23: 367–375. https://doi.org/10.1111/j.1365-2338.1993.tb01338.x

Edmardash YAE, Abdel Dayem MS, Gadallah NS (2011) The subfamily Cheloninae (Hymenoptera: Braconidae) from Egypt, with the description of two new species. ZooKeys 115: 85–102. https://doi.org/10.3897/zookeys.115.1186

Falcó JV, Monero J, Jiménez R (1993) Datos sobre Ciclostominos ibéricos. I. Braconinae (Hymenoptera: Braconidae). Boletin de la Associacion Espanola de Entomologia 17: 71–90.

Fernandez Triana J, Ward D (2015) Microgastrinae wasps of the world. http://microgastrinae.myspecies.info/ [accessed on 3 January 2018]

Fernandez Triana J, Shaw MR, Cardinal S, Mason PG (2014) Contributions to the study of the Holarctic fauna of Microgastrinae (Hymenoptera: Braconidae). I. Introduction and first results of transatlantic comparisons. Journal of Hymenoptera Research 37: 61–76. https://doi.org/10.3897/jhr.37.7186

Ferrière C (1947) Les espèces européennes du genre *Elasmus* Westw. (Hym. Chalc.). Mitteilungen der Schweizerischen Entomologischen Gesellschaft 20: 565–580.

Gebiola M, Bernardo U, Monti MM, Navone P, Viggiani G (2009) *Pnigalio agraules* (Walker) and *Pnigalio mediterraneus* Ferrière and Delucchi (Hymenoptera: Eulophidae): two closely related valid species. Journal of Natural History 43: 2465–2480. https://doi.org/10.1080/00222930903105088

Gibson GAP, Fusu L (2016) Revision of the Palaearctic species of *Eupelmus* (*Eupelmus*) Dalman (Hymenoptera: Chalcidoidea: Eupelmidae). Zootaxa 4081: 1–331. https://doi.org/10.11646/zootaxa.4081.1.1

Halperin J (1986) Braconidae (Hymenoptera) associated with forest and ornamental trees and shrubs in Israel. Phytoparasitica 14: 119–135. https://doi.org/10.1007/BF02980898

Haniotakis GE (2003) Olive pest control: present status and prospects. IOBC Bulletin 28: 1–9.

Hellenic Statistical Authority (2014) Hellenic Statistical Authority. http://www.statistics.gr/en/home/ [accessed on 26 March 2018]

Herz A, Hassan SA (2006) Are indigenous strains of *Trichogramma* sp. (Hymenoptera: Trichogrammatidae) better candidates for biological control of lepidopterous pests of the olive tree? Biocontrol Science and Technology 16: 841–857. https://doi.org/10.1080/09583150600827751

Herz A, Hassan SA, Hegazi E, Khafagi WE, Nasr FN, Youssef AA, Agamy E, Jardak T, Ksantini M, Mazomenos BE, Konstantopoulou MA, Torres L, Gonçalves F, Bento A, Pereira JA

(2005) Towards sustainable control of lepidopterous pests in olive cultivation. Gesunde Pflanzen 58: 117–128. https://doi.org/10.1007/s10343-005-0076-9

Inayatullah M, Naeem M (2004) An identification key to genera of Cheloninae (Braconidae: Hymenoptera) in the NWFP with new distributional records and taxonomic notes. Sarhad Journal of Agriculture 20: 143–147.

Jardak T (1980) Etudes bioécologiques de *Prays oleae* BERN (Lepidoptera: Hyponomeutidae) et de ses parasites oophages du genre *Trichogramma* (Hymenoptera: Trichogrammatidae): essais d'utilisation en lutte biologique. Thèse de 3éme cycle, Université d'Aix Marseille, Marseille, 42 pp.

Kapatos ET, Fletcher BS (1986) Mortality factors and life-budgets for immature stages of the olive fly, *Dacus oleae* (Gmel.) (Diptera: Tephritidae), in Corfu. Journal of Applied Entomology 102: 326–342. https://doi.org/10.1111/j.1439-0418.1986.tb00931.x

Kara K, Tschorsnig HP (2003) Host catalogue for the Turkish Tachinidae (Diptera). Journal of Applied Entomology 127: 465–476. https://doi.org/10.1046/j.0931-2048.2003.00786.x

Kavallieratos NG, Athanassiou, CG, Balotis GN, Tatsi GTh, Mazomenos BE (2005) Factors affecting male *Prays oleae* (Lepidoptera: Yponomeutidae) captures in pheromone-baited traps in olive orchards. Journal of Economic Entomology 98: 1499–1505. https://doi.org/10.1603/0022-0493-98.5.1499

Kittel RN, Austin AD (2014) Synopsis of Australian chelonine wasps (Hymenoptera: Braconidae: Cheloninae) with description of two new genera. Austral Entomology 53: 183–202. https://doi.org/10.1111/aen.12070

Li T, Yang ZQ, Sun SP, Wang R (2017) A new species of *Pnigalio* (Hymenoptera: Eulophidae) parasitizing *Eriocrana semipurpurea alpina* (Lepidoptera: Eriocraniidae) in China, with its biology and a key to Chinese known species. ZooKeys 687: 149–159. https://doi.org/10.3897/zookeys.687.14903

Madl M (2008) Zur Kenntnis der Familie Chalcididae (Hymenoptera: Chalcidoidea) in Österreich. Entomofauna 29: 69–80.

Nave A, Gonçalves F, Crespí AL, Campos M, Torres L (2016) Evaluation of native plant flower characteristics for conservation biological control of *Prays oleae*. Bulletin of Entomological Research 106: 249–257. https://doi.org/10.1017/S0007485315001091

Nave A, Gonçalves F, Teixeira R, Amaro Costa C, Campos M, Torres L (2017) Hymenoptera parasitoid complex of *Prays oleae* (Bernard) (Lepidoptera: Praydidae) in Portugal. Turkish Journal of Zoology 41: 502–512. https://doi.org/10.3906/zoo-1603-50

Nixon GE (1973) A revision of the north-western European species of the *vitripennis*, *pallipes*, *octonarius*, *triangulator*, *formosus*, *parasitellae*, *matacarpalis* and *circumscriptus*-groups of *Apanteles* Förster (Hymenoptera: Braconidae). Bulletin of Entomological Research 63: 169–228. https://doi.org/10.1017/S0007485300039006

Noyes JS (2017) Universal Chalcidoidea database. http://www.nhm.ac.uk/our-science/data/chalcidoids/introduction.html/ [accessed on 3 January 2018]

Papp J (1999) Redescription of F. Silvestri's two chelonine species (Hymenoptera: Braconidae: Cheloninae). Bollettino del Laboratorio di Entomologia Agraria Filippo Silvestri 55: 15–44.

Papp J (2007) Braconidae (Hymenoptera) from Greece, 6. Notes fauniques de Gembloux 60: 99–127.

Papp J (2012) A contribution to the braconid fauna of Israel (Hymenoptera: Braconidae), 3. Israel Journal of Entomology 41–42: 165–219.

Papp J (2014a) Faunistic contributions to the *Microchelonus* Szépligeti species of the Palaearctic region, with descriptions of two new species (Hymenoptera: Braconidae: Cheloninae). Acta Zoologica Hungaricae 60: 325–358. https:/real.mtak.hu/id/eprint/24326

Papp J (2014b) First survey of the Neotropical species of *Microchelonus* Szépligeti with descriptions of twenty-five new species (Hymenoptera: Braconidae: Cheloninae). Acta Zoologica Academiae Scientiarum Hungaricae 62: 217–344. https://dx.doi.org/10.17109/AZH.62.3.217.2016

Papp J (2014c) *Microchelonus deplanus* sp. n. from Canada and checklists of the Nearctic and Palaearctic species of the genus *Microchelonus* Szépligeti, 1908 (Hymenoptera: Braconidae: Cheloninae). Natura Somogyiensis 25: 115–140.

Penteado Dias AM, Fernandes LBR, Iemma LGR, Dias MM (2011) First occurrence of *Protapanteles (Protapanteles) enephes* (Nixon, 1965) (Hymenoptera: Braconidae: Microgastrinae) in Brazil and new biological data. Brazilian Journal of Biology 71: 735–738. http://dx.doi.org/10.1590/S1519-69842011000400019

Pereira JA, Bento A, Cabanas JE, Torres LM, Herz A, Hassan SA (2004) Ants as predators of the egg parasitoid *Trichogramma cacoeciae* (Hymenoptera: Trichogrammatidae) applied for biological control of the olive moth, *Prays oleae* (Lepidoptera: Plutellidae) in Portugal. Biocontrol Science and Technology 14: 653–664. https://doi.org/10.1080/09583150410001682386

Pérez Rodríguez J, Oltra Moscardó T, Peris Felipo FJ, Jiménez Peydró R (2013) Microgastrinae (Hymenoptera: Braconidae) in the forest state of Artikutza (Navarra: Spain): diversity and community structure. Insects 4: 493–505. http://doi.org/10.3390/insects4030493

Polaszek A (2009) Species diversity and host associations of *Trichogramma* in Eurasia. In: Consoli FL, Parra JRP, Zucchi RA (Eds) Egg parasitoids in agroecosystems with emphasis on *Trichogramma*. Springer, Dordrecht, 237–266. https://doi.org/10.1007/978-1-4020-9110-0_9

Rallo L, Díez CM, Morales Sillero A, Miho H, Priego Capote F, Rallo P (2018) Quality of olives: A focus on agricultural preharvest factors. Scientia Horticulturae 233: 491–509. http://doi.org/10.1016/j.scienta.2017.12.034

Ramos P, Panis A (1975) Les chalcidiens parasites de *Prays oleae* (Lepidoptera: Plutellidae) en Andalousie. Entomophaga 20: 225–227. https://doi.org/10.1007/BF02371946

Ramos P, Campos M, Ramos JM (1998) Long-term study on the evaluation of yield and economic losses caused by *Prays oleae* Bern. in the olive crop of Granada (southern Spain). Crop Protection 17: 645–647. http://doi.org/10.1016/S0261-2194(98)00065-9

Redolfi I, Campos M (2010) Developmental and reproductive biology of the ectoparasitoid, *Elasmus steffani*, in a substitute host, *Ephestia kuehniella*. Journal of Insect Science 10: 119. https://doi.org/10.1673/031.010.11901

Silvestri F (1908) La tignola dell'olivo (*Prays oleellus* Fabr.). Bollettino del Laboratorio di Zoologia Generale 2: 83–184.

Stavraki H (1970) Contribution a l'inventaire du complexe parasitaire de quelques insectes nuisibles a l'olivier en Grèce. Entomophaga 15: 225–231. https://doi.org/10.1007/BF02371000

Stavraki HG (1977) Results obtained from released of the oophagous parasites *Trichogramma* spp. against *Prays oleae* Bern. (Lepidoptera: Hyponomeutidae) over a four year period in Greece. Mededelingen van de Faculteit Landbouwwetenschappen, Rijksuniversiteit Gent 42:1361–1371.

Stavraki HG (1985) Use of *Trichogramma* spp. against the carpophagous generation of *Prays oleae* (Bern.) in Greece. In: Cavalloro R, Crovetti A (Eds) Proceedings of the CEC/FAO/IOBC international joint meeting, integrated pest control in olive-groves, Pisa (Italy), 3–6 April 1984, AA Balkema, Rotterdam, 242–246.

Telenga NA (1955) Braconidae, subfamily Microgasterinae, subfamily Agathinae. Fauna USSR, Hymenoptera 5: 311.

Texeira R, Bento A, Gonçalves M (2000) Avaliaçao da fauna auxiliar associada ao olival em produçao biológica em Trás-os-Montes. Boletín de Sanidad Vegetal. Plagas 26: 629–636.

Tobias VI, Belokobylskij SA, Kotenko AG, Medvedev G (1986) Family Braconidae. Keys to the insects of the European part of the USSR, Volume III. Part 5. Nauka Publisher, Leningrad, 500 pp.

Tolkanitz VI (2007) Ichneumon flies of the genus *Exochus* Gravenhorst (Hymenoptera: Ichneumonidae: Metopiinae) of the fauna of Palaearctic region. Russian Entomological Journal 16: 339–358.

Torres MR (2010) Parasitoides de plagas identificados en la provincia de Jaén (España). Boletín de la Sociedad Entomológica Aragonesa 46: 597–601.

Tschorsnig HP (2017) Preliminary host catalogue of Palaearctic Tachinidae (Diptera). http://www.nadsdiptera.org/Tach/WorldTachs/CatPalHosts/Cat_Pal_tach_hosts_Ver1.pdf [accessed on 12 June 2018]

van Achterberg K (2013) Fauna Europaea: Hymenoptera, Braconidae. Fauna Europaea version 2.6.2. http://faunaeur.org/ [accessed on 3 January 2018]

Velcheva N, Atanassov A, Karadjova O, Hubenov Z (2012) Parasitoid assemblages isolated from externally feeding lepidopterans and codling moth (*Cydia pomonella* L., Tortricidae) in a young apple orchard in West Bulgaria. Bulgarian Journal of Agricultural Science 18: 675–681.

Vidal S (1997) Determination list of entomophagous insects. No. 13. OILB, Avignon, 53 pp.

Wagener B, Reineke A, Löhr B, Zebitz CPW (2006) Phylogenetic study of *Diadegma* species (Hymenoptera: Ichneumonidae) inferred from analysis of mitochondrial and nuclear DNA sequences. Biological Control 37: 131–140. http://doi.org/10.1016/j.bopcontrol.2006.01.004

Walker AK, Huddleston T (1987) New Zealand chelonine braconid wasps (Hymenoptera). Journal of Natural History 21: 339–361. http://doi.org/10.1080/00222938700771061

Yamvrias C (1998) Insect pests of olives. Stamoulis, Athens, 126 pp.

Yu DS, van Achterberg C, Horstmann K (2012) Taxapad 2012, Ichneumonoidea 2011. Database on flash-drive. Ottawa, Ontario.