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Paraphyletic genus *Ditylenchus* Filipjev (Nematoda, Tylenchida), corresponding to the *D. triformis*-group and the *D. dipsaci*-group scheme

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Abstract

The genus *Ditylenchus* has been divided into 2 groups: the *D. triformis*-group, and the *D. dipsaci*-group based on morphological and biological characters. A total of 18 populations belong to 5 species of *Ditylenchus* was studied: *D. africanus*, *D. destructor*, *D. myceliophagus* and *dipsaci*, *D. weischeri*, the first 3 belong to the *D. triformis*-group, the last 2 the *D. dipsaci*-group. The species of *D. triformis*-group were cultured on fungi, while the species from *D. dispaci*-group cultured on excised roots of plant hosts in petri dish. DNA sequences of regions of the nuclear ribosomal first internal transcribed spacer (ITS1) and the small subunit 18S were PCR amplified, sequenced and the phylogenetic analyses also including the sequences of the closely related species from the GenBank. The randomly amplified polymorphisms of genomic DNA (RAPD) were also generated. Two clusters or clades corresponding to the 2 groups were consistently observed with significant statistical support from the 3 datasets. The phylogenetic analysis also revealed that the genus is paraphyletic, separating the 2 groups by species of *Anguina* and *Subanguina*.

Keywords
Ditylenchus, ITS, 18S ribosomal DNA, RAPD, genetic variations, mycophagous, plant parasitic nematodes, phylogeny

Introduction

The genus *Ditylenchus* Filipjev (1936) consists of 80-90 accepted species (Brzeski 1991) of either mycophagous, entomophlic or plant parasitic species. The genus includes some of the most destructive nematode pests, e.g. the mushroom spawn nematode *D. myceliophagus* Goodey 1958, the potato rot nematode *D. destructor* Thorne 1945, and the stem and bulb nematode *D. dipsaci* (Kühn, 1857) Filipjev 1936, the latter two are also internationally quarantined. As the climate change intensifies and international trade increases, invasive alien species such as nematode species are increasingly becoming serious problems, as demonstrated by the recent outbreak of the stem and bulb nematode in central Canada and the neighboring states of USA, (Yu et al. 2010, Qiao et al. 2013), and the recent finding of potato rot nematode in Ontario (Yu et al. 2012), which was the first finding on the continental Canada for the pest.

Taxonomy of the genus both above and below the rank has been confusing. The genus was first placed in the family Tylenchidae of Tylenchina (Filipjev 1936), moved to Anguillulina Schneider (1939) and moved again to Anguinidae (Paramonov 1970). The family has been moved between Hexatylina and Tylenchina (Siddiqi 1986, and 2000). Within the genus, species delimitation based on morphology has been rather arbitrary, since many morphometrical characters are highly variable and only a few were constant enough to be used for taxonomic purposes (Fortuner 1982). The species complex of *D. dipsaci* (Sturhan & Brzeski, 1991) makes this situation even more confusing. Recently applications of molecular methods have provided new tools for researchers to better understand the biology and taxonomy of the genus. For example, *D. weischeri* Chizhov, Borisov & Subbotin (2010) has been separated as a valid species from the *D. dipsaci* species complex, *D. gigas* Vovlas (2011) from the giant race of *D. dipsaci*, and *D. africanus* Wendt (1995) from *D. destructor*. Recent phylogenetic studies of ribosomal DNA indicated that the genus may be paraphyletic (Holterman et al. 2009; Giblin-Davis et al. 2010).

Two groups of the genus were recognized: the *D. triformis*-group and *D. dipsaci*-group (Siddiqi 1980). The *D. triformis*-group includes species with a rounded tail tip, lateral fields of six lines, and having mycophagous life cycle such as *D. destructor* and *D. myceliophagous*, while the *D. dipsaci*-group includes obligate plant parasites with a sharp-pointed tail tip and lateral fields of four lines. Those entomophlic species such as *D. halictus* are also mycophagous; belong to the *D. triformis*-group (Giblin-Davis et al. 2010).

The objective of the study was to use three molecular datasets, namely ITS1 and 18S fragment sequences of ribosomal DNA and RAPD polymorphisms of genomic DNA, to determine the phylogenetic relationships of the two groups of *Ditylenchus* species.

Material and methods

Nematode population

Live nematodes of eight populations of *D. destructor*, six populations of *D. dipsaci*, one of each *D. africanus*, *D. weischeri* and *D. myceliophagus* from different regions of three countries were collected (Table 1). Species identifications were confirmed using morphological and molecular methods.

Nematode culturing

Ditylenchus destructor, *D. myceliophagus* and *D. africanus* were cultured on *Fusarium oxysporium* on 10% potato dextrose agar (PDA). *Ditylenchus dipsaci* and *D. weischeri* were cultured on yellow pea and soybean excised roots on White's medium (White 1939) respectively but attempts were also made to culture *D. dipsaic*, and *D. weischeri* on *F. oxysporium*.

Sample preparation

PDA with fungus media and roots infested with nematodes were cut into small pieces and nematodes extracted using the Baermann funnel method (Baermann 1917).

DNA extraction

One or two extracted nematodes were subjected to DNA extraction. The nematodes were crushed in microtubes containing 40 µL 10×PCR buffer (100 mM Tris-HCl, pH 9.0 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$), 10 µL Proteinase K (1 mg/mL), 50 µL distilled water. The microtubes were incubated for 1.5 h at 65°C followed by 15 min at 95 °C and stored at -20 °C. DNA templates were quantified using a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE, USA).

Sequencing and alignment of ITS1 and 18S regions of nuclear rRNA

A region of the internal transcribed spacer 1 (ITS1) gene was amplified using the primers ITS-F (5'-TTGATTACGTCCCTGCCCTTT-3'), ITS-R (5'-ACGAGC-CGAGTGATCCACCG-3'). The amplification protocol was: initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation (30 s at 94 °C), annealing (45 s at 58 °C), and extension (2 min at 72 °C), with a final extension for 10 min at 72 °C. A region of the small subunit (SSU) 18S rRNA gene (18S) was amplified

Table 1. Origins, hosts and access numbers of *Ditylenchus* species and populations used in this study

Code	Species	Location	Host	Accession No.	
				ITS	18S
CH01	*D. destructor*	Inner Mongolia, China	Sweet potato	KJ567140	KJ492926
CH02	*D. destructor*	Jilin, China	Sweet potato	KJ567141	KJ492927
CH03	*D. destructor*	Henan, China	Sweet potato	KJ567142	KJ492928
CH04	*D. destructor*	Shandong, China	Sweet potato	KJ567143	KJ492929
CH05	*D. destructor*	Jiangsu, China	Sweet potato	KJ567144	KJ492930
CH06	*D. destructor*	Hebei, China	Sweet potato	KJ567145	KJ492931
CA01	*D. destructor*	Ontario, Canada	Sweet potato	KJ567146	KJ492932
CU01	*D. destructor*	Clemson University, USA	Sweet potato	KJ567147	KJ492933
CA02	*D. dipsaci*	Ontario, Canada	Onion	KJ567148	KJ492934
CU02	*D. dipsaci*	Clemson University, USA	Garlic	KJ567149	KJ492935
CA03	*D. dipsaci*	Ontario, Canada	Garlic	KJ567150	KJ492936
CA04	*D. dipsaci*	Ontario, Canada	Garlic	KJ567151	KJ492937
CA05	*D. dipsaci*	Ontario, Canada	Garlic	KJ567152	KJ492938
CA06	*D. dipsaci*	Ontario, Canada	Garlic	KJ567153	KJ492939
DA	*D. africanus*	South Africa	Peanut	KJ567154	KJ492940
DW	*D. weischeri*	Manitoba, Canada	Canada thistle	KJ567155	KJ492941
DM	*D. myceliophagus*	Ontario, Canada	Grass	KJ567156	KJ492942

using the primers 18S-F (5'-TTGGATAACTGTGGTTTAACTAG-3') and 18S-R (5'-ATTTCACCTCTCACGCAACA-3'). The amplification condition was: 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 45 s at 60 °C and 2 min at 72 °C, with final extension of 10 min at 72 °C. All PCR reactions were performed in 25 ul volumes including 10 ng DNA, 2.5 µl 10×PCR buffer, 1.5 µl 2.5 mM dNTPs, 0.2 ul 10 µM primers and 0.25 µl Titanium Taq DNA polymerase (supplier). The ITS and 18S fragments were sequenced in-house with an ABI Prism 377 sequencer (Perkin Elmer) in both directions and unambiguous consensus sequences obtained. The sequences were deposited into the genBank database. DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). The sequences were compared with those of the other nematode species available at the genBank sequence database using the BLAST homology search program. The model of base substitution was evaluated using MODELTEST (Posada and Crandall 1998; Huelsenbeck and Ronquist 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001) running the chain for 1 × 106 generations and setting the "burnin" at 1,000. We used the Markov Chain Monte Carlo (MCMC) method within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon 1999) using 50% majority rule.

RAPD (randomly amplified polymorphic DNA) and data analysis

Twenty seven random primers were used for RAPD analysis. These primers were previously shown to be suitable for inter-species comparison of *Ditylenchus* (Digby and Kempton 1987; Zouhar et al. 2007). All PCR reactions were performed in 25 µl volumes consisting of 1 µL of genomic DNA prepared earlier as described above, 2.5 µl of 10×PCR buffer, 1.25 µl of 2.5 mM dNTPs, and 0.25 µl of Titanium Taq DNA polymerase (Clontech Lab Inc.). Amplification conditions were as follows: an initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 1min, annealing/extension at 72 °C for 1min and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis (100V, 1h) in 2.0% agarose gels in TAE buffer with 180-200 ng DNA. The gels were stained with ethidium bromide, visualized and photographed under UV-light (Bio-rad DX, USA). All reactions were repeated twice for clear and stable banding patterns. The presence or absence of DNA fragments was scored as one or zero, respectively, in the binary matrix. Simple matching coefficients (SM) (Digby and Kempton 1987) and hierarchical cluster analysis were performed with NTSYS2.1 (Exeter Software, Setauket, NY). Cluster analysis, by the un-weighted pair method with arithmetic mean (UPGMA), was performed with the SAHN (sequential, agglomerative, hierarchical and nested clustering method). The robustness of the dendrogram was tested with 1000 bootstrap replicates using PAUP software (Swofford 2003).

Results

DNA sequences: Ribosomal DNA fragments of the internal transcribed spacer 1 (404 bp) and fragments of the 18S ribosomal RNA gene (902 bp) were amplified and sequenced and sequences deposited in GenBank (www.ncbi.nlm.nih.gov/genbank). GenBank accession numbers are listed in Table 1.

Phylogeny: Phylogenetic trees based on the ITS1 and 18S sequences of rDNA are shown in Figures 1 and 2 respectively. The results are consistent for both ITS and 18S with species separating into two clusters, one cluster comprising *D. destructor*, *D. africanus* and *D. myceliophagus*, and the second comprising *D. dipsaci*, *D. weischeri* and *D. gigas*, with the groupings corresponding well with the tail endings. The 2 clusters were separated by species of *Anguina*.

RAPD analysis: Among the 27 primers (excepting RAPD2, RAPD3, RAPD5, RAPD7, OPA17 and OPB16 which amplified no visible bands) 21 random primers produced clear and reproducible bands. A total of 212 bands ranging from 100-2000 bp in size were produced by the 21 primers. 121 and 42 polymorphic bands were obtained for *D. destructor* and *D. dipsaci* respectively, which suggests higher genetic variation among populations of the *D. destructor* than those of *D. dipsaci*. Figure 3 presents the RAPD profiles obtained from primers OPG-05 to exemplify the banding patterns observed.

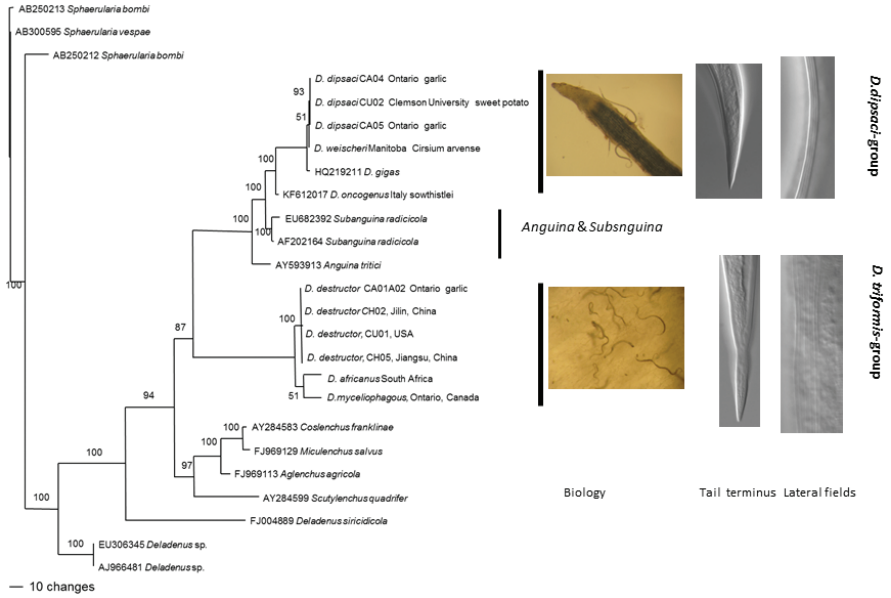

Figure 1. The 10001st Bayesian likelihood tree inferred from ITS sequences under GTR+I+G model (lnL = 9697.1895; freqA = 0.2646; freqC = 0.2062; freqG = 0.2602; freqT = 0.269; R(a) = 0.9399; R(b) = 3.4936; R(c) = 2.4954; R(d) = 0.5528; R(e) = 5.2698; R(f) = 1; Pinva = 0.4389; Shape = 0.7862). Posterior probability values exceeding 50% are given on appropriate clades.

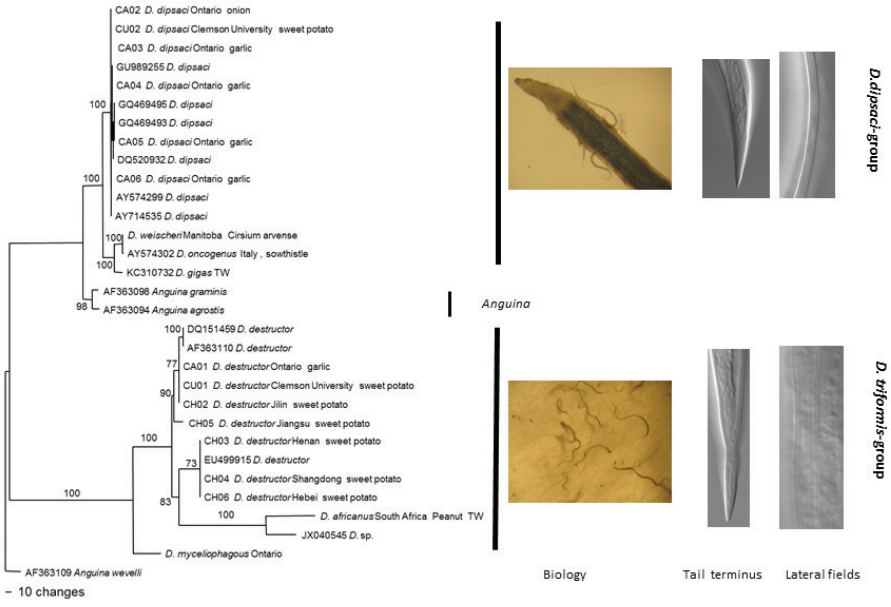

Figure 2. The 10001st Bayesian likelihood tree inferred from 18S sequences under GTR+I+G model (lnL = 9697.1895; freqA = 0.2646; freqC = 0.2062; freqG = 0.2602; freqT = 0.269; R(a) = 0.9399; R(b) = 3.4936; R(c) = 2.4954; R(d) = 0.5528; R(e) = 5.2698; R(f) = 1; Pinva = 0.4389; Shape = 0.7862). Posterior probability values exceeding 50% are given on appropriate clades.

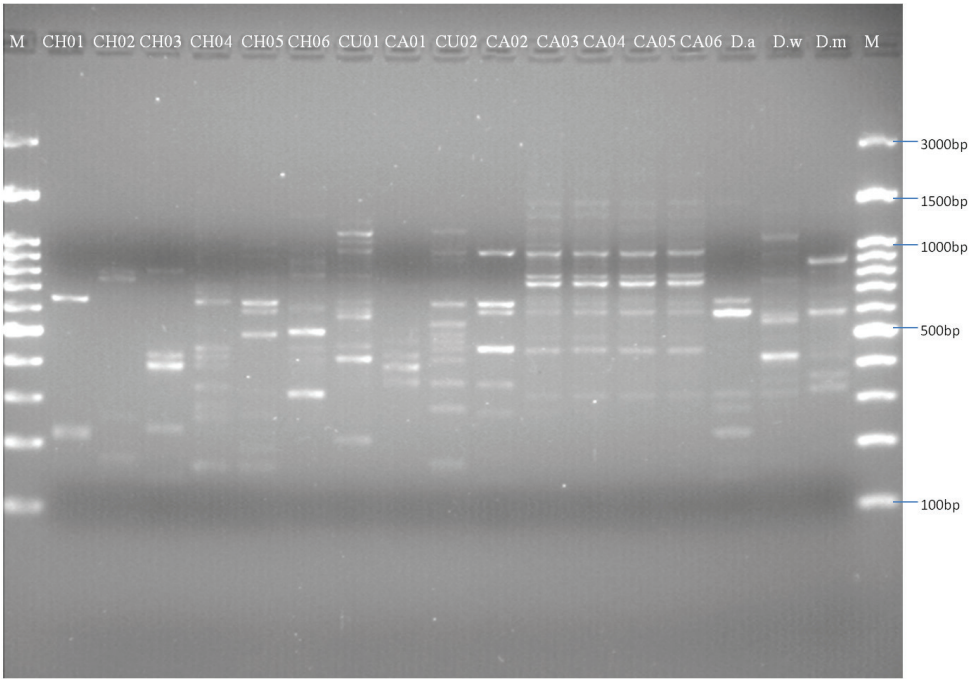

Figure 3. Random Amplified Polymorphic DNA (RAPD) profiles of all *Ditylenchus* species using primer OPG-05.

The RAPD binary data matrix and resulting simple matching coefficient (SM) are presented in Table 2. Figure 4 shows the dendrogram indicating the relationships among all collections. Species of *Ditylenchus* separated into two clusters consistent with the phylogenetic results based on the ITS1 and 18S sequences. *D. destructor*, *D. africanus*, and *D. myceliophagus* comprised one cluster and *D. dipsaci* and *D. weischeri* the second cluster. All *D. destructor* populations were in one cluster with similarity of 74.2%, and all six populations of *D. dipsaci* in the other cluster with a higher degree of genetic similarity (87%).

Conclusions

All three molecular data supports morphological schemes for this genus to be divided into two groups: *D. triformis*-group and *D. dipsaci*-group, and that the genus is paraphyletic dividing along the group line by *Anguina* and *Subanguina*.

Discussion

The results of the study provide strong evidence for divide the genus into 2 groups, one for *D. triformis*-group and *D. dipsaci*-group, and genus is paraphyletic. Paraphyletic

Table 2. Similarity matrix (Simple Matching Coefficient) among all *Ditylenchus* species obtained with 21 primers and based on shared DNA fragments.

	CH01	CH02	CH03	CH04	CH05	CH06	CA01	CU01	CA02	CU02	CA03	CA04	CA05	CA06	DA	DW	DM
CH01	1.000																
CH02	0.909	1.000															
CH03	0.909	0.818	1.000														
CH04	0.681	0.681	0.681	1.000													
CH05	0.773	0.773	0.773	0.909	1.000												
CH06	0.773	0.773	0.773	0.909	0.818	1.000											
CA01	0.727	0.727	0.727	0.772	0.773	0.864	1.000										
CU01	0.773	0.773	0.773	0.818	0.818	0.909	0.955	1.000									
CA02	0.409	0.409	0.409	0.455	0.455	0.455	0.500	0.455	1.000								
CU02	0.682	0.591	0.682	0.455	0.455	0.455	0.591	0.545	0.909	1.000							
CA03	0.500	0.500	0.500	0.545	0.545	0.545	0.591	0.545	0.909	0.818	1.000						
CA04	0.500	0.500	0.500	0.545	0.545	0.545	0.591	0.545	0.909	0.818	1.000	1.000					
CA05	0.500	0.500	0.500	0.545	0.545	0.545	0.591	0.545	0.909	0.818	1.000	1.000	1.000				
CA06	0.500	0.500	0.500	0.545	0.545	0.545	0.591	0.545	0.909	0.818	1.000	1.000	1.000	1.000			
DA	0.591	0.591	0.591	0.727	0.727	0.636	0.591	0.636	0.545	0.545	0.636	0.636	0.636	0.636	1.000		
DW	0.591	0.500	0.591	0.455	0.545	0.455	0.500	0.455	0.818	0.727	0.818	0.818	0.818	0.818	0.545	1.000	
DM	0.591	0.591	0.591	0.727	0.727	0.727	0.773	0.727	0.636	0.727	0.636	0.636	0.636	0.636	0.636	0.545	1.000

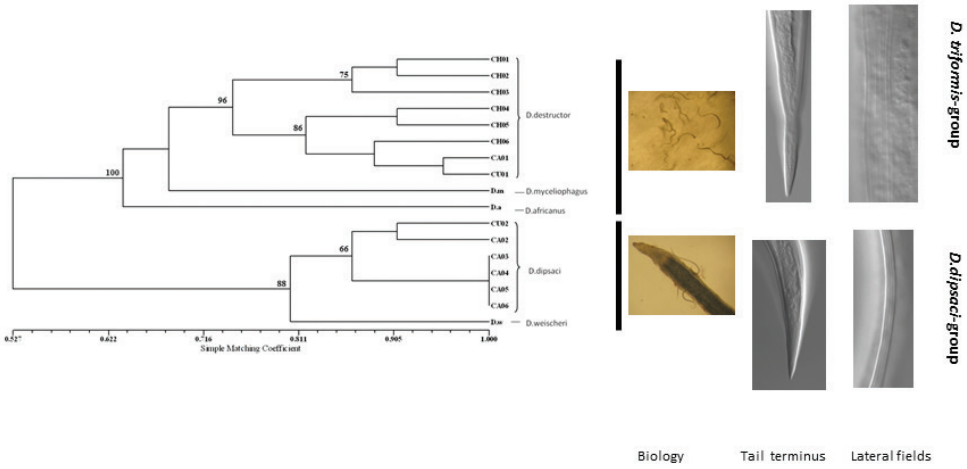

Figure 4. Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) tree showing estimated average genetic distances among all *Ditylenchus* species based on simple matching coefficient obtained from RAPD analysis.

and polyphyletic taxa are nothing new to biosystematics, even in nematoda several taxa have been found either paraphyletic or polyphyletic: such as *Hoplolaimus* is paraphyletic (Bae et al. 2008, Ma et al. 2011) and Aphelenchoididae polyphyletic (Kanzaki et al. 2009). It is debateable whether non-monophyletic taxa should be accepted. However as taxonomy advances from traditional to phylogenetic; however, more and more researchers would reject paraphyletic or polyphyletic taxa since they are inconsistent with evolution.

When the genus *Ditylenchus* was established by Filipjev (1936) by synonymizing *Tylenchus dipasci* to *D. dipsaci* it was placed in the family Tylenchidae (Nematoda: Tylenchida) as the sister genus to *Tylenchus*. Even today differences between species of the two genera are primarily morphometric, although now the genus is placed in the family of Anguinidae. There is some molecular evidence suggesting that one of the evolutionary paths of plant parasitism in nematodes is from algae-feeding nematodes *Tylenchus* to *Ditylenchus* (Holterman et al. 2009), which may be true for the obligate plant parasitic *Ditylenchus* species since the sharp-pointed tail tip is a feature in common for the two genera. Morphologically, the *D. triformis*-group is closely related with *Safianema*, and there is also molecular evidence (Giblin-Davis 2010) that they belong to one clade, that the species of *D. triformis*-group should be synonymized into *Safinema*, and there are also molecular evidences that *Safianema* and *D. triformis*-group are closely related to Neotylenchidae (suborder Hexatylina) than to Tylenchidae (suborder: Tylenchina) (Robin-Davis 2010), and a rounded tail tip (shared characteristic for both *D. triformis*-group and *Safianema*) and is a shared character in Hexatylina. To resolve the synonymization and the eventual high rank placement of the putatively synonymized *Safinema*, more studies are needed.

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RESEARCH ARTICLE

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Review of the planthopper genus *Neohemisphaerius* (Hemiptera, Fulgoroidea, Issidae) with description of one new species from China

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Abstract

The planthopper genus *Neohemisphaerius* Chen, Zhang & Chang, 2014 (Hemiptera: Fulgoroidea: Issidae) is reviewed to include 3 species: *N. wugangensis* Chen, Zhang & Chang, 2014 (China: Hunan), *N. yangi* Chen, Zhang & Chang, 2014 (China: Guangdong) and *N. guangxiensis* **sp. n.** (China: Guangxi). A revised generic diagnosis is given. The new species is described and all species illustrated. A key to these three species is also given. The species *Neohemisphaerius signifer* (Walker) is transferred back to *Hemisphaerius as H. signifer* Walker, **comb. revived**.

Keywords

Fulgoromorpha

Introduction

The genus *Neohemisphaerius* was erected by Chen, Zhang & Chang, 2014 for two new species (*N. wugangensis* and *N. yangi*) and *N. signifer* Walker, 1851, from China. In this paper, one new species of the genus *Neohemisphaerius* is described and illustrated from China, the generic characteristics are redefined and a checklist and key to the known

species of the genus are provided. In addition, *N. signifer* is removed from the genus; its placement was based on the identification by Fennah (1956) which has proven erroneous when compared to studied images of the type in the Natural History Museum, London. This type, which differs from *Neohemisphaerius* in lacking a median carina on the frons and in having the anteclypeus flat and hindwings well developed, is returned to *Hemisphaerius* as comb. revived, pending further studies. The non-type specimen from China figured by Fennah (1956) as *N. signifer* belongs to an unknown species.

Material and methods

The morphological terminology of the head and body follows Chan and Yang (1994), and the terminology of male genitalia follows Gnezdilov (2003). The genital segments of the examined specimens were macerated in 10% KOH and drawn from preparations in glycerin jelly using a light microscope. Photographs of the specimens were made using Zeiss stereo Discovery V8. Microscope with Zeiss Axio Cam HRc camera, images were produced using the software Axion Vision V4.8.2.0 and edited and enhanced using Adobe Photoshop CS4.0.

The type specimens of the new species are deposited in School of Life Sciences, Jinggangshan University.

Taxonomy

Genus *Neohemisphaerius* Chen, Zhang & Chang, 2014

Neohemisphaerius Chen, Zhang & Chang, 2014: 80

Type species. *Neohemisphaerius wugangensis* Chen, Zhang & Chang, 2014.

Diagnosis. Body hemispherical, head including eyes wider than pronotum. Vertex about 2.5–3.1 times wider than long, anterior margin more or less straight, posterior margin angulately concave, disc depressed, edges carinated. Frons longer than broad, with median carina, lateral margins slightly elevated. Clypeus convex on disc, distinctly tapering to apex. Pronotum depressed on disc, with two central pits, edges carinated. Mesonotum subtriangular, without median and lateral carinae. Forewings hemispherical, claval suture present. Hind wings rudimentary, veins indistinct. Hind tibiae with 2 lateral teeth. Spinal formula of the hind leg (9,10)-(4,5)-2.

Distribution. China (Guangdong, Guangxi, Hunan)

Discussion. The genus *Neohemisphaerius* is similar to *Hemisphaerius* Schaum, 1850 and *Gergithus* Stål, 1870, but it differs from *Hemisphaerius* in: frons with median carina; clypeus with a hump-shaped process in middle and forewings with claval suture present. It differs from *Gergithus* in: frons with median carina; forewings with claval suture present; hind wings rudimentary, shorter than half length of forewings.

Key to species of genus *Neohemisphaerius*

1 Forewings (Figs 1–2, 4–5) pale brown, with two black patches at costal margin; aedeagus (Figs 9–10) dorsally with a hump-shaped process, each side with birdhead-shaped processes ..... ***N. guangxiensis* sp. n.**

– Forewings (Figs 13–14) yellowish, with extensive black markings; aedeagus not as above **2**

2 Frons (Fig. 16) with distinct median carina; anal tube (Chen et al. 2014: fig. 2–35: H) in dorsal view with apical margin sinuate; aedeagus (Chen et al. 2014: figs 2–35: M, K) ventrally with short hooks, shorter than 1/5 length of aedeagus; spinal formula of hind leg10-4-2..... ***N. wugangensis***

– Frons (Fig. 20) with obscure median carina; anal tube (Chen et al. 2014: fig. 2-36: H) in dorsal view apical margin round; aedeagus (Chen et al. 2014: figs 2–36: L, K) ventrally with long hooks, longer than half length of aedeagus; spinal formula of hind leg 9-5-2 ..... ***N. yangi***

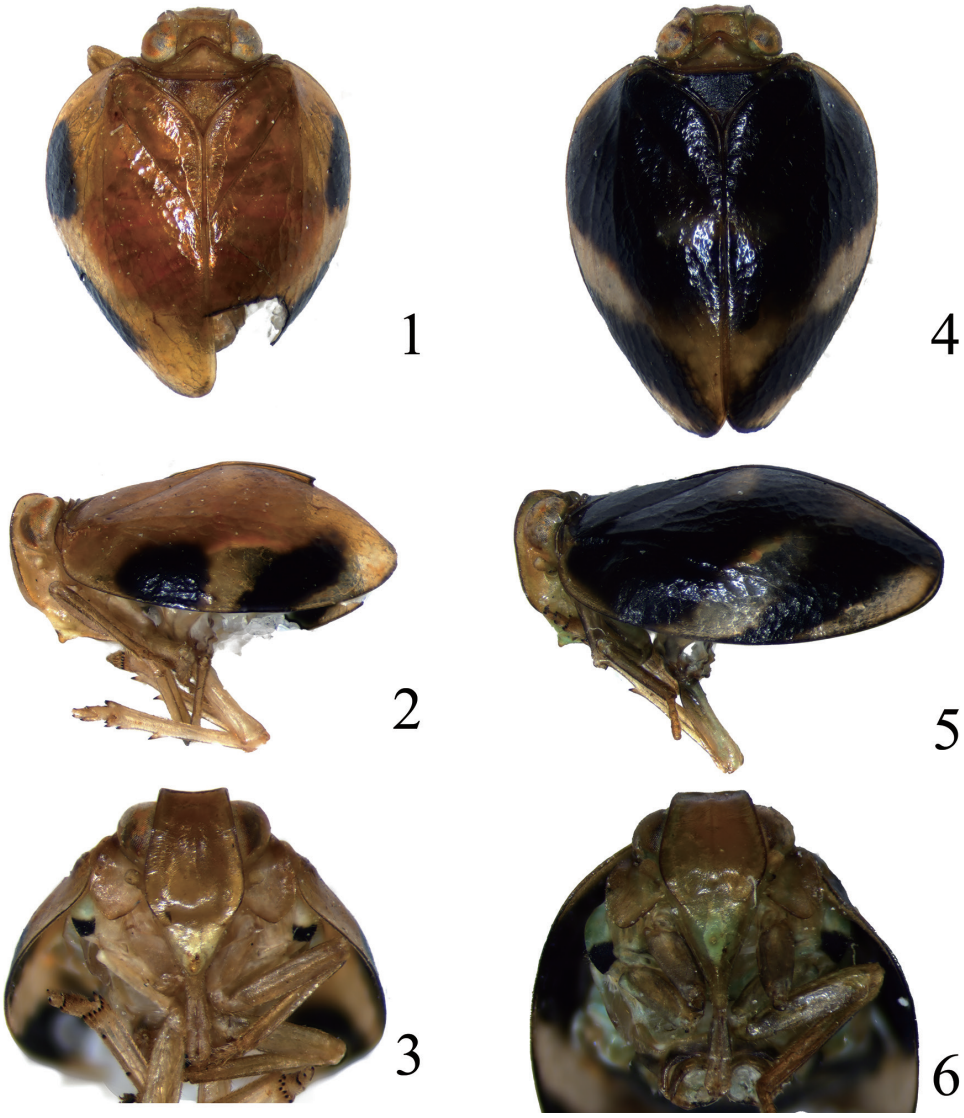
***Neohemisphaerius guangxiensis* sp. n.**
http://zoobank.org/9362854B-F612-4BDB-8A71-4859C8E76C4F
Figs 1–12

Type material. Holotype: ♂, China: Guangxi, Maoershan National Nature Reserve (E110°27'56.9", N25°54'43.5"), 1470 m, 18 July 2015, Z.G. Zhang; paratypes: 2 ♂♂, 5 ♀♀, same data as holotype.

Description. Body length (from apex of vertex to tip of forewing): male 4.63 mm, female 5.21 mm; Forewing: male 4.12 mm, female 4.60 mm.

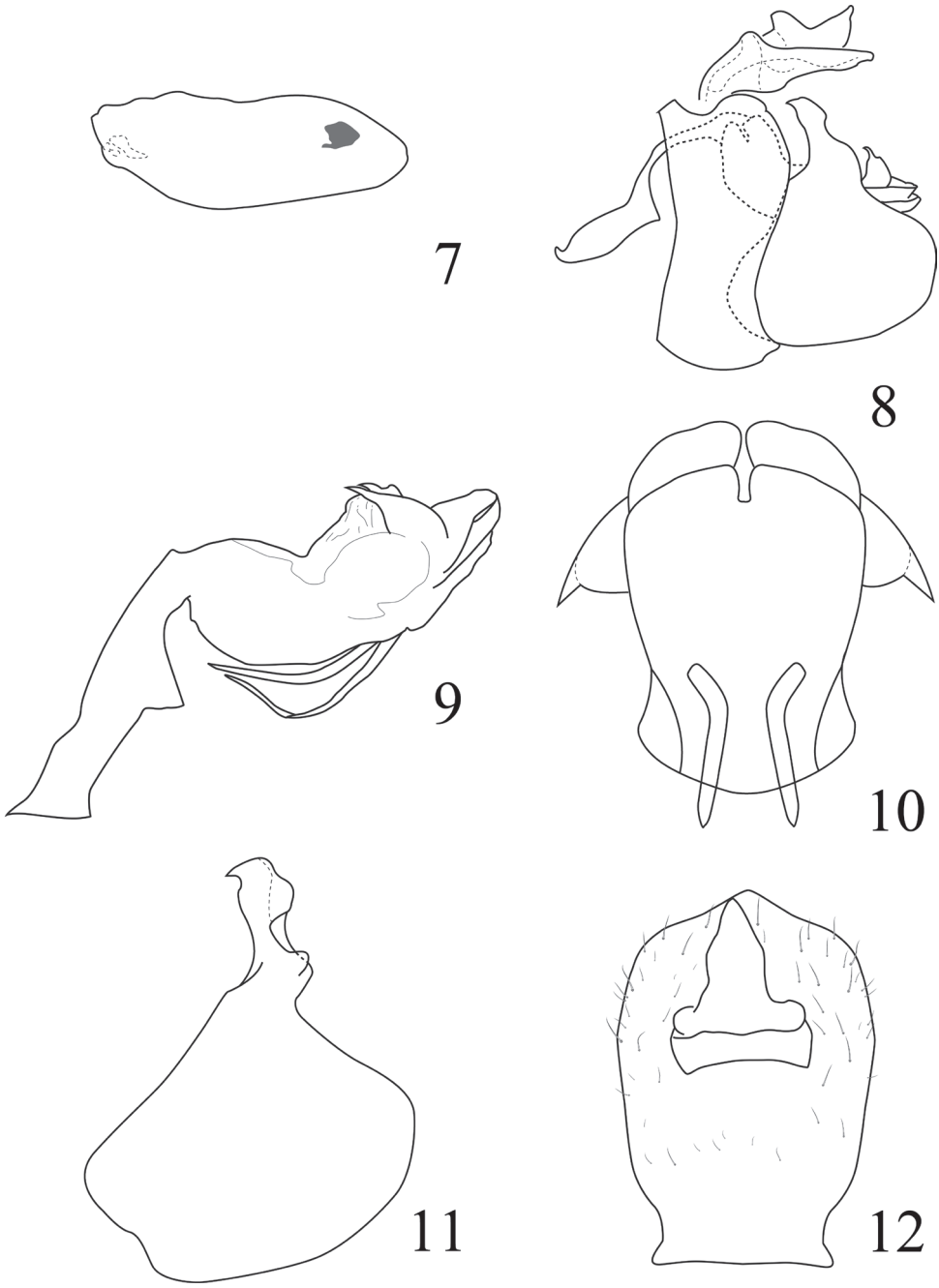
Coloration. Male: Vertex (Fig. 1) and frons (Fig. 3) brown, edges dark brown. Clypeus (Fig. 3) pale yellowish, rostrum (Fig. 3) dark brown, antenna brown (Figs 2–3). Pronotum (Fig. 1) brown with pale brown on disc, mesonotum (Fig. 1) brown with lateral angles dark brown. Forewings (Figs 1–2) brown with black markings near costal margin, hind wing pale brown. Legs pale brown. Female: Clypeus (Fig. 6) pale green near base, rostrum (Fig. 6) dark brown. Pronotum (Fig. 4) dark brown, mesonotum black brown. Forewings (Figs 4–5) with extensive irregular black markings, costal margin with pale brown spots at base and apex, pale stripe arising from middle of costal margin oblique to suture.

Head and thorax. Vertex (Fig. 1) quadrangular, about 3.14 times wider than long, anterior margin straight, posterior margin angulately concave. Frons (Fig. 3) narrow at base, widest between eyes, about 1.36 times longer than broad, median carina present, distinctly convex above frontoclypeal suture. Clypeus (Fig. 3) with a hump-like process. Pronotum (Fig. 1) with posterior margin straight, depressed on disc, with two central pits. Mesonotum (Fig. 1) subtriangular, about 1.94 times longer in midline than the length of pronotum. Forewings (Figs 1–2) hemispherical, claval suture present, with longitudinal veins. Hind wings rudimentary, veins obscure. Hind tibiae with 2 lateral teeth. Spinal formula of the hind leg (9,10)-(4,5)-2.

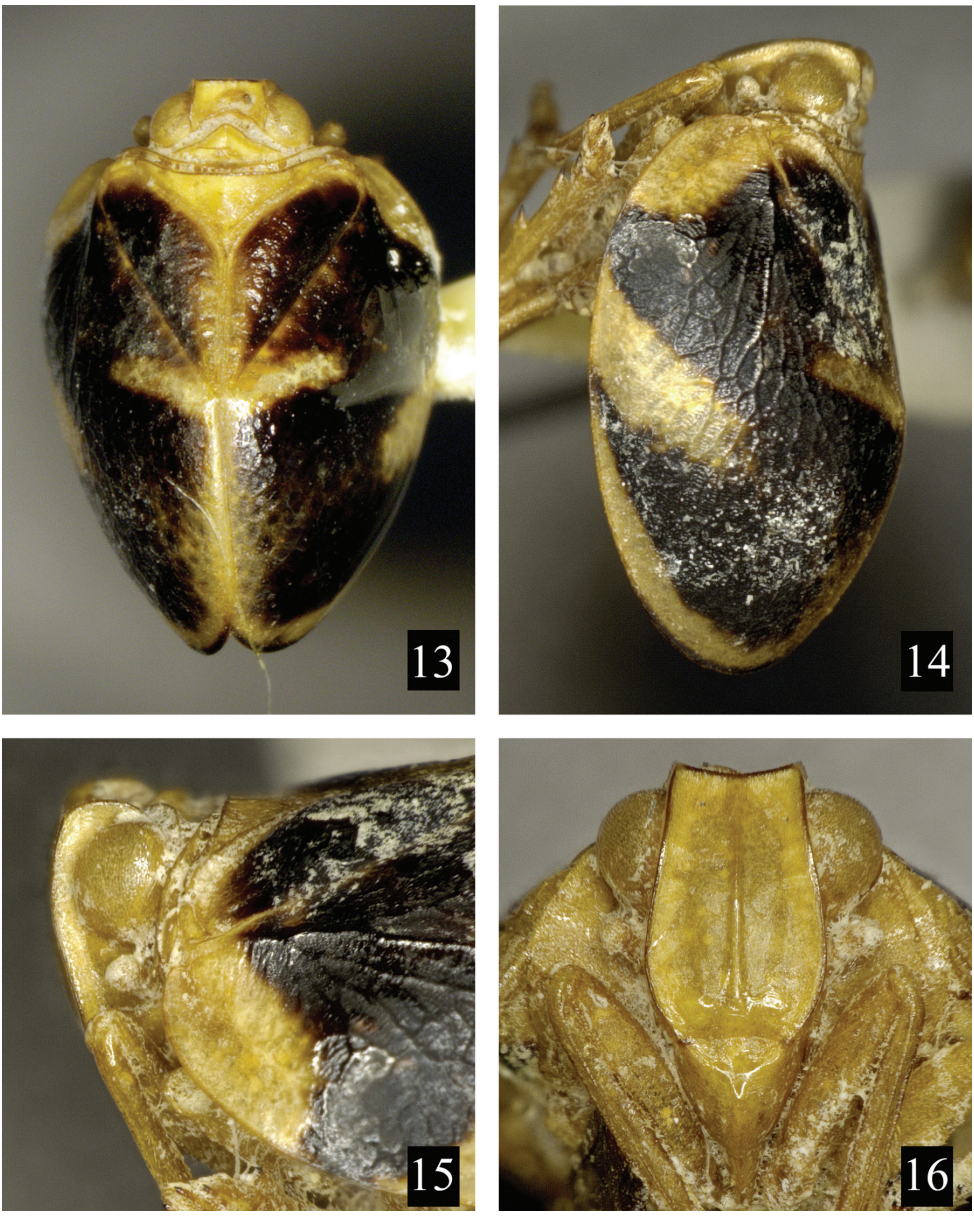


Figures 1–6. *Neohemisphaerius guangxiensis* sp. n. **1** Adult (male), dorsal view **2** Adult (male), in lateral view **3** Frons and clypeus (male), in front view **4** Adult (female), in dorsal view **5** Adult (female), in lateral view **6** Frons and clypeus (female), in front view.

Male genitalia. Anal tube (Fig. 12) relatively short, oval in dorsal view. Anal column relatively long, located at 1/3 basad of anal tube. Pygofer (Fig. 8) in lateral view, with anterior margin moderately concave, posterior margin raised near base. Aedeagus dorsally (Fig. 9) with a hump-shaped process near mid-length, each side with a bird-head-shaped process at 1/3 distance from apex, process acute apically, directed cephalad; dorso-lateral lobes obtuse apically, aedeagus ventrally with a pair of convergent



Figures 7–12. *Neohemisphaerius guangxiensis* sp. n. **7** Hind wing **8** Male genitalia, in lateral view **9** Aedeagus, in left view **10** Aedeagus, ventral view **11** Genital style, in profile view **12** Anal tube, in dorsal view.

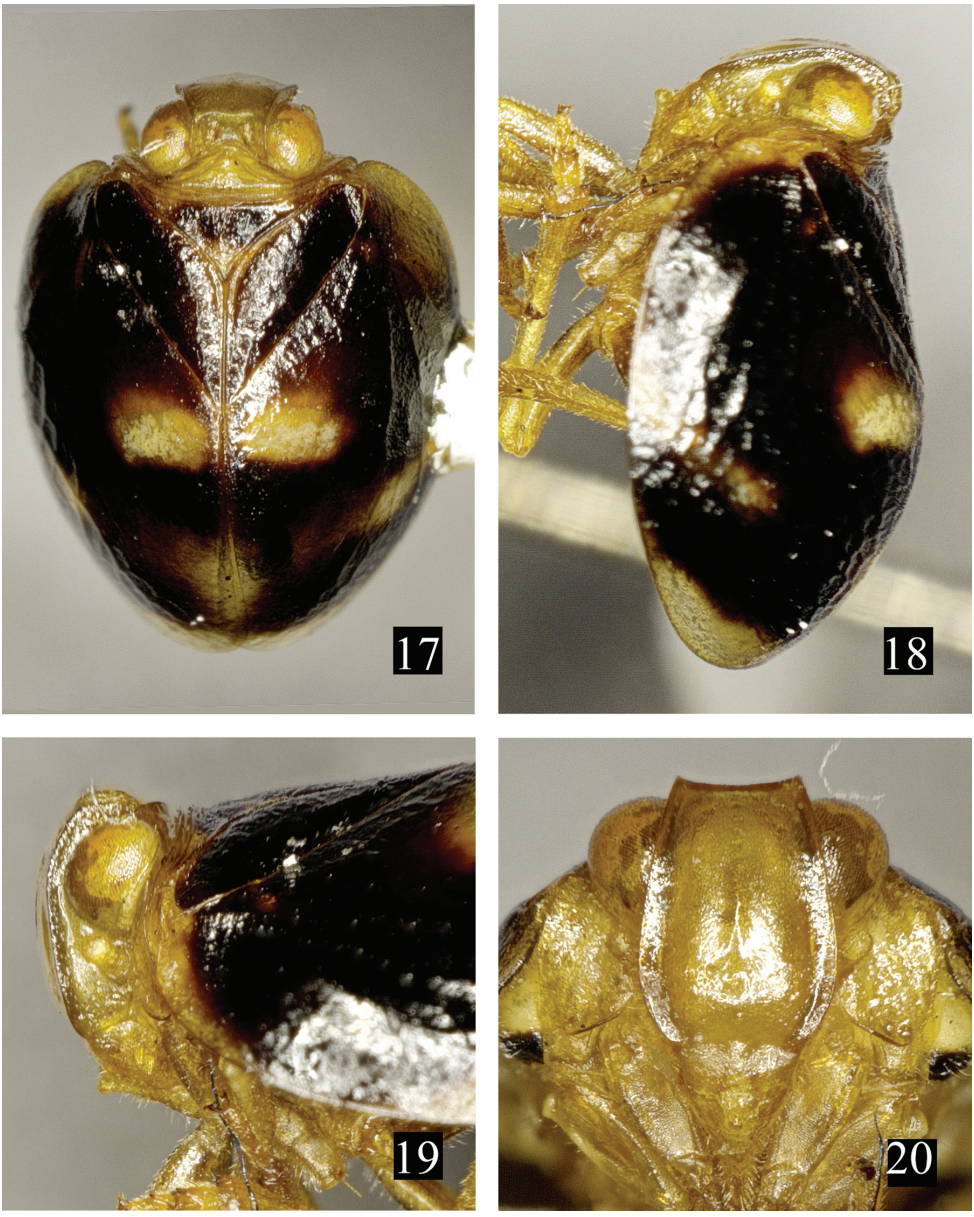


Figures 13–16. *Neohemisphaerius wugangensis* Chen, Zhang & Chang, 2014. **13** Adult (male), in dorsal view **14** Adult (male), in lateral view **15** Head (male), in lateral view **16** Frons and clypeus (male), in front view.

hook-like processes, apical margin of ventral lobe (Fig. 10) with a notch in middle. Style (Fig. 11) with a strongly convex hind margin and capitulum narrowing apically.

Etymology. The specific name refers to the locality, Guangxi province, China.

Host plant. Unknown.



Figures 17–20. *Neohemisphaerius yangi* Chen, Zhang & Chang, 2014. **17** Adult (male), in dorsal view **18** Adult (male), in lateral view **19** Head (male), in lateral view **20** Frons and clypeus (male), in front view.

Distribution. China (Guangxi).

Remarks. This species is similar to *N. wugangensis*, but differs in: (i) Anal tube (Fig. 12) longer than broad, with apical margin not expanded (in *wugangensis* anal tube about as long as broad, apical margin expanded (see Chen et al. 2014: figs 2–35: H); (ii) Aedeagus (Fig. 9) with a bird-head-shaped subapical process in each side, ven-

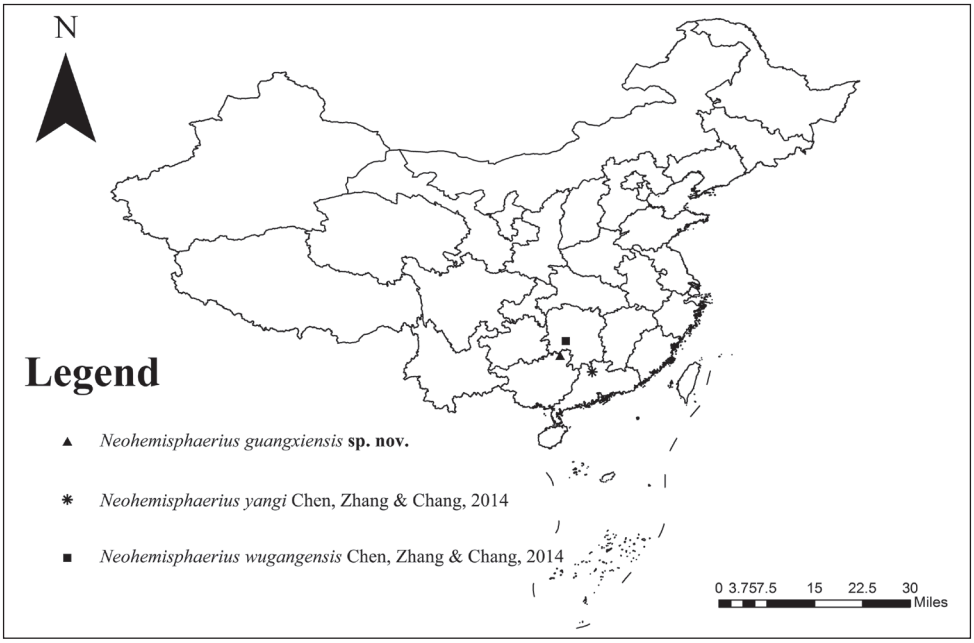


Figure 21. Geographic distribution of *Neohemisphaerius* species in China.

trally with pairs of long hooks near mid-length (in *wugangensis* processes of aedeagus different and ventrally with pairs of short hooks 1/3 from base); (iii) Apical margin of ventral lobe (Fig. 10) with a notch in middle (in *wugangensis* ventral lobe with apical margin convex in middle (see Chen et al. 2014: fig. 2–35: K).

Neohemisphaerius wugangensis Chen, Zhang & Chang, 2014

Figs 13–16

Neohemisphaerius wugangensis Chen, Zhang & Chang, 2014: 80: figs 2–35.

Material examined. 1♂4♀♀, Yunshan National Forest Park, Wugang city, Hunan Province, China

Neohemisphaerius yangi Chen, Zhang & Chang, 2014

Figs 17–20

Neohemisphaerius yangi Chen, Zhang & Chang, 2014: 83: figs 2–36.

Material examined. 2♂♂7♀♀, Nanling National Nature Reserve, Guangdong Province, China.

Acknowledgments

We are grateful to Ms. M.L. Chan (National Museum of Natural Science, Taichung Taiwan) and Dr. V.M. Gnezdilov (Zoological Institute, Russian Academy of Science, Russia) for providing valuable references. This work was supported by the Natural Science Foundation of China (31460157), the Natural Science Foundation of Jiangxi Province, China (20114BAB214013), Science Research Fund of Jiangxi Provincial Education Department (GJJ14562), the Scientific Research Foundation for Doctors of Jinggangshan University (JZ10039) and High Level Discipline of Biology, Jiangxi Province.

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RESEARCH ARTICLE

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Revision of *Mandarella* Duvivier from Taiwan, with a new species, new synonymies and identities of highly variable species (Insecta, Chrysomelidae, Galerucinae, Alticini)

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Abstract

Taiwanese species of *Mandarella* Duvivier are compared on the basis of morphological and molecular evidence. Only three of eleven morphospecies are considered to be valid. *Mandarella uenoi* (Kimoto, 1969) is transferred from the genus *Luperus* Geoffroy. *Stenoluperus taiwanus* Kimoto, 1991 and *S. kimotoi* Döberl, 2001 are synonymized with *M. uenoi*. Taiwanese records of *Stenoluperus tibialis* Chen, 1942, *S. nipponensis* Laboissière, 1913, and *S. potanini* (Weise, 1889) were based on misidentifications and represent *M. uenoi*. The Taiwanese population previously erroneously identified as *S. pallipes* Gressitt and Kimoto, 1963 is here described as a new species, *M. tsoui* **sp. n.**, *Stenoluperus esakii* Kimoto, 1969, *S. matsumurai* Takizawa, 1978, and *M. taiwanensis* Medvedev, 2012 are synonymized with *M. flaviventris* (Chen, 1942).

Keywords

Flea beetles, alpine, molecular, taxonomic revision

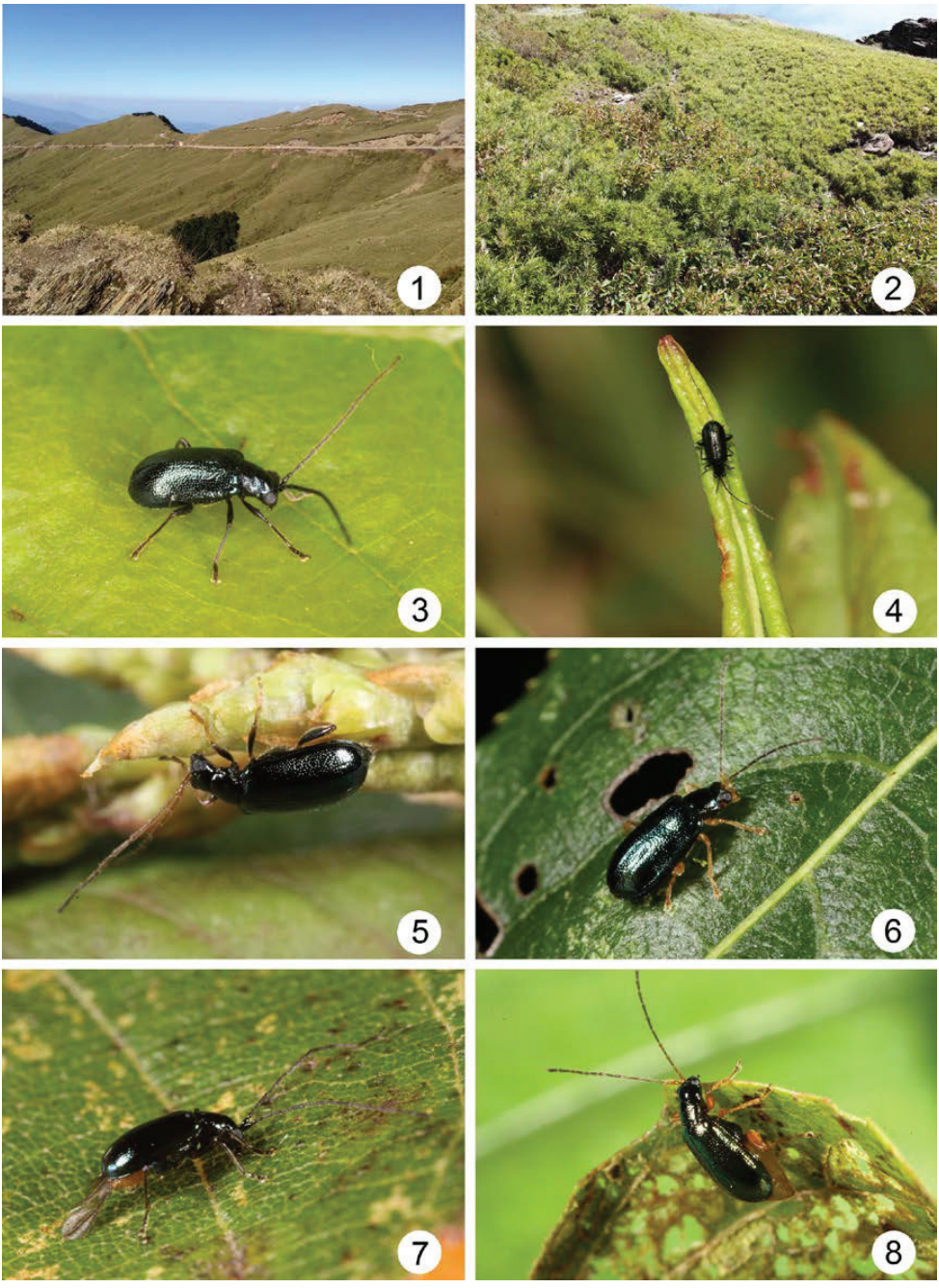
* Authors contributed equally

Introduction

Mandarella Duvivier, 1892 is a small genus of flea beetles containing five species (Döberl 2010). *Stenoluperus* Ogloblin, 1936 was a larger genus (33 species) and was proposed as a junior synonym of *Mandarella* Duvivier by Medvedev (2012). This synonym is confirmed after/by examination of type species of both genera (Konstantinov, personal communication). Thus, *Mandarella* now contains 34 valid species limited to the Palaearctic region.

More than 250 mountains exceed 3000 meters in Taiwan. Localities higher than 3000 m present cold and windy montane habitats (Figs 1–2) where few insects can survive, including leaf beetles. However, members of *Mandarella* are adapted to these habitats and display extensive morphological diversity (Figs 3–8). Based on morphological characteristics of body color and antennomeres, 11 species, mostly from limited montane areas, have been reported from Taiwan. Chûjô (1965) recorded the first two species, *Stenoluperus flaviventris* Chen, 1942 and *S. tibialis* Chen, 1942. Kimoto (1969) described *S. esakii*, reported *S. pallipes* Gressitt and Kimoto, 1963 and *S. nipponensis* Laboissière, 1913. Kimoto (1974) subsequently recorded *S. potanini* (Weise, 1889). Takizawa (1978) described *S. matsumurai*. Kimoto (1991a) described *S. taiwanus*. At the same year, Kimoto (1991b) described *S. minor* and *S. itoi*. However, *S. minor* Kimoto 1991b was a primary junior homonym of *S. minor* Kimoto, 1977 and was replaced as *S. kimotoi* by Döberl (2001). *Stenopluperus itoi* Kimoto, 1991b was a secondary junior homonym of *Mandarella itoi* Chûjô, 1966 and was replaced as *M. taiwanensis* by Medvedev (2012). We have discovered that *Luperus uenoi* Kimoto, 1969 is also a member of *Madarella* based on examination of the type specimens.

Although these *Mandarella* species can be separated by color patterns and relative lengths of antennomeres (Kimoto and Takizawa 1997), only three forms of male aedeagi were found during our study. In addition, previous authors have noted that variations in body color and antennomeres may be the result of local adaptation that is not indicative of species boundaries (Kurachi et al. 2002, Nahrung and Allen 2005, Quinzin and Mardulyn 2014). Molecular approaches have been applied broadly in systematics of various insects and have fueled taxonomic debates about species recognition, especially when morphological characters are insufficient (Brown et al. 2012, Kumar et al. 2012; Lee et al. 2013, Lees et al. 2014, Park et al. 2011, Tsai et al. 2014). Sequences data from cytochrome oxidase subunit I (COI), a small fragment of mitochondrial DNA, have been viewed as efficient markers in Chrysomelidae and have been exploited to resolve debates in identification (Germain et al. 2013, Kubisz et al. 2012, Lopes et al. 2015) and elucidate species complex phylogenetics (Quinzin and Mardulyn 2014). Nie et al. (2012) also used COI to clarify two closely related leaf beetles with color variation in elytra and pronota. In the present study, mtDNA COI markers are used to examine the taxonomy of 11 species of *Mandarella* leaf beetles that vary locally in morphology and color.



Figures 1–8. Field photographs. **1** Alpine habitat, Hohuanshan **2** Microhabitat **3** *Mandarella uenoi* form C **4** *M. uenoi* form B **5** *M. uenoi* form D **6** *M. tsoui* sp. n. **7** *M. flaviventris* form G **8** *M. flaviventris* form I.

Methods

Depositories of material examined

BPBM Bernice P. Bishop Museum, Hawaii, USA [James Boone];
CAS California Academy of Sciences, California, USA [David H. Kavanaugh];
EIHU Systematic Entomology, The Hokkaido University Museum, Sapporo, Japan [Masahiro Ôhara];
EUMJ Entomological Laboratory, Faculty of Agriculture, Ehime University, Matsuyama, Japan [Hiroyuki Yoshitomi];
KMNH Kitakyushu Museum of Natural History and Human History, Kitakyushu, Japan [Yûsuke Minoshima];
KUEC Faculty of Agriculture, Kyushu University, Fukuoka, Japan [Osamu Tadauchi];
NMNS National Museum of Natural Science, Taichung, Taiwan [Ming-Luen Jeng];
TARI Taiwan Agricultural Research Institute, Taichung, Taiwan.

Exact label data are cited for type specimens and voucher ones of the described species; a double slash (//) divides the data on different labels and a single slash (/) divides the data in different rows. Other comments and remarks are in square brackets: [p] – preceding data are printed, [h] – preceding data are handwritten, [w] – white label, [y] – yellow label, [b] – blue label, [r] – red label, and [y] – yellow label.

Specimens and sampling

Approximately 2500 specimens were examined for this study. Most of them either belong to the historic collections at TARI or were collected recently as part of a long term project "inventorying chrysomelids of Taiwan" by the Taiwan Chrysomelid Research Team (TCRT). One hundred and thirty-five specimens were collected for DNA analysis. These specimens were classified into morphospecies, including two specimens of *Mandarella nipponensis* collected from Japan. *Dercetina itoi* Kimoto, 1969 and *D. shirozui* Kimoto, 1969 were used as outgroups for convenience since they fed on the same host plants as *M. tsoui* sp. n.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from the meta-femora via QuickExtract DNA extraction kits (Epicenter Biotechnologies, Madison, WI). The protocol was modified according to Tsai et al. (2014). The primer sets used to amplify the mitochondrial COI gene are listed (Table 1). Polymerase Chain Reaction was conducted in a volume of 25 μl and the programing conditions were 94 °C for 2 min for initial denaturation, 35 cycles of 94 °C for 40 s, 45 °C for 40 s, and 72 °C for 40 s, then 72 °C for 10 min

Table 1. Primers and their amplification size in this study.

Gene	Primer	Sequence 5'→3'	Size (bp)	References
COI	COI-Chry_F (+)	ACYAAYCAYAAAGAYATWGG	689	In this study
	COI-49_Chrysomelidae_F	CATAAAGATATTGGHACHTT	683	
	COI-64_Chrysomelidae_F	ACHYTRTAYTTYATTTTYGG	668	
	CI-731Coleoptera (-)	CCAAAAAATCAAAATAAATGTTG		Tsai et al. (2014)

"+" and "-" are upstream and downstream primers, respectively.

as a final extension. The upstream primer COI-49_Chrysomelidae_F and COI-64_Chrysomelidae_F were used for COI if COI-Chry_F was not successful in achieving amplification. Purification of PCR products was conducted via QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany) from 1% agarose gel. DNA products were sequenced using Taq dye terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 377A sequencer.

Phylogenetic analyses

Sequences were edited in Bioedit 7.0 (Hall 1999) and then aligned using Muscle Multiple Alignment option in SeaView4 (Gouy et al. 2010). Genetic divergences among species were analyzed using MEGA 6.0 via p-distance (Tamura et al. 2013).

Phylogenetic inference of COI was conducted using neighbor-joining clustering (NJ) and Bayesian inference (BI). For NJ, Kimura two-parameter (K2P) was selected as the substitution model and 1000 replications of bootstrapping analyses were applied. For BI, the evolutionary hypothesis of nucleotide substitution inferred in jModelTest 0.1 (Posada 2008) using Bayesian Information Criterion (BIC) for the best-fit models of COI was TIM3 + I + G. BI of the COI gene was analyzed in MrBayes v. 3.2 (Ronquist et al. 2012) with three heat chains and one cold chain, and MCMCMC searches were conducted for 1×10^7 generations with sampling every 100 generations. Analysis was finished with the average standard deviation of split frequencies below 0.01. The initial 25% of trees were discarded as burn-in, and then the remaining trees were used to generate a consensus tree.

Results

A total of 131 specimens of *Mandarella* flea beetles were successfully amplified for COI, with 584 bp in this study. The average nucleotide compositions for G, A, T, C are 18.4%, 28.0%, 32.8%, 20.8%, respectively. All sequences have been deposited in GenBank (Suppl. material 1).

Phylogenetic inferences based on COI gene using neighbor-joining (NJ) and Bayesian inference (BI) reveal that montane *Mandarella* flea beetles are monophyletic, with three lineages, each including several morphospecies (Fig. 9). In the *M. uenoi*

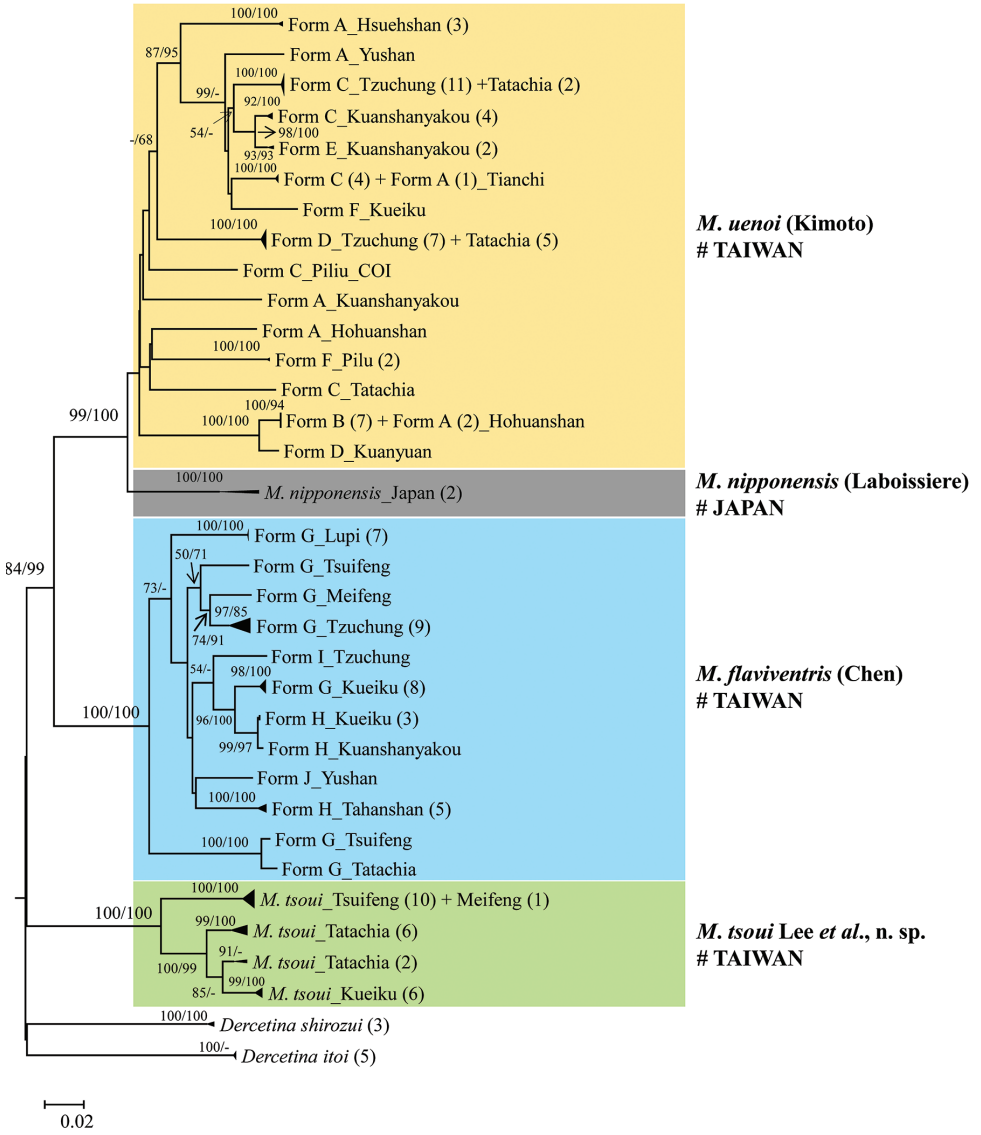


Figure 9. Neighbor-joining (NJ) inference based on COI gene using Kimura two-parameter (K2P) substitution model. Both bootstrapping values of NJ (left) and those of posterior probabilities from Bayesian inference (BI) (right) more than 50% are shown at nodes.

lineage, Japanese populations of *M. nipponensis* diverge separately, while the other morphology-based Taiwanese taxa (i.e., *M. kimotoi*, *M. nipponensis*, *M. potanini*, *M. taiwana*, *M. tibialis*) collected from different montane areas overlap with each other. A similar situation exists in the *M. flaviventris* lineage, with four morphospecies, *M. flaviventris*, *M. esakii*, *M. matsumurai*, and *M. taiwanensis*, from different localities over-

lapping and mixing. The third lineage includes several flea beetles in a separate group that occur only in mainland China. Therefore, specimens collected from Tsuifeng, Tatachia and Kueiku forming a separate lineage should be considered a new species, i.e. *M. tsoui* sp. n.

Among *Mandarella* flea beetles, the interspecific genetic distances range from 16.2–22.6%, while the intraspecific divergence is 0.0–14.4%.

Systematics

***Mandarella uenoi* (Kimoto, 1969), comb. n.**

Stenoluperus tibialis: Chûjô 1965: 98 (Taiwan); Kimoto 1969: 40 (additional records in Taiwan); Kimoto 1991c: 13 (additional record in Taiwan). **Misidentification**

Mandarella tibialis: Medvedev 2012: 427.

Luperus uenoi Kimoto, 1969: 39 (Taiwan).

Stenoluperus nipponensis: Kimoto 1969: 40 (Taiwan); Kimoto 1989: 253 (additional records in Taiwan); Kimoto 1991c: 12 (additional records in Taiwan). **Misidentification**

Stenoluperus potanini: Kimoto 1974: 26 (Taiwan); Kimoto 1989: 253 (additional records in Taiwan); Kimoto 1991c: 12 (additional records in Taiwan). **Misidentification**

Stenoluperus taiwanus Kimoto, 1991a: 14. **New synonym**

Mandarella taiwana: Medvedev 2012: 427.

Stenoluperus minor Kimoto, 1991b: 117 (nec Kimoto, 1977).

Stenoluperus kimotoi Döberl, 2001: 383. (replacement name for *Stenoluperus minor* Kimoto, 1991). **New synonym**

Type material. *Luperus uenoi*. Holotype ♂ (KUEC): "(Taiwan) / Mt. Nan-hu-ta Shan [南湖大山] / 3,580 m / Tái-chung Hsien [h, w] // 17.VI. [h] 19 [p] 61 [h] / S. Ueno [p, w] // *Luperus* / *uenoi* / Kimoto, sp. n. [h, w] // HOLOTYPE [p, r]". Paratypes: 1♂, 1♀, (KMNH): "(Taiwan) / Mt. Nan-hu-ta Shan [南湖大山] / 3,580 m / Tái-chung Hsien [h, w] // 17.VI. [h] 19 [p] 61 [h] / S. Ueno [p, w] // *Luperus* / *uenoi* / Kimoto, sp. n. [h, w] // PARATOPOTYPE [p, b]".

Stenoluperus minor. Paratype: 1♂ (KMNH): "Mt. YUSHAN [玉山] / TAIWAN / 8. VI. 1980 / N. ITO [p, y] // *Stenoluperus* / *minor* / Kimoto, sp. n. [h] / Det. S. Kimoto, 19 [p] 91 [h, w] // PARATOPOTYPE [p, b]".

Stenoluperus taiwanus. Paratypes: 1♀ (KMNH): "(Taiwan) / Yuanfeng [鳶峰], 2800m / -- Kunyang [昆陽], 3100m / Nantou Hsien [p, w] // *Stenoluperus* / *taiwanus* / Kimoto, sp. n. [h] // Det. S. Kimoto, 19 [p] 91 [h, w] // *Stenoluperus* / *tibialis* / Chen ? [h] / Det. S. Kimoto, 19 [p] 75 [h, w] // PARATYPE [p, b] // Japan-U. S. / Co-op. Sci. / Programme [p, y] // 1.VI.1965 / T. Nakane [h, w]"; 1♂ (KMNH): "Mt. Ho Huan Shan, [合歡山] / (3200m) / M-Taiwan / 28.V.1989 / Col. K. Baba [p, w] //

Stenoluperus / *taiwanus* / Kimoto, sp. n. [h] // Det. S. Kimoto, 19 [p] 91 [h, w] // PARATYPE [p, b]"; 1♀ (KMMH): " M.t Wu Kon Shan, [五公山] / near Liu kuei, / S-Taiwan / 3.VI.1989 / Col. K. Baba [p, w] // *Stenoluperus* / *taiwanus* / Kimoto, sp. n. [h] // Det. S. Kimoto, 19 [p] 91 [h, w] // PARATOPOTYPE [p, b]".

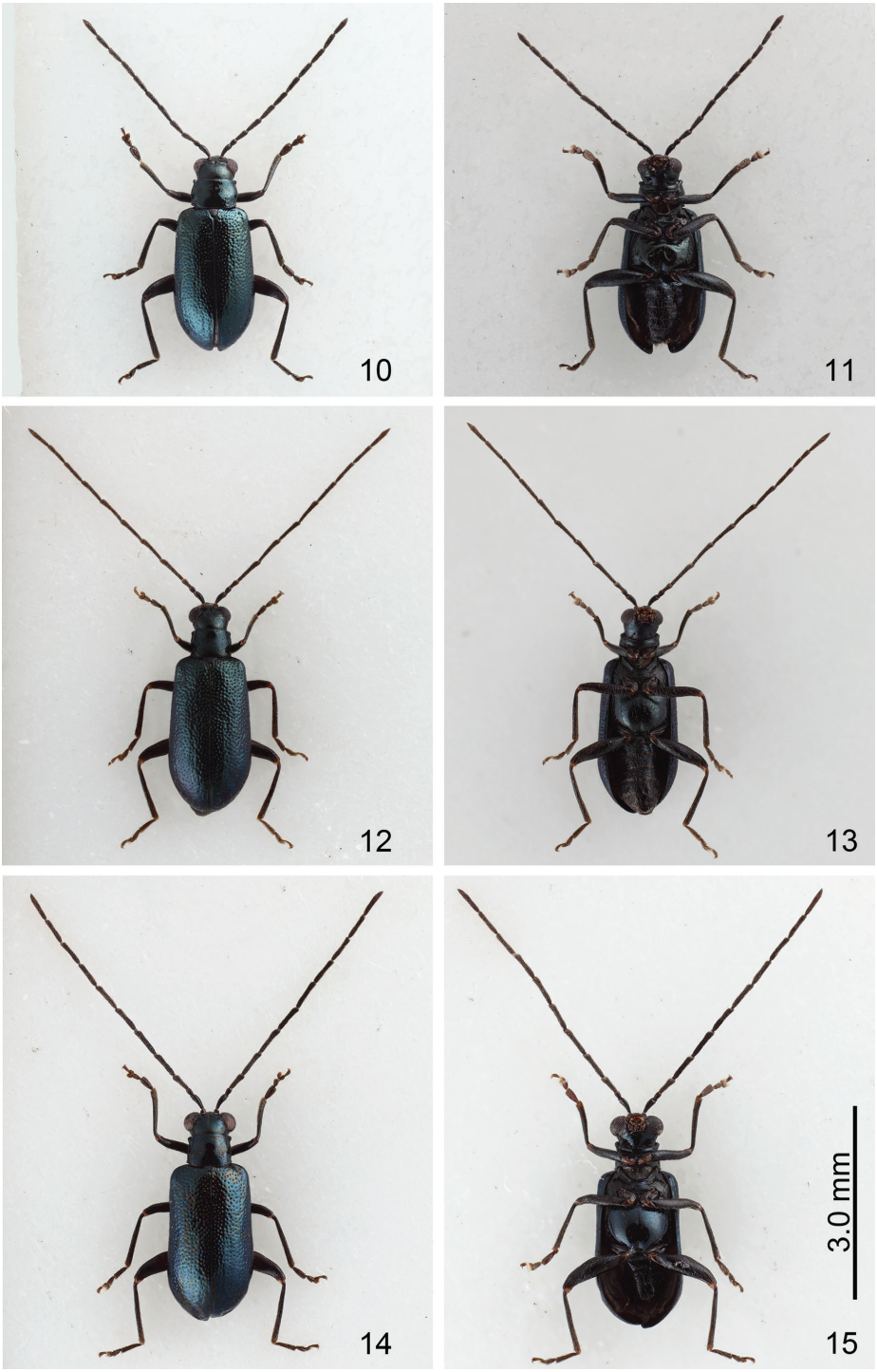
Voucher specimens. *Stenoluperus nipponensis*. 1♂ (KMNH): "(Taiwan) / Mt. Nanhu-pei Shan [南湖北山] / 3,500 m / I-lan Hsien [h, w] // 17.VI. [h] 19 [p] 61[h] / S. Ueno [p, w]"; 1♂ (KMNH): "Mt. Hsüeh Shan [雪山] / 3,400~3,600 m / Tái-chung Hsien [h, w] // 22.VI. [h] 19 [p] 61 [h] / S. Ueno [p, w]" (it belong to form *uenoi*); 1♂ (KMNH): "(Taiwan) / Alishan [阿里山] / Yushankou [玉山口] / Chiai Hsien [p, w] // May [p] 26 [h] .1971 [p] / K. Kanmiya [p, w]"; 1♀ (KMNH): "Ya Kou, [啞口] / Alt. Ca. 2800m. / Kao Hsiung Hsien, / S-Taiwan / 1.VIII.1986 / Col. K. Baba [p, w]"; they were determined by Kimoto in 1968. 1♀ (EUMJ): "(TAIWAN) / Tsuifeng [翠峰] / Nantou Hsien / 28. VI, 1970 / Y. Hor [p, w]"; it was determined by Kimoto in 1991.

Stenoluperus potanini. 1♀ (CAS): "Szechuan, W. China / Omei Shan: Shin-kai / -sze, 1,500 M. Aug. / 9. 1940. L. Gressitt [p, w]"; 1♂ (BPBM): "near Mupin / 7000-1300 ft [p] / Jul.6-8, [h] '29 [p, w] // Szechuan / CHINA / DCGraham [p, w] // US [p, w]"; 1♂ (BPBM): "nr Mupin / Jul.7.1929 / 12,300 ft. [p, w] // Szechuan / CHINA / DCGraham [p, w] // ILL [p, y] // N48 [h, w]"; 1♀ (BPBM): "Washan [p] / 7-26-25 [h] / Szechuan [p, w] // China / Alt [p] 11,000 ft [h, w] // DCGraham / collector [p, w] // US [p, w]"; 1♀ (BPBM): "Szechuan, W. China / Omei Shan: S. side. / 2,000-1,000 M. Aug. / 12. 1940. L. Gressitt [p, w] // N62 [h, w] // ILL [p, y]"; 1♀ (BPBM): "Szechuan, W. China / Nien-hwo-shih to / summit. Omei Shan / 2,000-3,060 M. Aug / 10. 1940. L. Gressitt [p, w]"; they was determined by Gressitt & Kimoto in 1962. 1♂ (KMNH): "Mt. HOHUAN [合歡山] / TAIWAN / 3.V.1982 / T. ITO [p, y]; 1♀ (KMNH): "Ho Huan Shan, [合歡山] / Alt. 3200m. / Nan Tow Hsien. / M-Taiwan / 6.VIII.1986 / Col. K. Baba [p, w]" (it should belong to form *uenoi*); both were determined by Kimoto in 1991. 1♂, 1♀ (KMNH): "(FORMOSA) / Lake Yenyanfu [鴛鴦湖] / Ilan Hsien / 29, IV 1982 / N. Ohbayashi leg. [p, w]"; both were determined by Kimoto in 1987.

Stenoluperus tibialis. 1♂ (CAS): "Szechuan, W. China / Omei Shan: below / Shin-kai-sze, alt. 1,400-1,000 M. Aug. / 17. 1940. L. Gressitt [p, w]"; 1♀ (BPBM): "Szechuan, W. China / Nien-hwo-shih to / summit. Omei Shan / 2,000-3,060 M. Aug / 10. 1940. L. Gressitt [p, w]"; both were determined by Gressitt & Kimoto in 1962. 2♂♂, 2♀♀ (KMNH): "(Taiwan) / Alishan [阿里山] / Chiai Hsien [p, w] // May [p] 25 [h] .1971 [p] / K. Kanmiya [p, w]"; they were determined by Kimoto in 1973

Description. Male. Body size, relative lengths of antennomeres, and color patterns extremely variable, separated into six forms:

Form A (formerly identified as *M. uenoi*): Length 3.1–3.5 mm. General color metallic blue except antenna, leg, and abdomen black (Figs 10–11). Elytron with longitudinal ridges. Antenna 0.8X as long as body, four apical antennomeres wide, ratio of length of antennomeres II to XI about 0.6 : 1.0 : 1.2 : 1.4 : 1.4 : 1.5 : 1.5 : 1.4 : 1.2 : 1.6; ratio of length to width from antennomeres II to XI about 1.8 : 3.0 : 3.6 : 4.0 : 3.7 : 3.4 : 2.7 : 3.0 : 2.9 : 3.9 (Fig. 20). Some individuals with longer antenna, as long



Figures 10–15. *Mandarella uenoi*, color variations, all at same scale. **10** Form A, dorsal view **11** Same, ventral view **12** Form B, dorsal view **13** Same, ventral view **14** Form C, dorsal view **15** Same, ventral view.

as body, four apical antennomeres slender, ratio of length of antennomeres II to XI about 0.6 : 1.0 : 1.3 : 1.6 : 1.3 : 1.6 : 1.4 : 1.4 : 1.3 : 1.7; ratio of length to width from antennomere III to XI 1.8 : 3.0 : 4.0 : 4.7 : 4.1 : 4.6 : 4.2 : 4.2 : 3.9 : 4.1 (Fig. 21).

Form B (formerly identified as *M. potanini*): Similar to form A, but body larger, length 3.7–4.1 mm; elytron without longitudinal ridges (Figs 12–13). Antennae longer, 1.2X longer than body, antennomere II subequal to III, antennomeres III-XI extremely slender, ratio of length of antennomeres II to XI about 0.7 : 1.0 : 2.6 : 2.7 : 2.5 : 2.5 : 2.5 : 2.3 : 2.2 : 2.3; ratio of length to width from antennomeres II to XI 1.6 : 2.2 : 6.2 : 6.5 : 6.2 : 6.1 : 6.1 : 6.6 : 6.2 : 6.5 (Fig. 22).

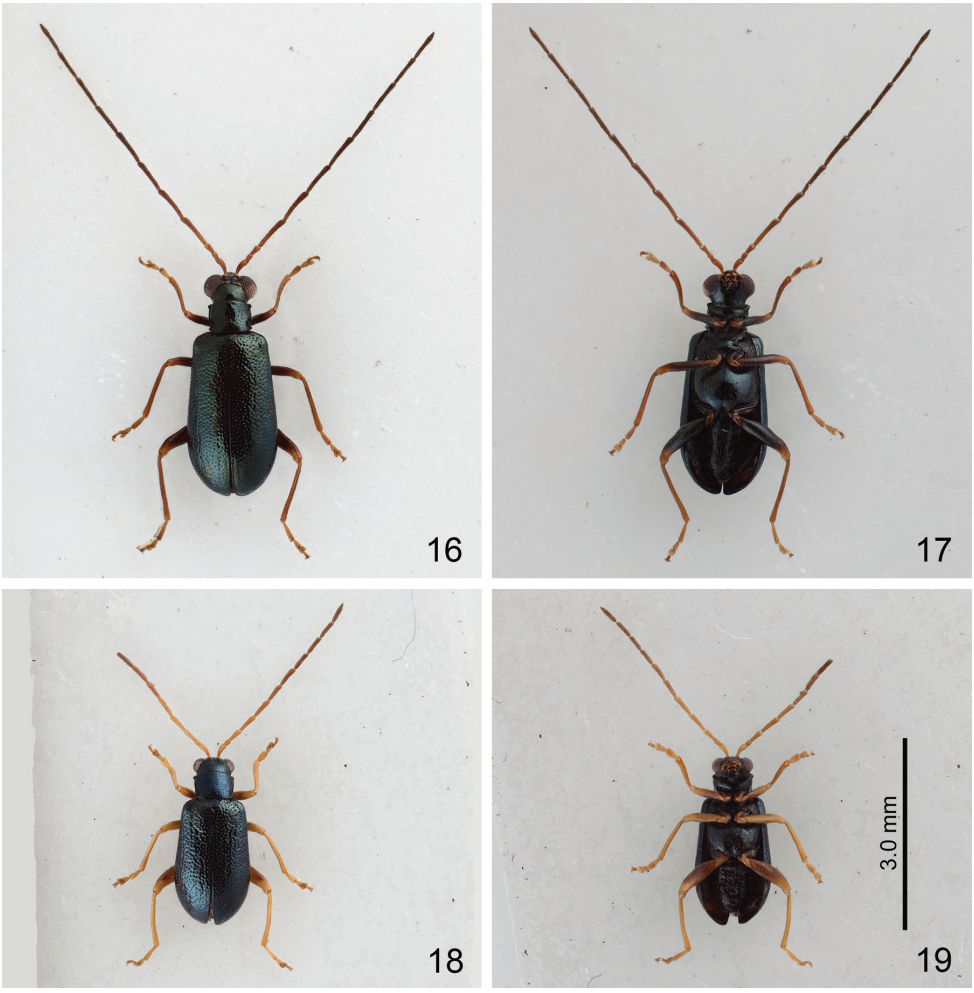
From C (formerly identified as *M. nipponensis*): Length 3.8–4.1 mm. Similar to form B (Figs 14–15); antennae 1.2X longer than body, antennomere III much longer than II, III to XI extremely slender, ratio of length of antennomeres II to XI about 0.4 : 1.0 : 1.5 : 1.7 : 1.6 : 1.6 : 1.5 : 1.5 : 1.4 : 1.5; ratio of length to width from antennomere II to XI 1.7 : 4.1 : 6.1 : 6.9 : 7.0 : 7.0 : 6.8 : 6.7 : 6.4 : 6.8 (Fig. 23).

Form D (formerly identified as *M. tibialis*): General color metallic blue but antennae and legs yellowish brown, coxa and femora metallic blue except apex (Figs 16–17). Length 3.4–3.7 mm. Antennae extremely long, about 1.5X longer than body, antennomere II subequal to III, III to X extremely slender; ratio of length of antennomeres II to XI 1.0 : 1.0 : 3.9 : 3.8 : 3.8 : 3.9 : 3.8 : 3.9 : 3.5 : 3.9; ratio of length to width from antennomeres II to XI 1.7 : 1.7 : 6.5 : 6.3 : 7.3 : 7.5 : 7.3 : 7.5 : 6.8 : 7.5 (Fig. 24). Some individuals with antennomere III much longer than II, III to X extremely slender; ratio of length of antennomeres II to XI 0.5 : 1.0 : 1.4 : 1.5 : 1.4 : 1.5 : 1.5 : 1.5 : 1.4 : 1.6; ratio of length to width from antennomeres II to XI 1.8 : 3.9 : 6.3 : 6.5 : 6.1 : 6.5 : 7.5 : 7.5 : 6.7 : 8.0 (Fig. 25).

Form E (formerly identified as *M. kimotoi*): General color metallic blue but antennae and legs yellowish brown (Figs 18–19). Elytra with longitudinal ridges. Length 3.0–3.1 mm, antennae 0.9X as long as body, ratio of length of antennomeres II to XI about 0.5 : 1.0 : 1.3 : 1.6 : 1.5 : 1.5 : 1.5 : 1.4 : 1.3 : 1.6; ratio of length to width from antennomeres II to XI 1.2 : 2.9 : 4.1 : 4.8 : 4.5 : 4.7 : 4.5 : 4.2 : 4.1 : 4.8 (Fig. 26).

Form F (formerly identified as *M. taiwana*): Similar to form E, but larger, length 3.4–3.7 mm. Antennae longer, about 1.1X longer than body; antennomere III much longer than III, III to X extremely slender; ratio of length of antennomeres II to XI 0.4 : 1.0 : 1.5 : 1.6 : 1.5 : 1.6 : 1.5 : 1.5 : 1.4 : 1.6; ratio of length to width from antennomeres II to XI 1.5 : 3.9 : 6.1 : 6.4 : 6.5 : 6.7 : 6.3 : 7.1 : 6.7 : 6.7 (Fig. 27).

Pronotum 1.4–1.6 times as broad long, quadrate, disc with scattered fine punctures, sometimes with feeble lateral depressions. Elytra 1.7–1.8 times as long as broad, parallel-sided, disc with dense, irregular, coarse punctures. First tarsomeres of front and middle legs extremely variable, extremely swollen, either elongate swollen or apically swollen. Posterior margin of last abdominal ventrite rounded, with two small incisions. Penis (Figs 28–29) extremely slender, about 9.1 times as long as broad; parallel-sided; tectum well sclerotized and apically tapering; almost straight in lateral view, curved near apex, apex truncate; endophallus with one longitudinal sclerite, apex bifurcate and forming two acute process, medially narrow.



Figures 16–19. *Mandarella uenoi*, color variations, all at same scale. **16** Form D, dorsal view **17** Ditto, ventral view **18** Form E, dorsal view **19** Ditto, ventral view.

Females. Length 3.5–3.7 mm, width 1.5–1.7 mm. Similar to male; head weakly constricted behind eyes. First tarsomeres of front and middle legs normal and not swollen. Gonocoxae (Fig. 30) slender, each gonocoxa apically widened, apex with eight setae; gonocoxae connected at middle, base abruptly and extremely widened. Ventrite VIII (Fig. 31) weakly sclerotized; apical margin with several short setae, disc with one pair of oblique dark stripes connected at apex, with several setae along outer margins of dark stripes; spiculum extremely long. Spermathecal receptaculum (Fig. 32) extremely swollen; pump strongly curved; sclerotized spermathecal duct long, weakly projecting into receptaculum.

Diagnosis. *Mandarella nipponensis*, *M. tibialis*, and *M. potanini* were misidentified as *M. uenoi*. *Mandarella nipponensis* possesses a wide and asymmetric penis (Figs

33–34). The penis of *M. tibialis* is wider in lateral view with a pair of stout acute processes at the base of the endophallic sclerites (Figs 35–36). The penis of *M. potanini* is wider and straight apically, and possesses a pair of serrate sclerites at the middle of the endophallic sclerites (Figs 37–38).

Host plants. Adults are abundant at high altitudes during spring and summer. The first author collected more two hundred specimens in three hours in Yuanfeng, Nantou county on June 12, 2015. They were resting on leaves of various plants and produced very small feeding scars.

Distribution. Endemic to Taiwan.

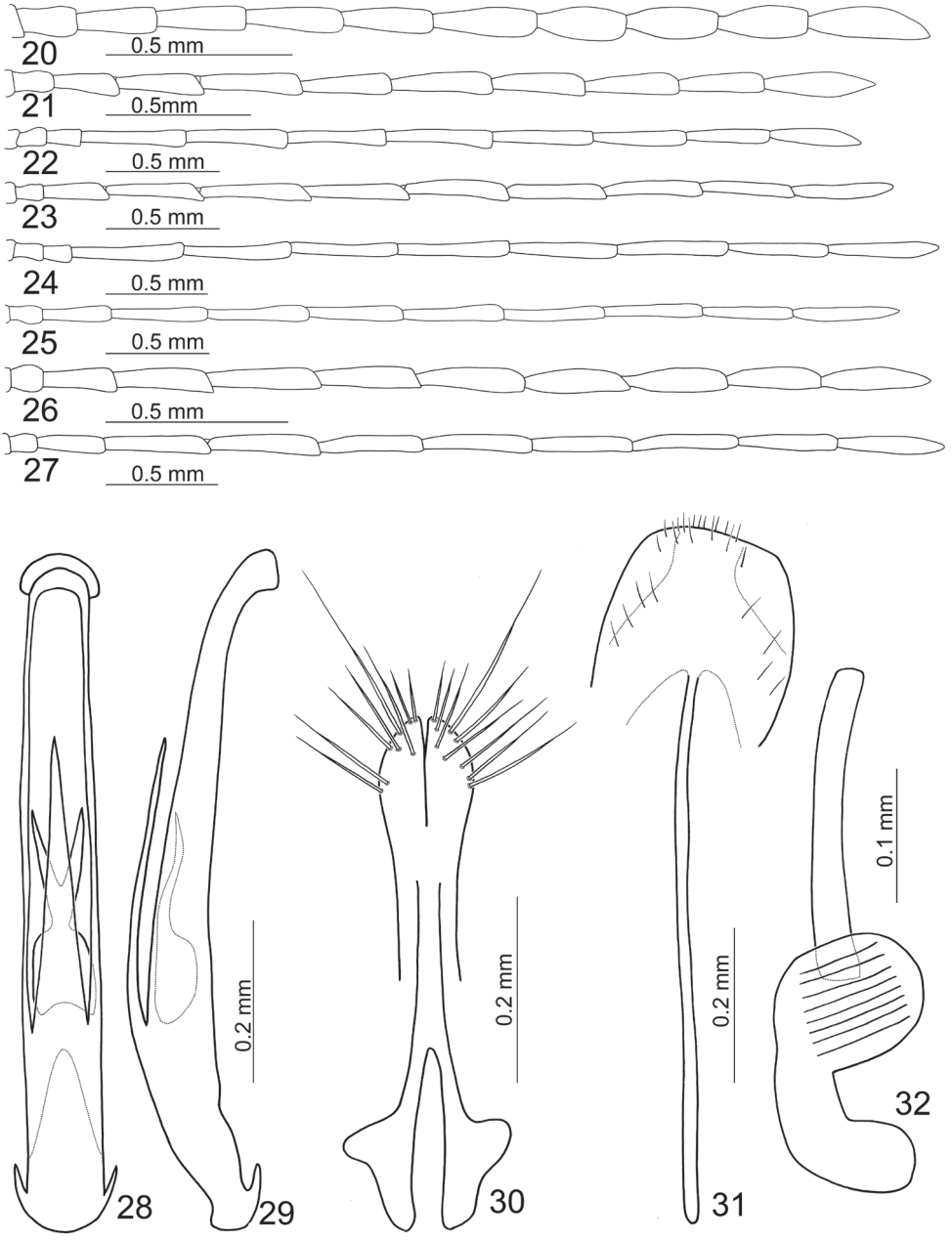
Other material examined. A total of 1008 specimens was examined (Supplementary file 2: *Mandarella uenoi*, specimens examined).

***Mandarella tsoui* sp. n.**
http://zoobank.org/546B8B49-6115-4E64-A683-6B70DAFD8075

Stenoluperus pallipes: Kimoto, 1969: 39 (Taiwan); Kimoto, 1989: 253 (additional records in Taiwan). **Misidentification**

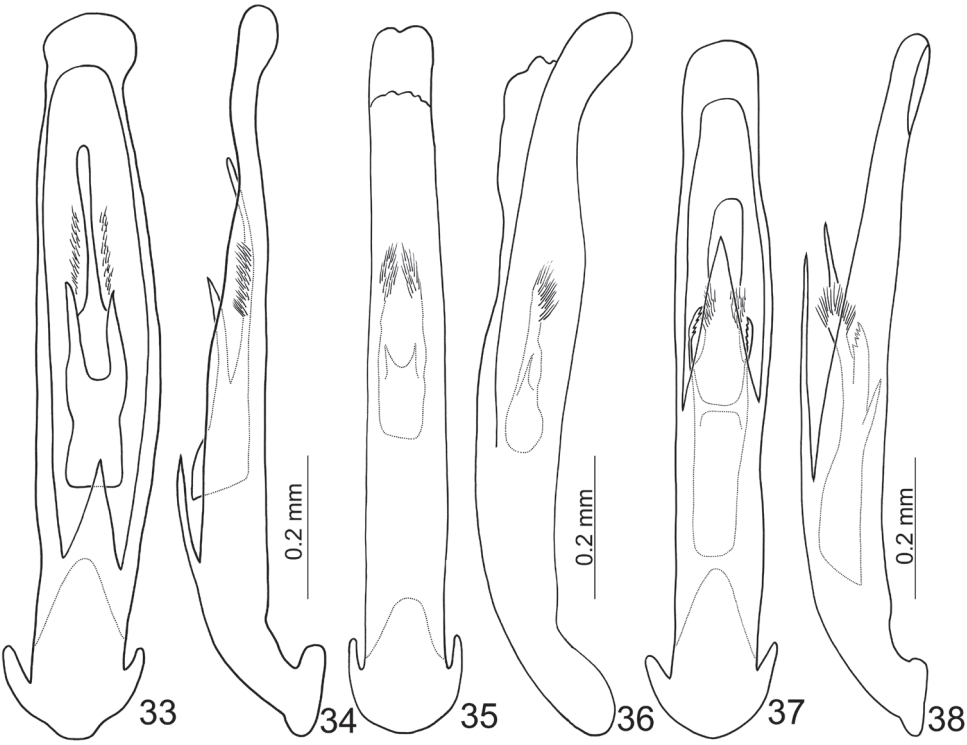
Type material of *Sternoluperus pallipes*. Holotype ♂ (CAS): "Suisapa, 1000 M. / Lichuan Distr. / W. Hupeh, China / VII- [p] 26 [h] -48 [p, w] // Gressitt & / Djou Collrs. [p, w] // HOLOTYPE [p] / Stenoluperus / pallipes ♂ [h] // Gressitt & Kimoto [p, r] // Stenoluperus / pallipes / G & K [h] / J. L. Gressitt det. [p, w] // NO34 [p, w]".

Type material (n = 354). Holotype ♂ (TARI): Nantou, Tsuifeng (翠峰), 2374 m, 21.IV.2015, leg. C.-F. Lee (TARI). Paratypes: **Chiayi**: 2♂♂, 1♀, Alishan (阿里山), 2216 m, 5-9.VIII.1981, leg. L. Y. Chou & S. C. Lin (TARI); 1♂, Fenchihu (奮起湖), 1400 m, 18.V.2014, leg. W.-C. Liao (TARI); 2♂♂, 5♀♀, Shihshan channel (石山引水道), 2300 m, 23.XI.2013, leg. W.-C. Liao (TARI); **Hsinchu**: 2♂♂, Kuanwu (觀霧), 2000 m, 30.IV.2010, leg. M.-H. Tsou (TARI); 1♂, Mamei (馬美), 1560 m, 18.V.2008, leg. S.-F. Yu (TARI); **Hualien**: 1♂, Kuanyuan (關原), 2374 m, 2.VII.2008, leg. M.-H. Tsou (TARI); 1♀, Piliu (碧綠), 2150 m, 13.VI.2014, leg. C.-F. Lee (TARI); 1♂, 1♀, Tayuling (大禹嶺), 2560 m, 9-16.VI.1980, leg. K. S. Lin & B. H. Chen (TARI); 1♂, same locality, 12-15.IX.1980, leg. K. S. Lin & C. H. Wang (TARI); 4♂♂, 1♀, same locality, 6-9.IX.1983, leg. L. Y. Chou & K. C. Chou (TARI); 1♀, Tzuen (慈恩), 2000 m, 12.VII.2014, leg. M.-H. Tsou (TARI); **Ilan**: 6♂♂, 2♀♀, Ssuyuanyakou (思源啞口), 1948 m, 28.IV.2009, leg. M.-H. Tsou (TARI); **Kaohsiung**: 3♀♀, Chungchihkuan (中之關), 1930 m, 16.IV.2012, leg. L.-P. Hsu; 1♀, same locality, 13.X.2012, leg. L.-P. Hsu (TARI); 1♀, Tengchih (藤枝), 1550 m, 2-5.VI.2008, leg. C.-F. Lee (TARI); 1♂, same locality, 26.V.2009, leg. C.-F. Lee (TARI); 3♂♂, 1♀, same locality, 13.IV.2013, leg. W.-C. Liao (TARI); 1♂, same locality, 8.VI.2013, leg. W.-C. Liao (TARI); 1♂, Tona (多納), 500 m, 3.II.2013, leg. W.-C. Liao (TARI); **Nantou**: 1♂, Chingching (清境), 1750 m, 27.VII.2013, leg. W.-C. Liao (TARI); 1♀, Meifeng (梅峰), 2100 m, 20-22.VI.1979, leg. K. S. Lin & B. H.



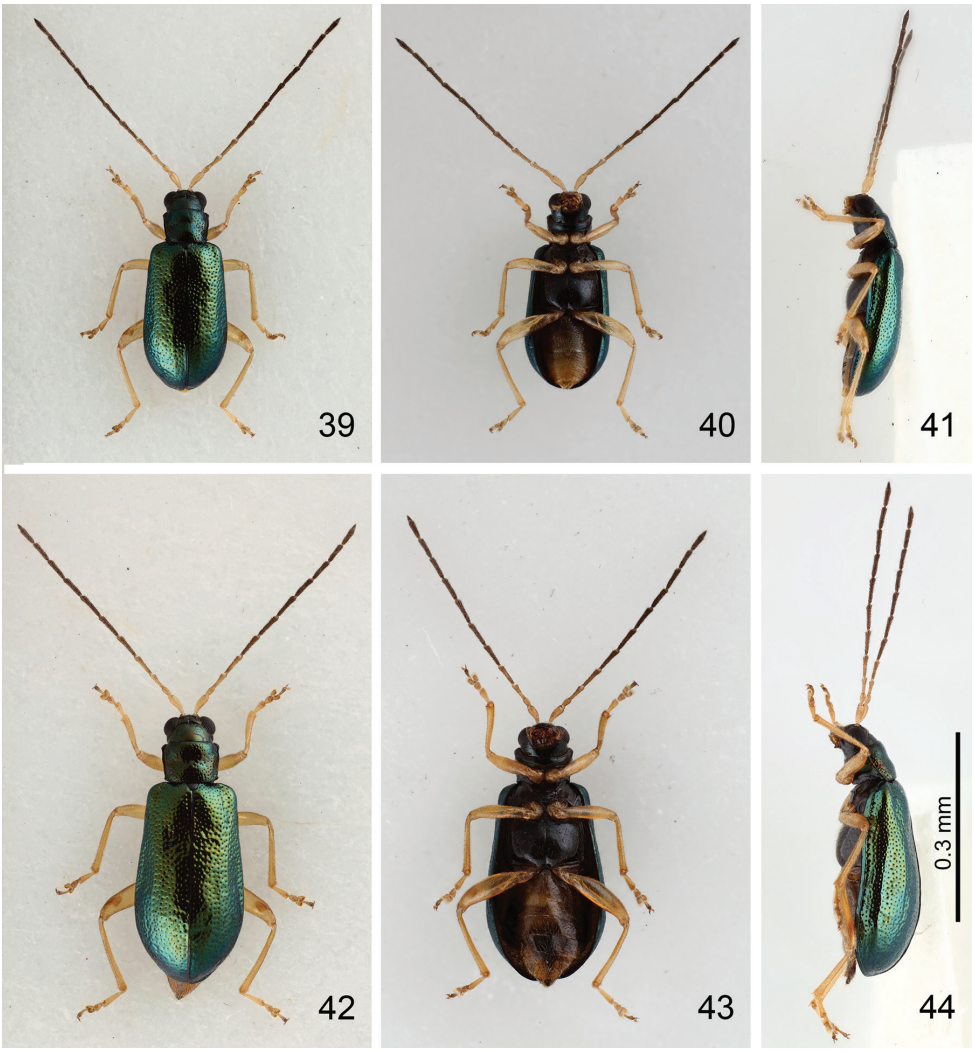
Figures 20–32. Diagnostic characters of *Mandarella uenoi*. **20** Male antenna, form A, typical **21** Male antenna, form A, elongate **22** Male antenna, form B **23** Male antenna, form C **24** Male antenna, form D, typical **25** Male antenna, form D, variation **26** Male antenna, form E **27** Male antenna, form F **28** Penis, dorsal view **29** Penis lateral view **30** Gonocoxae **31** Ventrite VIII **32** Spermatheca.

Chen (TARI); 1♂, same locality, 26.VIII.1980, leg. K. S. Lin & C. H. Wang (TARI); 2♂♂, same locality, 28-29.VIII.1981, leg. L. Y. Chou & S. C. Lin (TARI); 1♂, 1♀, same locality, 8-11.V.1984, leg. K. C. Chou & C. C. Pan (TARI); 3♂♂, same locality, 17.VI.2010, leg. C.-F. Lee (TARI); 1♂, 2♀♀, same locality, 3.VII.2008, leg. M.-H. Tsou (TARI); 1♂, same locality, 20.IV.2011, leg. C.-F. Lee (TARI); 3♂♂, Nenkao trail (能高古道), 2600 m, 12.VII.2014, leg. J.-C. Chen (TARI); 2♂♂, 2♀♀, Nenkaoshan (能高山), 2860 m, 18.X.2011, leg. J.-C. Chen (TARI); 1♂, 3♀♀, Sungkang (松岡), 2000 m, 15-17.VIII.1984, leg. K. C. Chou (TARI); 1♂, 1♀, same locality, 13-15.IX.1984, leg. K. S. Lin & S. C. Lin (TARI); 1♂, same locality, 2.VII.2008, leg. M.-H. Tsou (TARI); 6♂♂, 9♀♀, Tatachia (塔塔加), 2610 m, 5.X.2008, leg. M.-H. Tsou (TARI); 1♂, 1♀, same locality, 9.VI.2009, leg. C.-F. Lee (TARI); 2♂♂, 1♀, 20.VII.2009, leg. S.-F. Yu (TARI); 1♂, same locality, 21.IX.2009, leg. M.-H. Tsou (TARI); 1♂, same locality, 30.X.2009, leg. C.-F. Lee (TARI); 4♂♂, 1♀, same locality, 17.XI.2009, leg. C.-F. Lee (TARI); 2♂♂, same locality, 19.XI.2009, leg. H. Lee (TARI); 2♂♂, same locality, 29.XII.2009, leg. M.-H. Tsou (TARI); 1♂, same locality, 27.IV.2010, leg. C.-F. Lee (TARI); 2♂♂, same locality, 9.VII.2014, leg. C.-F. Lee (TARI); 1♂, same locality, 13.VII.2014, leg. W.-C. Liao (TARI); 1♀, same locality, 1.VII.2015, leg. J.-C. Chen (TARI); 3♂♂, 2♀♀, Tsuifeng (翠峰), 2374 m, 21.VI.1979, leg. K. S. Lin & B. H. Chen (TARI); 3♂♂, 1♀, same locality, 3.VI.1980, leg. L. Y. Chou & C. C. Chen (TARI); 2♂♂, 5♀♀, same locality, 8.V.1981, leg. K. S. Lin & S. C. Lin (TARI); 15♂♂, 21♀♀, same locality, 25-27.VI.1981, leg. K. S. Lin & W. S. Tang (TARI); 2♂♂, 3♀♀, same locality, 1-3.VIII.1981, leg. T. Lin & W. S. Tang (TARI); 5♂♂, 1♀, same locality, 27.VIII.1981, leg. L. Y. Chou & S. C. Lin (TARI); 1♂, 1♀, same locality, 8.XI.1981, leg. S. C. Lin & W. S. Tang (TARI); 1♀, same locality, 23.V.1982, leg. L. Y. Chou (TARI); 7♂♂, 1♀, same locality, 1-3.IX.1982, leg. L.-Y. Chou & K. C. Chou (TARI); 2♂♂, same locality, 20.IV.1983, K. C. Chou & S. P. Huang (TARI); 3♂♂, same locality, 9.V.1984, leg. K. C. Chou & C. C. Pan (TARI); 2♂♂, 1♀, same locality, 5.VIII.1984, leg. K. S. Lin (TARI); 4♂♂, 6♀♀, same locality, 15-16.VIII.1984, leg. K. C. Chou (TARI); 1♂, same locality, 9.IV.2014, leg. C.-F. Lee (TARI); 1♂, Tungpu (東埔), 1200 m, 25-29.IX.1980, leg. L. Y. Chou & T. Lin (TARI); 7♂♂, 9♀♀, 28.IV.-2.V.1981, leg. T. Lin & C. J. Lee (TARI); 1♀, same locality, 22-25.XI.1982, leg. K. C. Chou & S. P. Huang (TARI); 1♂, 2♀♀, same locality, 20-24.VI.1983, leg. K. C. Chou & C. Y. Wong (TARI); 6♂♂, 3♀♀, same locality, 16-20.IV.1984, leg. K. C. Chou & C. H. Yung (TARI); 1♂, same locality, 23-27.VII.1984, leg. K. C. Chou & C. H. Yang (TARI); 1♀, Wushe (霧社), 1148 m, 30.VIII.-2.IX.1982, leg. L. Y. Chou & K. C. Chou (TARI); 13♂♂, 15♀♀, 19-22.IV.1983, leg. K. C. Chou & S. P. Huang (TARI); 1♂, 3♀♀, same locality, 7.V.1984, leg. K. C. Chou & C. C. Pan (TARI); **Pingtung**: 1♂, Jinshuiying (浸水營), 1450 m, 22.IX.2011, leg. J.-C. Chen (TARI); 1♀, Machia (瑪家), 1070 m, 17.III.2013, leg. W.-C. Liao (TARI); 1♂, Peitawushan (北大武山), 1100 m, 13.V.2010, leg. J.-C. Chen (TARI); 3♂♂, same locality, 21.III.2011, leg. J.-C. Chen (TARI); 1♂, same locality, 22.IX.2012, leg. J.-C. Chen (TARI); 1♂, Tahanshan (大漢山), 1200 m, 16.II.2013, leg. Y.-T. Chung (TARI); 2♂♂, Wutai (霧台), 1000 m,



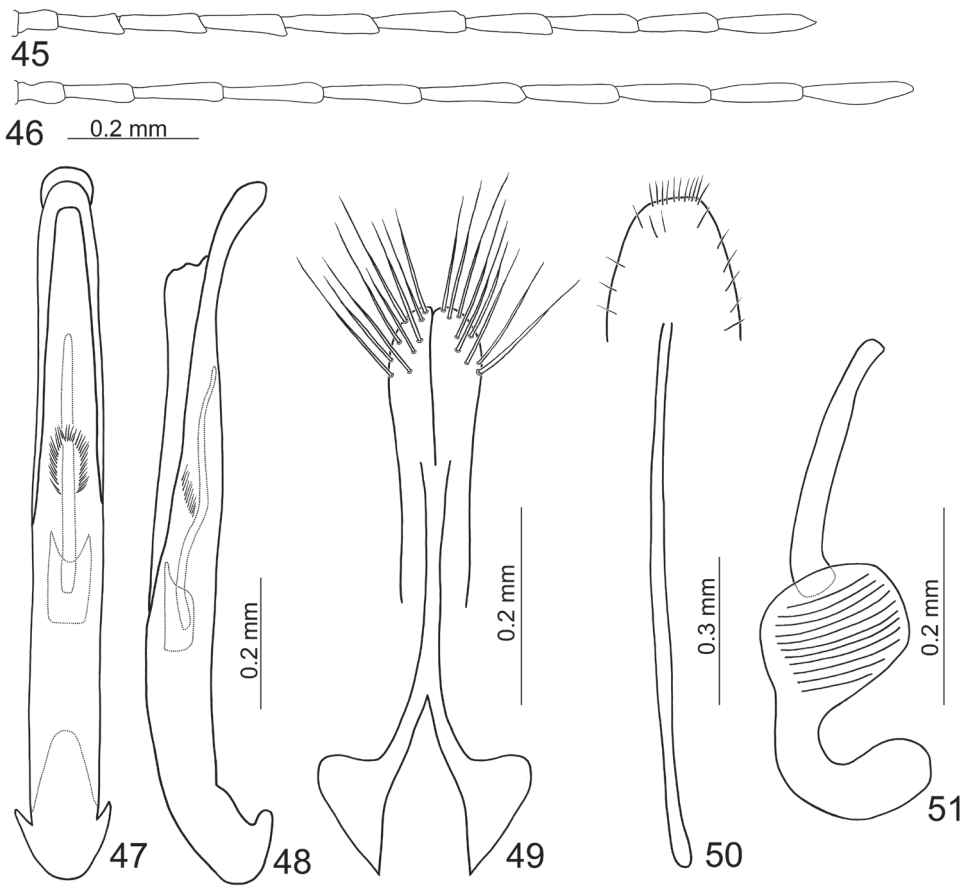
Figures 33–38. Penis of *Mandarella* species. **33** *M. nipponensis*, dorsal view **34** Same, lateral view **35** *M. tibialis*, dorsal view **36** Same, lateral view **37** *M. potanini*, dorsal view **38** Same, lateral view.

22.III.2011, leg. J.-C. Chen (TARI); **Taichung**: 5♂♂, 3♀♀, Kukuan (谷關), 730 m, 14-17.X.1980, leg. K. S. Lin & C. H. Wang (TARI); 4♂♂, 13♀♀, Lishan (梨山), 2000 m, 26.VI.1979, leg. K. S. Lin & L. Y. Chou (TARI); 1♂, 2♀♀, Tahsuehshan (大雪山), 2600 m, 22.IX.2007, leg. M.-H. Tsou (TARI); 1♂, 1♀, Wuling (武陵), 1900 m, 27-29.VI.1979, leg. K. S. Lin & L. Y. Chou (TARI); **Taitung**: 1♀, Hsiang-yang (向陽), 2320 m, 31.V.2011, leg. J.-C. Chen (TARI); 1♂, 1♀, same locality, 12.VII.2012, leg. J.-C. Chen (TARI); 1♂, 2♀♀, same locality, 9.V.2013, leg. J.-C. Chen (TARI); 2♀♀, same locality, 28.VI.2013, leg. J.-C. Chen (TARI); 1♂, 2♀♀, same locality, 22.XII.2013, leg. W.-C. Liao (TARI); 1♂, same locality, 28.III.2014, leg. J.-C. Chen (TARI); 1♀, same locality, 18.VII.2014, leg. W.-C. Huang (TARI); 4♂♂, 2♀♀, Liyuan (栗園), 1793 m, 8.VII.2010, leg. J.-C. Chen (TARI); 2♂♂, 1♀, 19.VI.2013, leg. B.-X. Guo (TARI); 5♂♂, 4♀♀, same locality, 19.VI.2013, leg. Y.-T. Chung (TARI); 1♂, same locality, 19.IV.2014, leg. W.-C. Huang (TARI); 7♂♂, 6♀♀, Motien (摩天), 1546 m, 5.X.2010, leg. C.-F. Lee (TARI); 1♂, same locality, 23.V.2011, leg. C.-F. Lee (TARI); 2♂♂, same locality, 19.VI.2011, leg. C.-F. Lee (TARI). 1♂ (EUMJ), labeled: "(TAIWAN) / Sungkang~ / Meifeng (2044~2127) / Nantow Co. / 18.V.1969 / S. Hisamatsu [p, w] // 松崗~梅峰 [h, w] // Sternoluperus / pallipes / Gressitt & Kimoto [h] / Det. S. Kimoto, 19 [p] 90 [h, w]".



Figures 39–44. *Mandarella tsoui* sp. n., all at the same cale. **39** Male, dorsal view **40** Same, ventral view **41** Same, lateral view **42** Female dorsal view **43** Same, ventral view **44** Same, lateral view.

Description. Male. Length 3.3–4.1 mm, width 1.3–1.7 mm. General color (Figs 39–41) bluish metallic; mouth parts, legs and abdomen yellowish; antennae dark brown but three or four basal antennomeres paler. Head weakly constricted behind eyes; antennae (Fig. 45) filiform and extremely slender, 1.2 times as body; ratio of length of antennomeres II to XI 0.6 : 1.0 : 1.3 : 1.3 : 1.5 : 1.4 : 1.3 : 1.4 : 1.3 : 1.6; ratio of length to width from antennomeres II to XI 2.0 : 3.2 : 4.3 : 4.2 : 4.7 : 4.6 : 4.3 : 4.4 : 4.0 : 5.3. Pronotum 1.3 times as broad long, quadrate, disc with scattered, coarse punctures, and with lateral depressions. Elytra 1.8 times as long as broad, parallel-sided, disc with dense, irregular, coarse punctures, and with depression at sides,



Figures 45–51. Diagnostic characters of *Mandarella tsoui* sp. n. **45** Male antenna, **46** Female antenna **47** Penis, dorsal view **48** Penis lateral view 49 Gonocoxae **50** Ventrite VIII **51** Spermatheca.

and longitudinal ridge present along depression. First tarsomeres of front and middle legs swollen. Posterior margin of last abdominal ventrite rounded, with two small incisions, basal margin irregularly serrate. Penis (Figs 47–48) extremely slender, about 10.3 times as long as broad; parallel-sided; tectum membranous; almost straight in lateral view, weakly curved near apex, apex narrowly rounded; endophallus with one extremely elongate sclerite, sinuate in lateral view; base dorsally covered by one transverse sclerite.

Female. Length 4.3–4.7 mm, width 1.8–1.9 mm. Similar to male (Figs 42–44); ratio of length of antennomeres II to XI 0.6 : 1.0 : 1.3 : 1.5 : 1.4 : 1.5 : 1.4 : 1.4 : 1.3 : 1.6; ratio of length to width from antennomeres II to XI 2.1 : 3.6 : 4.8 : 5.4 : 5.2 : 5.4 : 5.1 : 4.9 : 4.9 : 5.0 (Fig. 46). First tarsomeres of front and middle legs normal and not swollen. Gonocoxae (Fig. 49) slender, each gonocoxa apically widened, apex with nine setae; gonocoxae connected at middle, base abruptly and extremely widened. Ventrite

VIII (Fig. 50) weakly sclerotized; apical margin with several short setae, several long setae along lateral margin; spiculum extremely long. Spermathecal receptaculum (Fig. 51) extremely swollen; pump strongly curved; sclerotized spermathecal duct long, shallowly projecting into receptaculum.

Diagnosis. This new species is similar to *M. pallipes* but the latter lacks lateral depressions and ridges on the elytra.

Host plant. Adults are closely associated with *Stachyurus himalaicus* Hook. f. & Thomson ex Benth. (Stachyuraceae), which is sympatric with *Dercetina itoi* Kimoto, 1969 and *D. shirozui* Kimoto, 1969.

Etymology. This new species is named after Mr. Mei-Hua Tsou, a member of the TCRT and the first to collect this new species.

Distribution. Endemic to Taiwan.

Mandarella flaviventris (Chen, 1942)

Stenoluperus flaviventris Chen, 1942: 67 (China: Jiangxi); Gressitt and Kimoto 1963: 580 (China: Fujian); Chûjô 1965: 98 (Taiwan); Kimoto 1969: 40 (additional records in Taiwan); Kimoto 1987: 189 (additional records in Taiwan); Kimoto 1989: 253 (additional records in Taiwan); Kimoto 1991c: 12 (additional records in Taiwan).
Stenoluperus esakii Kimoto, 1969: 40. **New synonym**
Stenoluperus matsumurai Takizawa, 1978: 128. **New synonym**
Mandarella matsumurai: Medvedev 2012: 427.
Stenoluperus itoi Kimoto, 1991b: 116 (nec *Stenoluperus itoi* Chûjô, 1966).
Mandarella taiwanensis Medvedev, 2012: 427 (replacement name for *Stenoluperus itoi* Kimoto, 1991). **New synonym**

Type material. *Stenoluperus flaviventris*. The holotype was reportedly deposited at the Institute of Zoology, Chinese Academy of Sciences, China but could not be found (Yong-Ying Ruan, pers. comm. 8 October 2015).

Sternoluperus esakii. Holotype ♀ (KUEC): "[Formosa] / Hassenzan [八仙山] (Taichû-shû) / 13. Vii. 1932 / Teiso Esaki [p, w] // *Stenoluperus* / *esakii* / Kimoto, sp. n. [h, w] // HOLOTYPE [p, r]".

Stenoluperus itoi. Paratype: 1♂ (KMNH): "Mt. YUSHAN [玉山] / TAIWAN / 19. V. 1981 / N. ITO [p, y] // *Stenoluperus* / *itoi* / Kimoto, sp. n. [h] / Det. S. Kimoto, 19 [p] 91 [h, w] // PARATOPOTYPE [p, b]".

Stenoluperus matsumurai. Holotype ♂ (EIHU), holotype glued on the top of a triangular card; one front tibia and tarsi, antenna, and aedeagus also on the card: "Type [h, red letters, underside of triangular card] // Formosa / Matsumura [p, w] // (Japanese characters) / 21/IV1907 [h, underside of previous label] // Holo [h] –type [p] / *Stenoluperus* / *matsumurai* / Takizawa [h, r] // **Holotype** / Appended label by / ÔHARA, INARI, KANBE / SUZUKI and HIRONAGA / 2007 [p, w, with red band

along right side] / 0000003054 / Sys. Ent / Hokkaido Univ. / Japan [SEHU] [p, w]. Paratypes: 1♂, 1♀, glued on tops of triangular cards, mounted with the same pin as holotype, the male has the blackish abdomen.

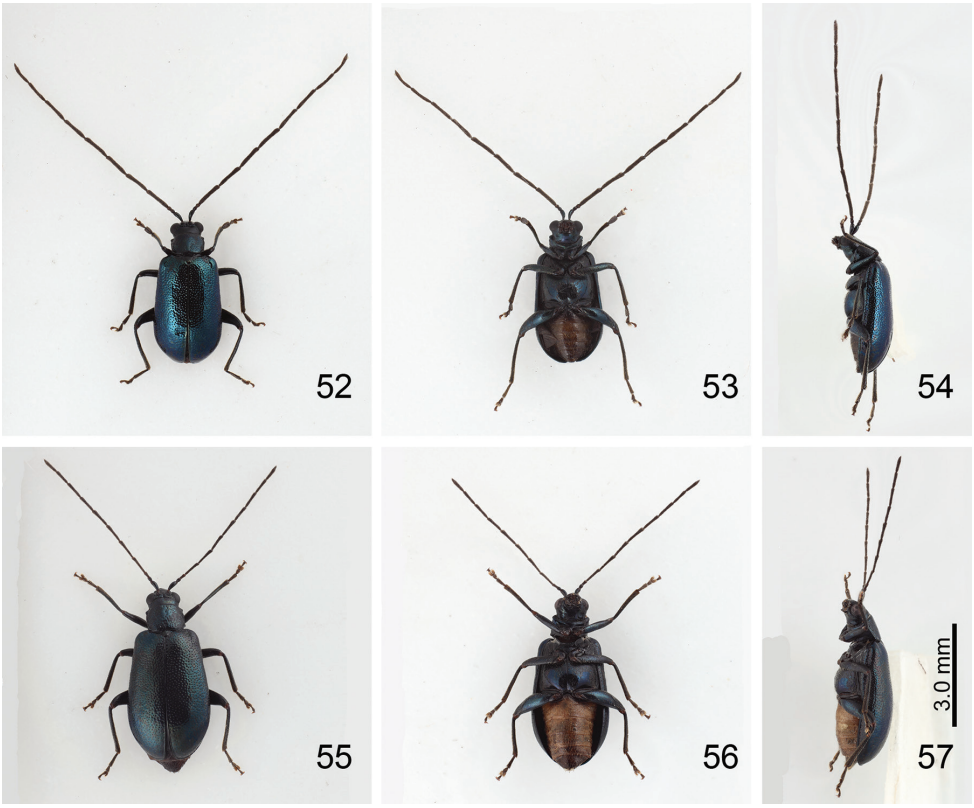
Voucher specimens. 1♀ (CAS): "FUKIEN, S. China / Shaowu: Tachulan / 1000 m. T. Maa [p, w] // Apr.30.1942 [h, w]"; 1♂, 1♀ (CAS): "FUKIEN, S. China / Shaowu: Tachulan / 1000 m. T. Maa [p, w] // Apr.17,1943 [h, w]"; 1♂ (BPBM): "FUKIEN, S. China / Shaowu: Tachulan [p] / IV.20. [h] 194 [p] 3 [h] T. Maa [p, w] // N52 [h, w] // ILL [p, w]"; 4♂♂ (BPBM), 2♀♀ (CAS): "FUKIEN, S. China / Shaowu: Tachulan / 1000 m. T. Maa [p, w] // Apr.27,1943 [h, w]"; they were determined by Gressitt & Kimoto in 1962. 1♂ (KMNH): " (Taiwan) / Alishan, [阿里山] 2300m / Chiayi Hsien [p, w] // 9. [h] iv.1965 [p] / Y. Hirashima [p, w] // Japan-U. S. / Co-op. Sci. / Programme [p, y]"; 1♀ (KMNH): " (Taiwan) / Meifeng [梅峰] / Nantou Hsien [h, w] // 18.V.1965 / B. S. Chang [h, w]"; 1♂ (CAS): "FORMOSA: / Arisan. [阿里山] / VIII-18-1947 / J. L. Gressitt [p, w] // L. Gressitt / Collection [p, w]"; they were determined by Kimoto in 1968. 1♂ (KMNH): " (Taiwan) / Alishan [阿里山] / Hsien [p, w] // May [p] 25 [h] .1971 [p] / K. Kanmiya [p, w]"; it was determined by Kimoto in 1973. 1♂ (KMNH): "ALISHAN [阿里山] / TAIWAN / 3. V. 1983 / T. ITO [p, y]", it was determined by Kimoto in 1987. 1♀ (EUMJ): "(TAIWAN) / Sungkang~ / Meifeng (2044~2127) / Nantow Co. / 18.V.1969 / S. Hisamatsu [p, w] // 松崗~梅峰 [h, w]"; it was determined by Kimoto in 1991.

Description. Color patterns and relative lengths of antennae separated into four forms:

Form G (formerly identified as *M. flaviventris*): General color (Figs 52–57) bluish metallic; antennae black and abdomen yellow. In male, antennae (Fig. 62) filiform and extremely slender, 1.4 times as body; ratio of length of antennomeres II to XI 1.2 : 1.0 : 4.5 : 5.0 : 5.0 : 5.4 : 5.2 : 5.2 : 4.5 : 5.0; ratio of length to width from antennomeres II to XI 1.5 : 1.1 : 5.0 : 5.6 : 5.6 : 6.0 : 5.8 : 5.8 : 5.7 : 6.4. In female, antennae shorter, as long as body (Fig. 63), antennomeres III relatively longer, ratio of length of antennomeres II to XI 0.9 : 1.0 : 2.6 : 2.8 : 2.6 : 2.7 : 2.4 : 2.4 : 2.1 : 2.4; ratio of length to width from antennomeres II to XI about 1.8 : 2.0 : 5.3 : 5.8 : 5.4 : 5.6 : 5.3 : 5.5 : 5.0 : 5.2.

Form H (formerly identified as M. *esakii*): Similar to form G, but antennae and legs dark brown (Figs 58–59).

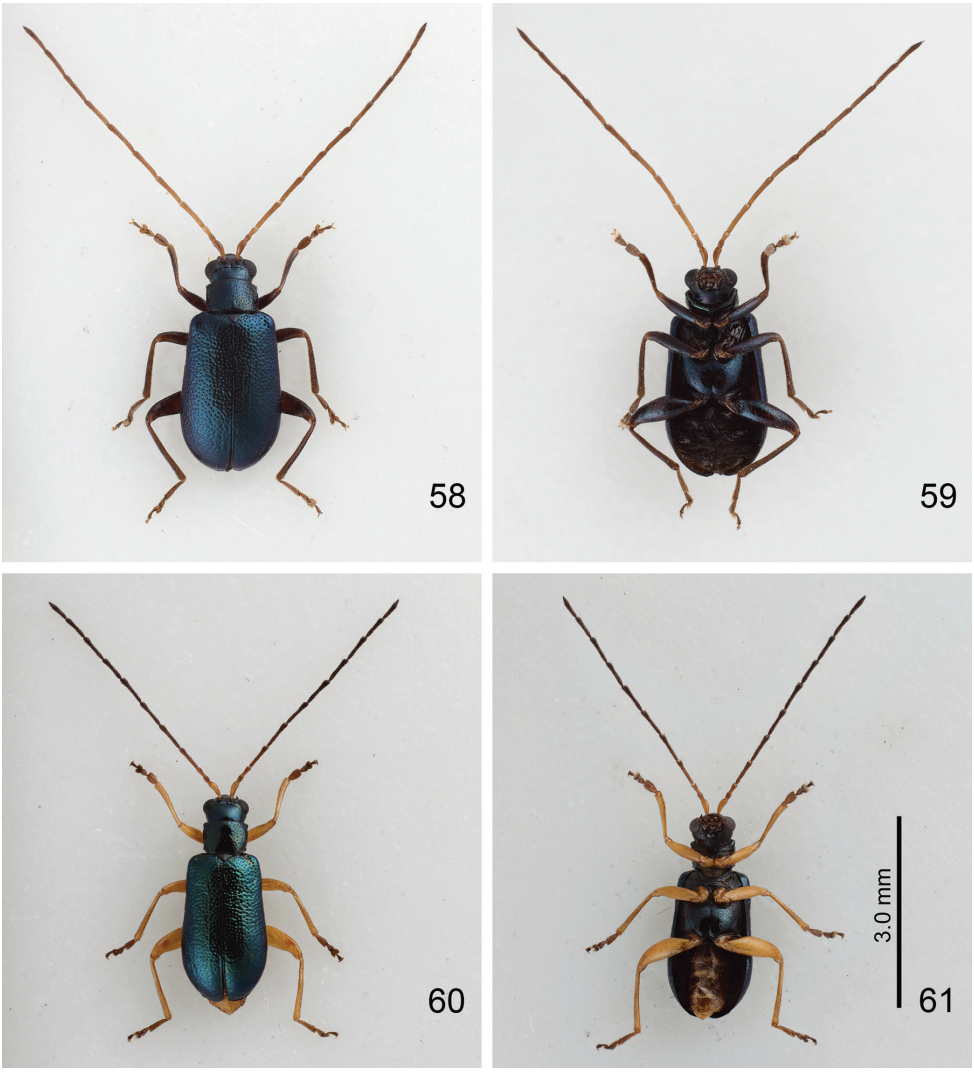
Form I (formerly identified as *M. matsumurai*): General color metallic blue but antennae, legs, and abdomen yellowish brown; seven apical antennomeres darkened antennomeres (Figs 60–61). In male, antennae (Fig. 64) 1.3× longer than body, antennomere II a little longer than III, III to X extremely slender, ratio of length of antennomeres II to XI 0.7: 1.0 : 2.1 : 2.4 : 2.2 : 2.3 : 2.1 : 2.2 : 1.9 : 2.1; ratio of length to width from antennomeres II to XI 1.7 : 2.6 : 5.6 : 6.2 : 5.7 : 6.1 : 5.6 : 5.8 : 5.0 : 5.4. In female, antennae shorter (Fig. 65), 0.9 times as long as body, antennomeres III relatively longer, ratio of length of antennomeres II to XI 0.8 : 1.0 : 2.0 : 2.2 : 2.1 : 2.1 : 1.8 : 1.8 : 1.5 : 1.9; ratio of length to width from antennomeres II to XI 2.0 : 2.4 : 4.7 : 5.3 : 5.1 : 4.9 : 4.5 : 4.5 : 3.9 : 4.7.



Figures 52–57. *Mandarella flaviventris*, form G, all at same cale. **52** Male, dorsal view **53** Same, ventral view **54** Same, lateral view **55** Female dorsal view **56** Same, ventral view **57** Same, lateral view.

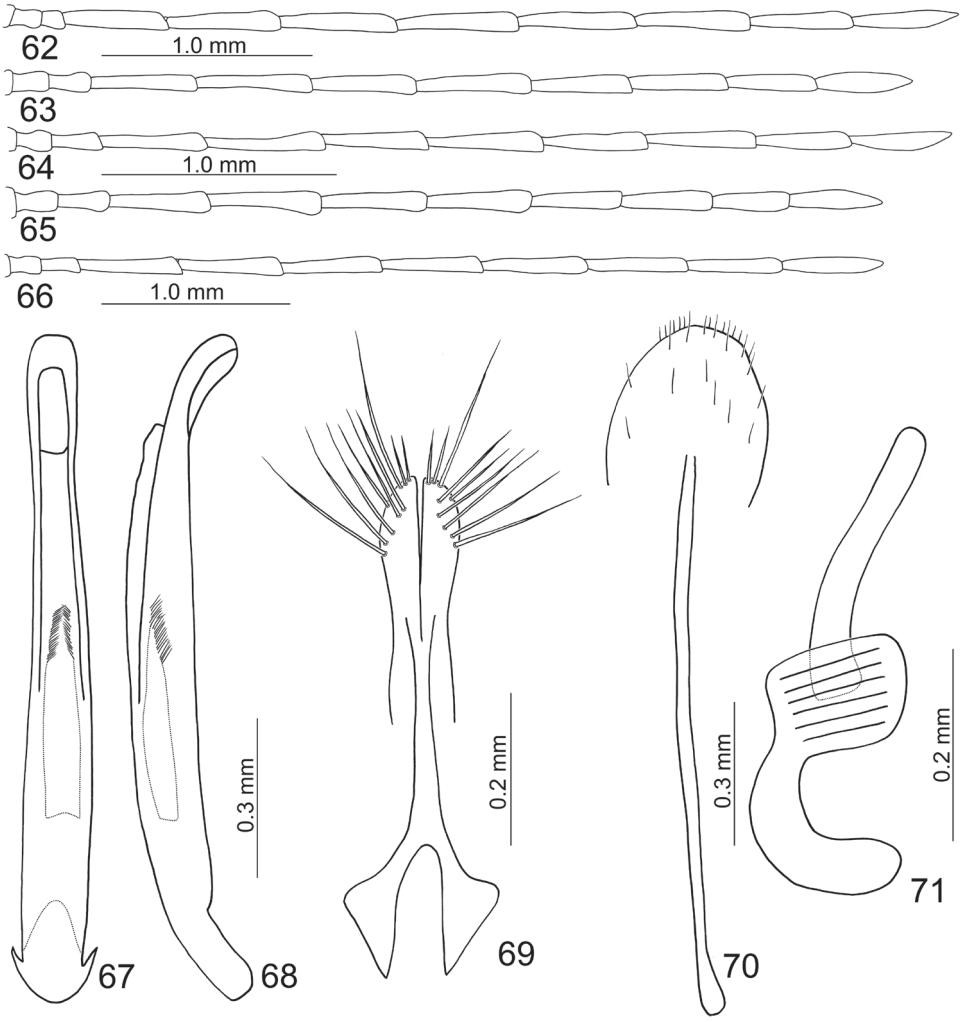
Form J (formerly identified as *M. taiwanensis*): Color pattern similar to form I, but abdomen blackish brown. In male, antennae 1.3X longer than body (Fig. 66), antennomere II a little longer than III, III to X extremely slender, ratio of length of antennomeres II to XI about 0.8 : 1.0 : 2.8 : 2.8 : 2.7 : 2.7 : 2.8 : 2.7 : 2.5 : 2.7; ratio of length to width from antennomeres II to XI 1.7 : 2.3 : 6.4 : 6.4 : 6.1 : 6.1 : 6.4 : 6.1 : 5.7 : 6.1.

Male. Length 3.6–4.6 mm, width 1.6–2.1 mm. Head strongly constricted behind eyes. Pronotum 1.4 times as broad long, quadrate, disc with dense and coarse punctures as on elytra, lacking lateral depressions. Elytra 1.7 times as long as broad, parallel-sided, disc with dense, irregular, coarse punctures. First tarsomeres of front and middle legs swollen. Posterior margin of last abdominal ventrite truncate, with two small incisions. Penis (Figs 67–68) extremely slender, about 10.1 times as long as broad; parallel-sided; tectum membranous; almost straight in lateral view, weakly curved near apex, apex narrowly rounded, ventral disc depressed near apex; endophallus with one elongate sclerite, weakly sclerotized, with dense setae above middle, and small teeth at middle; straight in lateral view.



Figures 58–61. *Mandarella flaviventris*, color variation, all at same scale. **58**. Form H, male, dorsal view **59** Same, ventral view **60** Form I, male, dorsal view **61** Same, ventral view.

Female. Length 3.9–5.4 mm, width 1.9–2.6 mm. Similar to male; but head weakly constricted behind eyes. First tarsomeres of front and middle legs normal and not swollen. Gonocoxae (Fig. 69) slender, each gonocoxa apically widened, apex with nine setae; gonocoxae connected at middle, base abruptly and extremely widened. Ventrite VIII (Fig. 70) weakly sclerotized; apical margin with several short setae, disc with several long setae scattered; spiculum extremely long. Spermathecal receptaculum (Fig. 71) extremely swollen; pump strongly curved; sclerotized spermathecal duct long, deeply projecting into receptaculum.



Figures 62–71. Diagnostic characters of *Mandarella flaviventris*. **62** Male antenna, form G **63** Female antenna, form G **64** Male antenna, form I **65** Female antenna form I **66** Male antenna, form J **67** Penis, dorsal view **68** Same, lateral view **69** Gonocoxae **70** Ventrite VIII **71** Spermatheca.

Diagnosis. Although *Mandarella flaviventris* is highly variable in color patterns, it is characterized by the small third antennomere (3rd antennomeres ≤1.3 times as long as 2nd antennomere). Some black individuals of *M. uenoi* also have small 3rd antennomeres, similar to *M. flaviventris* but their abdomens are black (yellow abdomens in *M. flaviventris*).

Host plants. Like *Mandarella uenoi*, adults rested on leaves of various plants and left small feeding scars.

Distribution. China (Fujian, Jiangxi), Taiwan.

Other material examined. Totally 717 specimens were studied (Suppl. material 3: *Mandarella flaviventris*, specimens examined).

Key to the Taiwanese species of *Mandarella* Duvuvier

1 Lateral depression and ridge present on each elytron; antennae, legs, and abdomen yellow, and antennomere III much longer than antennomere II (1.6 times) ..... ***M. tsoui* sp. n.**

– Lateral depression and ridge absent from each elytron; individuals with yellow antennae, legs, and abdomen, antennomere III slightly longer than antennomere II (1.3 times) (form I of *S. flavivientris*) **2**

2 Individuals with black or blackish legs, abdomen black, in males antennomere III from slightly longer to much longer than antennomeres II (≥1.3 times); individuals with yellow legs, antennomere III much longer than antennomere II (≥2.0 times); tectum of penis apically tapering, apex of endophallic sclerite bifurcate and acute..... ***M. uenoi* (Kimoto, 1969)**

– Individuals with black or blackish legs, abdomen yellow, in males antennomere III shorter than antennomere II (0.8 times); individuals with yellow, antennomere III slightly longer than antennomere II (1.3 times); tectum of penis membranous and invisible, apex of endophallic sclerite with dense marginal setae ..... ***M. flaviventris* (Chen, 1942)**

Discussion

A total of 11 species within *Mandarella* has been reported from Taiwan. Molecular analyses based on the COI sequences and morphological studies, including male aedeagi, revealed that only three species exist in Taiwan (Fig. 9). Variations exist in color patterns and ratios of the lengths between the antennomeres II and III, and these two morphological characteristics can be employed as diagnostic characters, as shown in identification key mentioned above, to distinguish the three major lineages. But, non-overlapping genetic distances of COI, i.e. >16.2% (interspecies) and <14.4% (intraspecies), for the three flea beetle lineages also are useful for delineation.

Morphological characters (i.e., body sizes, color patterns, relative lengths between antennomeres) used previously to identify different morphospecies likely are a reflection of their altitudinal distributions induced by local adaptations. For example, in the Hohuanshan mountains, the black forms A/B was mainly collected at an elevation of 3422 m (Hohuanshan Mt. Peak), while at 2756 m (Yuanfeng), the form D with yellow legs and darkened basal femora, is dominant with only seven out of 239 specimens representing the form A. Phylogenetic inferences of these *Mandarella* flea beetles also revealed local adaptation. The variable morphological forms recognized in the *M. uenoi* and *M. flaviventris* lineages may represent a more complicated scenario and related to evolutionary processes. Additional specimens from different montane areas are required to elucidate their phylogeographic histories and address the local adaptations of body size, body color, and the length of antennomeres.

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Supplementary material 1

Collection information, taxon ID, and accession numbers of COI gene for each flea beetle

Authors: Chi-Feng Lee, Cheng-Lung Tsai, Alexander Konstantinov, Wen-Bin Yeh

Data type: DNA alignment

Explanation note: DNA Submission in GenBank.

Supplementary material 2

***Mandarella uenoi*, other material examined**

Authors: Chi-Feng Lee, Cheng-Lung Tsai, Alexander Konstantinov, Wen-Bin Yeh

Data type: Occurence

Explanation note: 1008 specimens were examined. The localities, dates, and depositories were recorded.

Supplementary material 3

***Mandarella flaviventrites*, other material examined**

Authors: Chi-Feng Lee, Cheng-Lung Tsai, Alexander Konstantinov, Wen-Bin Yeh

Data type: Occurence

Explanation note: 707 specimens were examined. The localities, dates, and depositories were recorded.

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RESEARCH ARTICLE

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A new species and additional records of *Lobrathium* Mulsant & Rey (Coleoptera, Staphylinidae, Paederinae) from South China

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Abstract

Material of the genus *Lobrathium* Mulsant & Rey, 1878 from the Chinese provinces Fujian, Hunan, Sichuan, Guangdong and Guangxi is examined. Six species are identified, four of them described previously and two undescribed. *Lobrathium kedian* Peng & Li, **sp. n.** (Guangxi: Shiwangda Shan) is described and illustrated. One probably undescribed species remains unnamed. The female sexual characters of *L. flexum* Assing, 2014 are described and illustrated for the first time. The genus is now represented in mainland China by 43 species.

Keywords

Coleoptera, Staphylinidae, *Lobrathium*, new species, new records, China

Introduction

Until today, 42 species of the genus *Lobrathium* Mulsant & Rey have been reported from mainland China and 20 species from Taiwan (Assing 2010, 2012, 2013, 2014; Li et al. 2013; Li et al. 2013a, b, c; Lü and Li 2014). With a total of 14 described species, the *Lobrathium* fauna of Sichuan is currently more diverse than that of any of the other Chinese provinces, followed by Yunnan (8 species), Shaanxi (7 species), Guizhou (6 species) and Zhejiang (6 species) (Assing 2012, 2013, 2014; Li et al. 2013; Li et al. 2013a, b, c; Lü and Li 2014).

A study of *Lobrathium* material from southern China yielded a species new to science and additional records of *L. configens* Assing, 2012, *L. flexum* Assing, 2014, *L. hebeatum* Zheng, 1988 and *L. hongkongense* Bernhauer, 1931.

Material and methods

The following abbreviations are used in the text, with all measurements in millimeters:

Body length (BL) from the anterior margin of the labrum to the abdominal apex; forebody length (FL) from the anterior margin of the labrum to the posterior margin of the elytra; head length (HL) from the anterior clypeal margin to the occipital constriction; head width (HW): maximum width of head; length of antenna (AnL); length of pronotum (PL) along midline; maximum width of pronotum (PW); elytral length (EL) at the suture from the apex of the scutellum to the posterior margin of the elytra (at the sutural angles); maximum width of the elytra (EW); length of aedeagus (AL) from the apex of the dorsal plate to the base of the aedeagal capsule.

The type material is deposited in the Insect Collection of Shanghai Normal University, Shanghai, China (SNUC).

Results

Lobrathium configens Assing, 2012

Fig. 4

Material studied. China: Sichuan: 8 ♂♂, 2 ♀♀, Xiaojin County, Jiajin Shan, 30°48'49"N, 102°42'55"E, 2500 m, 20.VII.2015, Jiang, Peng, Tu & Zhou leg. (SNUC).

Comment. *Lobrathium configens* was previously known from the Chinese provinces Shaanxi, Sichuan, Qinghai, Hubei, Yunnan and Zhejiang (Assing 2012, 2013, 2014; Li et al. 2013a, b). For illustrations of *L. configens* see Assing (2012: figures 153–165) and Li et al. (2013a: figure 4).

Lobrathium flexum Assing, 2014

Figs 1A, 2A–C, 5

Material studied. China: Hunan: 1 ♂, 1 ♀, Yanling County, Nanfengmian, 26°18'N 114°00'E, 1600 m, 06.VI.2015, Peng, Shen, Tu & Zhou leg. (SNUC).

Comment. The original description is based on a male from Jiangxi. The previously unknown female sexual characters are as follows: posterior margin of tergite VIII convex (Fig. 2A); sternite VIII (Fig. 2B) weakly transverse, posteriorly broadly convex;

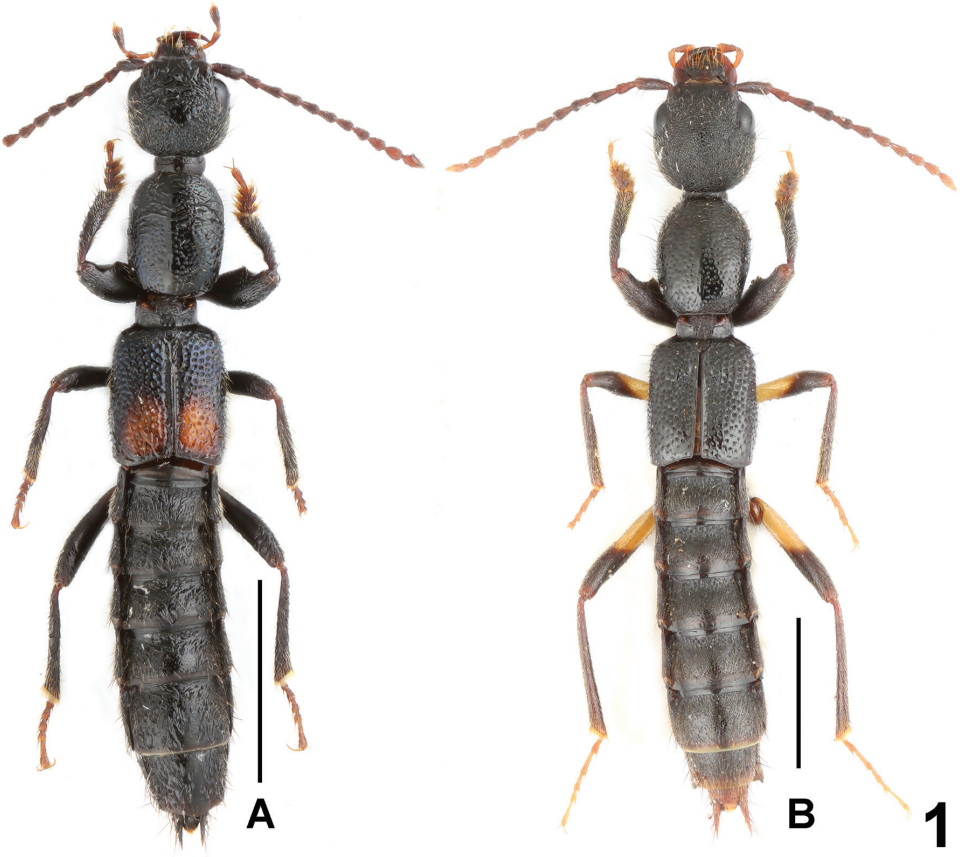


Figure 1. Habitus: **A** *Lobrathium flexum* **B** *Lobrathium kedian*. Scale bars: 2.0 mm.

tergite IX (Fig. 2C) undivided anteriorly. The above record from Hunan represents a new province record. For illustrations of the habitus and the male sexual characters see Assing (2014: figures 12–16).

Lobrathium hebeatum Zheng, 1988

Fig. 6

Material studied. China: Sichuan: 1 ♂, 3 ♀♀, Dayi County, Xiling Xueshan, 30°41'59"N, 103°12'10"E, 2150 m, 29.VII.2015, Jiang, Peng, Tu & Zhou leg. (SNUC).

Comment. The previously known distribution of *L. hebeatum* included the Chinese provinces Shaanxi, Sichuan, Yunnan, Henan and Ningxia (Assing 2012, 2013, 2014; Li et al. 2013a, b; Zheng 1988). For illustrations of *L. hebeatum* see Assing (2012: figures 142–147) and Li et al. (2013a: figure 9).

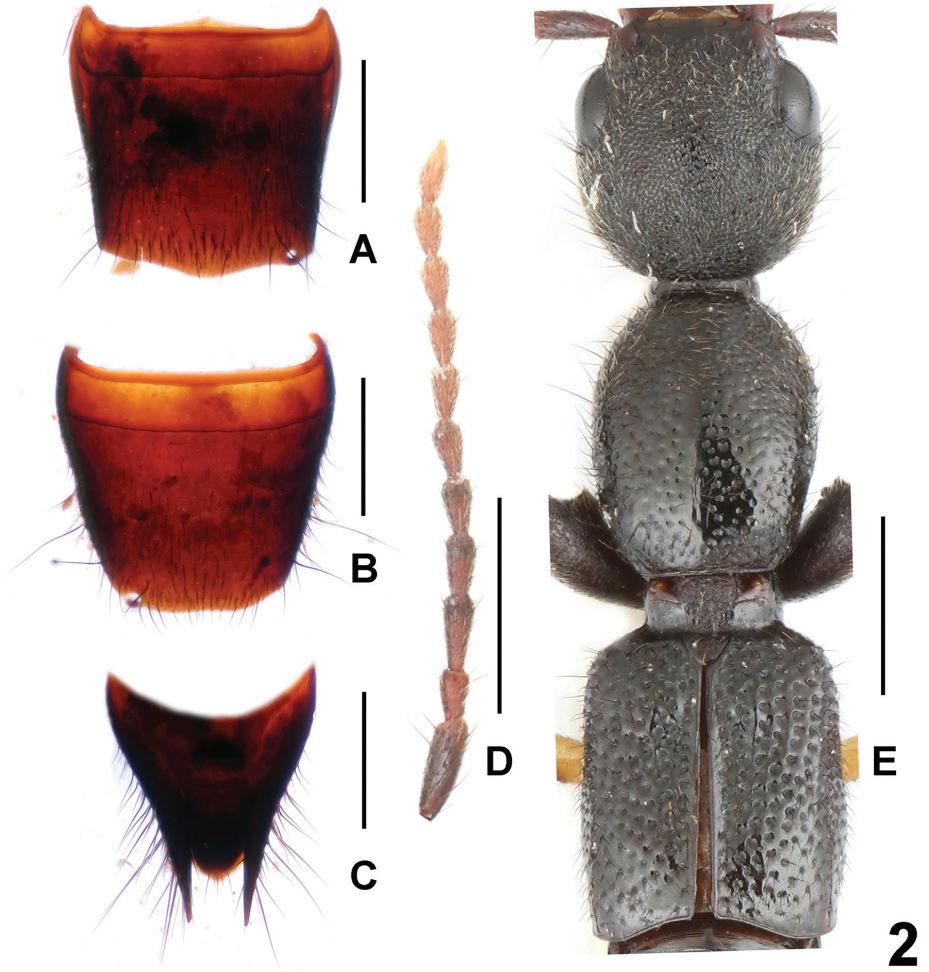


Figure 2. *Lobrathium flexum* (**A–C**) and *Lobrathium kedian* (**D–E**). **A** female tergite VIII **B** female sternite VIII **C** female tergites IX–X **D** antenna **E** forebody. Scale bars: 0.5 mm (**A–C**), 1.0 mm (**D–E**).

Lobrathium hongkongense Bernhauer, 1931

Figs 5, 8

Material studied. China: Fujian: 3 ♂♂, Nanping, Mangdang Shan, 26°41'51"N, 118°07'00"E, 400 m, 10.IX.2015, Yan & Tang leg. (SNUC). Hunan: 2 ♂♂, 1 ♀, Yanling County, Nanfengmian, 26°18'N 114°00'E, 1600 m, 06.VI.2015, Peng, Shen, Tu & Zhou leg. (SNUC). Guangdong: 1 ♂, 1 ♀, Ruyuan County, Nanling Nature Reserve, Qingshuigu, 24°54'57"N, 113°01'55"E, 900 m, 04.V.2015, Peng, Tu & Zhou leg. (SNUC); 1 ♀, Jieyang, Puning, Wufeng Shan, 500 m, 08.VI.2015, Aranyu leg. (SNUC).

Comment. *Lobrathium hongkongense* was previously known from Japan, Hong Kong, Taiwan and the Chinese provinces Fujian, Guizhou, Zhejiang, Jiangsu, Sichuan,

Yunnan, Guangxi, Hubei and Shaanxi (Assing 2012, 2013; Li et al. 2013a, b). The specimens represent the first record from Hunan and Guangdong. For illustrations of *L. hongkongense* see Assing (2012: figures 125–132) and Li et al. (2013a: figure 10).

***Lobrathium kedian* Z. Peng & L.-Z. Li, sp. n.**
http://zoobank.org/546F9FE5-C3BA-469F-A96A-8FC2117C7634
Figs 1B, 3, 7

Type material. Holotype: ♂, labelled 'China: Guangxi Prov., Shangsi County, Shiwanda Shan, 300–500 m, 21°54'N, 107°54'E, 25–IV–2011, Peng & Zhu leg.' (SNUC). Paratypes: 8 ♂♂, 8 ♀♀, same label data as holotype (SNUC).

Description. Measurements (in mm) and ratios: BL 9.88–10.20, FL 5.49–5.62, HL 1.37–1.42, HW 1.36–1.44, AnL 3.13–3.20, PL 1.57–1.63, PW 1.24–1.30, EL 1.35–1.39, EW 1.39–1.46, AL 1.13–1.20, HL/HW 0.97–1.00, HW/PW 1.06–1.10, HL/PL 0.85–0.88, PL/PW 1.25–1.27, EL/PL 0.84–0.87.

Habitus as in Fig. 1B. Coloration: body black, mandibles dark brown, labial palpi light brown; antennae dark brown to light brown; legs with blackish brown profemora and protibiae, basal halves of meso- and metafemora yellowish brown, distal halves gradually infuscate.

Head as wide as long, widest behind eyes; punctation coarse and very dense; interstices without microsculpture. Antenna as in Fig. 2D.

Pronotum distinctly longer than wide, with impunctate midline; punctation coarse and dense, but distinctly sparser than that of head; interstices glossy.

Elytra distinctly broader than pronotum; punctation coarse, arranged in irregular series only laterally. Hind wings approximately 1.85–2.02 times as long as elytra.

Abdomen somewhat narrower than elytra; punctation fine and dense; posterior margin of tergite VII with palisade fringe.

Male. Sternite VII (Fig. 3D) strongly transverse and with shallow median impression posteriorly, without modified setae, posterior margin broadly concave; sternite VIII (Fig. 3E) posteriorly with deep impression, this impression with a cluster of numerous short peg-setae, postero-laterally with a cluster of short black setae; posterior excision large, deep and U-shaped; aedeagus (Figs 3F, G) with apically bifid ventral process in ventral view and broad dorsal plate.

Female. Posterior margin of tergite VIII (Fig. 3A) convex; sternite VIII (Fig. 3B) weakly transverse, posterior margin broadly convex; tergite IX (Fig. 3C) slender and undivided anteriorly.

Distribution and natural history. The type locality is situated in Shiwangda Shan to the south of Shangsi, southern Guangxi. The specimens were sifted from leaf litter in broad-leaved forests at altitudes of 300–500 m (Fig. 7).

Etymology. The specific name is the Chinese noun "kedian" (punctation) in apposition. It refers to the punctation of the head of *L. kedian*, which is denser than that of other species known from Guangxi.

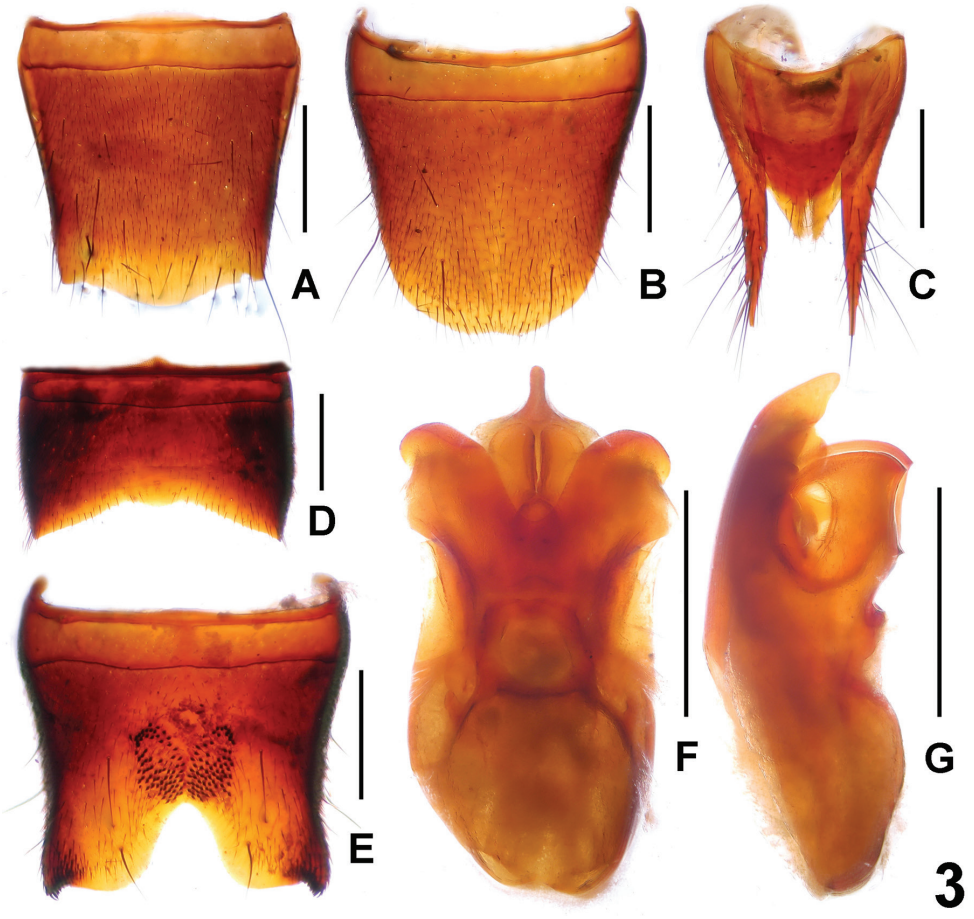
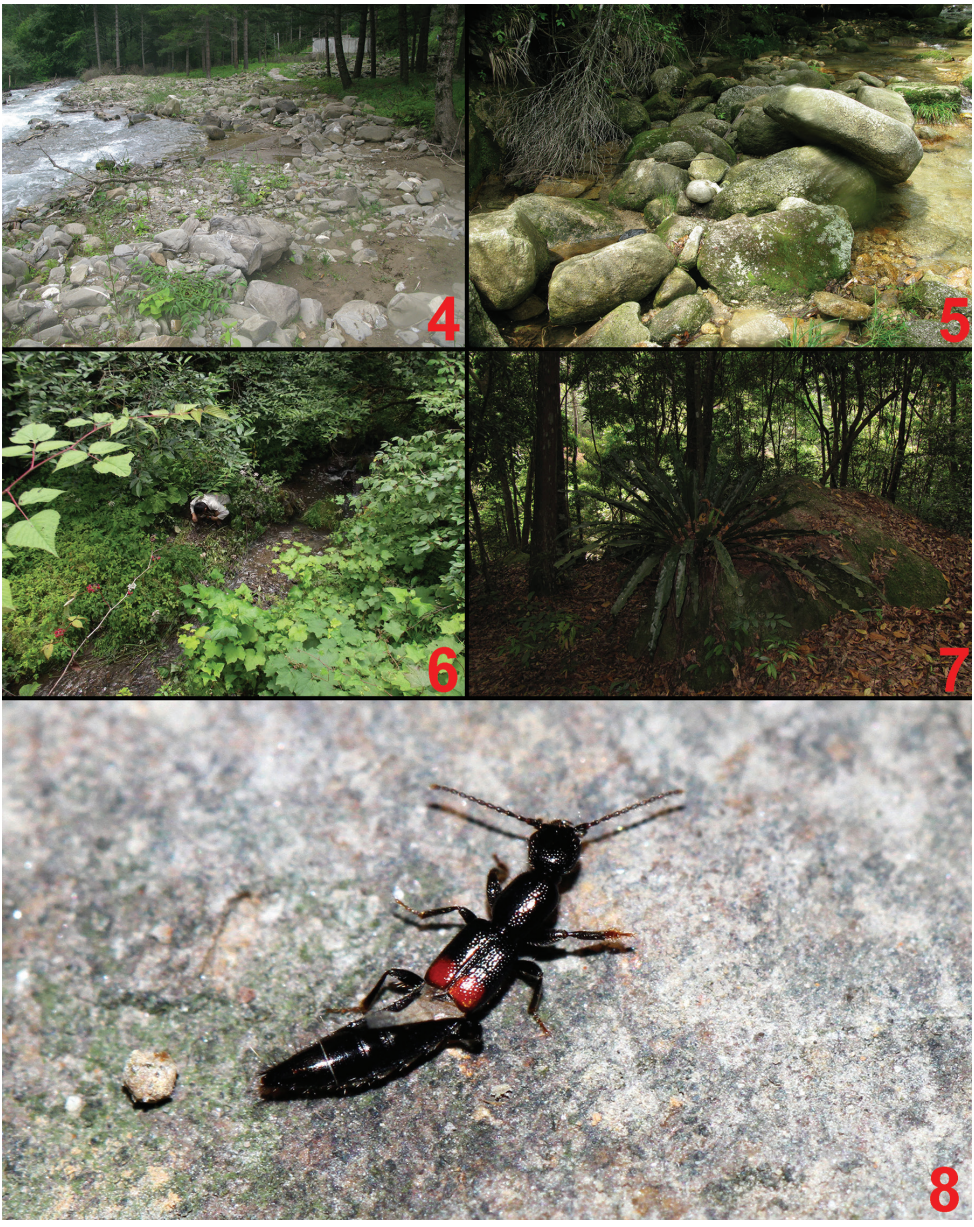


Figure 3. *Lobrathium kedian*. **A** female tergite VIII **B** female sternite VIII **C** female tergites IX–X **D** male tergite VIII **E** male sternite VIII **F** aedeagus in ventral view **G** aedeagus in lateral view. Scale bars: 0.5 mm.

Comparative notes. *Lobrathium kedian* shares a bifid ventral process with *L. digitatum* Assing, 2010 from Taiwan, but differs from it in many respects, particularly by larger body size, the shape and chaetotaxy of the male sternite VIII and by the shape of the aedeagus. For illustrations of *L. digitatum* see Assing (2010: figures 203–210).

***Lobrathium* sp.**
Fig. 6

Material studied. China: Sichuan: 1 ♀, Dayi County, Xiling Xueshan, 30°41'59"N, 103°12'10"E, 2150 m, 29.VII.2015, Jiang, Peng, Tu & Zhou leg. (SNUC).



Figures 4–8. Habitats of *Lobrathium.* **4** Jiajin Shan, alt. 2500 m (*L. configens*) **5** Nanfengmian, alt. 1600 m (*L. flexum* and *L. hongkongense*) **6** Xiling Xueshan, alt. 2150 m (*L. hebeatum* and *Lobrathium* sp.) **7** Shiwangda Shan, alt. 300–500 m (*L. kedian* sp. nov.) **8** *Lobrathium hongkongense* walking on the stone.

Comment. This species is similar and probably closely related to *L. daxuense* Assing, 2012. The female represents an undescribed species distinguished from its congeners particularly by the light brown coloration, large body size (8.34 mm), much denser punctation of the head, a slender pronotum, and the female secondary sexual characters.

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Lü Z-K, Li L-Z (2014) Two new species of *Lobrathium* Mulsant & Rey (Coleoptera, Staphylinidae, Paederinae) from China. ZooKeys 447: 125–131. doi: 10.3897/zookeys.447.8217

Zheng F-K (1988) Five new species of the genus *Lobrathium* Mulsant et Rey from China (Coleoptera: Staphylinidae: Paederinae). Acta Entomologica Sinica 31: 186–193.

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RESEARCH ARTICLE

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Phylogenetic radiation of the greenbottle flies (Diptera, Calliphoridae, Luciliinae)

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Abstract

The subfamily Luciliinae is diverse and geographically widespread. Its four currently recognised genera (*Dyscritomyia* Grimshaw, 1901, *Hemipyrellia* Townsend, 1918, *Hypopygiopsis* Townsend 1916 and *Lucilia* Robineau-Desvoidy, 1830) contain species that range from saprophages to obligate parasites, but their pattern of phylogenetic diversification is unclear. The *28S rRNA*, *COI* and *Period* genes of 14 species of *Lucilia* and *Hemipyrellia* were partially sequenced and analysed together with sequences of 11 further species from public databases. The molecular data confirmed molecular paraphyly in three species-pairs in *Lucilia* that hamper barcode identifications of those six species. *Lucilia sericata* and *L. cuprina* were confirmed as mutual sister species. The placements of *Dyscritomyia* and *Hypopygiopsis* were ambiguous, since both made *Lucilia* paraphyletic in some analyses. Recognising *Hemipyrellia* as a genus consistently left *Lucilia* s.l. paraphyletic, and the occasionally-recognised (sub)genus *Phaenicia* was consistently paraphyletic, so these taxa should be synonymised with *Lucilia* to maintain monophyly. Analysis of a matrix of 14 morphological characters scored for adults of all genera and for most of the species included in the molecular analysis confirmed several of these findings. The different degrees of parasitism were phylogenetically clustered within this genus but did not form a graded series of evolutionary stages, and there was no particular relationship between feeding habits and biogeography. Because of the ubiquity of hybridization, introgression and incomplete lineage sorting in blow flies, we recommend that using a combination of mitochondrial and nuclear markers should be a procedural standard for medico-criminal forensic identifications of insects.

Keywords

Lucilia sericata, *Lucilia cuprina*, molecular systematics, parasitism, myiasis

Introduction

All four genera of the subfamily Luciliinae are reported to exhibit parasitism in the form of myiasis – the infestation of humans' and other animals' living tissues by fly larvae (Stevens 2003) – ranging from facultative secondary necrophagous myiasis in species like *Lucilia sericata* (Meigen, 1826) to obligate primary carnivorous myiasis in species such as *Lucilia bufonivora* Moniez, 1876. *Lucilia cuprina* (Wiedemann, 1830) and *L. sericata* are noted veterinary pests. Molecular approaches to the management of these flies' populations can be built on a phylogenetic analysis of the species, but such analyses based on morphological data (Stevens and Wall 1997, Otranto and Stevens 2002, Stevens 2003) have found no evolutionary pattern underlying the radiation of feeding behaviours in *Lucilia* Robineau-Desvoidy, 1830, and biogeographical patterns in the different forms of myiasis have yet to be studied. Furthermore, several taxonomic questions remain regarding the subfamily, from the molecular identification of its species to the definitions of its genera.

At the highest taxonomic level, Rognes (1991) suggested that the genera *Dyscritomyia* Grimshaw, 1901, *Hemipyrellia* Townsend, 1918, *Hypopygiopsis* Townsend 1916, and *Lucilia* Robineau-Desvoidy, 1830 should be united in the subfamily Luciliinae. Several phylogenetic studies have placed species of *Hemipyrellia* within *Lucilia* (Wells et al. 2007, Park et al. 2009, Liu et al. 2011, McDonagh and Stevens 2011). Evidence of whether *Dyscritomyia* is related to *Lucilia* or nested within it has depended on which gene was analysed (Wells et al. 2007, McDonagh and Stevens 2011). The definitions and relationships of these genera therefore need attention.

Several other genera have been included in the Luciliinae, such as *Bufolucilia* Townsend, 1919, *Francilia* Shannon, 1924, *Acrophagella* Ringdahl, 1942, *Phumonesia* Villeneuve, 1914 and *Viridinsula* Shannon, 1926 but most of these are now treated as synonyms of *Lucilia*. *Lucilia* itself has been variously divided into subgenera (Malloch 1926) or genera (Hall 1948), respectively. *Phaenicia* Robineau-Desvoidy, 1863 has been the most used of these names and its use persists (e.g. Park et al. 2009) even though its validity has been challenged regularly (Aubertin 1933, Zumpt 1965, Stevens and Wall 1996). A phylogenetic study of *Lucilia* presents an opportunity to assess this matter.

The largest genus in the subfamily, *Lucilia* has received few quantitative phylogenetic studies (Aubertin 1933, Stevens and Wall 1996, 1997, Wells et al. 2007, Park et al. 2009, DeBry et al. 2012, Sonet et al. 2012), with research generally focusing on species of medical, veterinary or forensic interest in specific geographic regions (Stevens and Wall 2001, Chen et al. 2004, Wallman et al. 2005, Harvey et al. 2008, Reibe et al. 2009, Liu et al. 2011, Boehme et al. 2012, DeBry et al. 2012, Nelson et al. 2012, Sonet et al. 2013). The most comprehensive revision of the genus was published by Aubertin (1933), who recognised 27 species. Since then revisions of the genus and keys for the identification of its species have been produced, but only for specific geographic regions (Hall 1948, James 1971, Rognes 1980, 1991, Smith 1986, Whitworth 2006, 2010). Most species of *Lucilia* are limited to particular continents or islands and very few, such as *L. sericata*, are cosmopolitan. It is difficult to assess relationships and biogeographical patterns when studies are taxonomically geographically fragmented.

At the species level, *L. sericata* and *L. cuprina* have been referred to as sister-species (Ash and Greenberg 1974) because they are very similar morphologically and each is often misidentified as the other. They are now both found in Australia, New Zealand, South Africa, large parts of Asia, Europe and North America (Waterhouse and Paramonov 1950, Rognes 1980, 1994, Norris 1990, Bishop 1991, 1995, Holloway 1991, Fischer 2000, Harvey et al. 2003a, 2003b, 2008, Chen et al. 2004, Heath and Bishop 2006, Park et al. 2009, Liu et al. 2011, Boehme et al. 2012, GilArriortua et al. 2013). They have each received intensive biological investigation, and it would benefit comparative studies if it could be confirmed that they are actually sister species.

Several studies have established that natural hybrids of *L. sericata* and *L. cuprina* exist (Stevens and Wall 1996, Stevens et al. 2002, Wallman et al. 2005, Tourle et al. 2009, DeBry et al. 2010, Williams and Villet 2013). Two other species pairs, *Lucilia coeruleiviridis* Macquart, 1855 and *L. mexicana* Macquart, 1843, and *L. caesar* (Linnaeus, 1758) and *L. illustris* (Meigen, 1826), also show molecular paraphyly (DeBry et al. 2012, Sonet et al. 2012, 2013), possibly due to introgressive hybridisation or incomplete lineage sorting. The frequency and phylogenetic distribution of this phenomenon in the genus is of general interest because of its implications for understanding speciation and diversification in the group.

The aims of this study are therefore to confirm if *L. sericata* and *L. cuprina* are sister-species; to explore if *L. coeruleiviridis* (Macquart, 1855) / *L. mexicana* Macquart, 1843 and *L. caesar* (Linnaeus, 1758) / *L. illustris* (Meigen, 1826) are paraphyletic species; to examine the relationships between the species of *Lucilia* and clarify the taxonomic status of *Phaenicia*; to estimate the relationships of *Dyscritomyia*, *Hemipyrellia*, *Hypopygiopsis* and *Lucilia*; and to assess the geographical and phylogenetic patterns of myiasis-causing behaviour in these flies.

Materials and methods

DNA data

Adult *Lucilia* flies were obtained from around the world (Table 1). *Hemipyrellia fernandica* (Macquart, 1855) were obtained from Benin, South Africa and Tanzania, and *Calliphora vicina* Robineau-Desvoidy, 1830 were obtained from France and used as an outgroup (Table 1). Identifications were made by the donors based on morphology and verified using published keys (Aubertin 1931, 1933, Smith 1986, Holloway 1991, Whitworth 2006, 2010). All flies were kept in separate 1.5 ml Eppendorf tubes in 96% ethanol or as dried pinned specimens and deposited with the Durban Natural Science Museum after analysis.

One hind leg of each fly was used for DNA analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Three genes were chosen for sequencing: *28S* rRNA (*28S*), a nuclear gene that has been used in previous studies and would allow comparison with other studies

Table 1. Specimen locality data for sequences added to GenBank. (Accession numbers starting KF are new sequences from this study).

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
Calliphora vicina	CV_FRC_01(F)	Montferrier-Sur-Lez	France	JN792781	KF839531	KF839562
	CV_FRC_02(M)	Montferrier-Sur-Lez	France	KF839506		
Hemipyrellia fernandica	H_BEN_01(M)	Contonou	Benin	KF839511	KF839539	KF839567
	H_BEN_02(M)	Contonou	Benin	KF839512	KF839540	KF839568
	H_SA_DBN_01(F)	Durban	South Africa	KF839513	KF839541	KF839569
	H_TAN_01(M)	Mkuraja	Tanzania	KF839514	KF839542	KF839570
	H_TAN_02(M)	Mkuraja	Tanzania	KF839515	KF839543	KF839571
Lucilia caesar	Ca_FRC_01(M)	Montferrier-Sur-Lez	France	JN792782	JN792858	KF839556
	Ca_FRC_02(F)	Montferrier-Surz-Lez	France	KF839501	KF839532	KF839557
Lucilia coeruleiviridis	Co_CAN_01(M)	Windsor	Canada	KF839502	KF839533	KF839558
	Co_CAN_02(M)	Windsor	Canada	KF839503		KF839559
	Co_USA_03(F)	Putnam Co. Missouri	United States of America	KF839504	KF839534	KF839560
	Co_USA_04(F)	Martinstown, Missouri	United States of America	KF839505		KF839561
Lucilia cuprina	C_AUS_01 (M)	Sydney	Australia	KF856254		JN792622
	C_EGT_01 (F)	Alexandria	Egypt	JN792706	JN792784	JN792625
	C_SA_CT_02 (F)	Cape Town	South Africa	JN792713	JN792791	JN792632
	C_SA_DBN_01(F)	Durban	South Africa	JN792724	JN792802	JN792642
	C_THA_02 (F)	Chiang Mai	Thailand	JN792741	JN792819	JN792661
	C_THA_03 (F)	Chiang Mai	Thailand	JN792742	JN792820	JN792662
	C_ZIM_02 (F)	Matobos	Zimbabwe	JN792745	JN792823	JN792667
Lucilia eximia	Ex_CSR_01(F)	Santo Domingo	Costa Rica	KF839507	KF839535	KF839563
	Ex_CSR_02(F)	Santo Domingo	Costa Rica	KF839508	KF839536	KF839564
Lucilia fayeae	Fa_DOM_01(F)	Calibishie	Dominica	KF839509	KF839537	KF839565
	Fa_DOM_02(F)	Calibishie	Dominica	KF839510	KF839538	KF839566
Lucilia illustris	IL_CAN_01(F)	Windsor	Canada	KF839516	KF839544	KF839572
	IL_CAN_02(F)	Windsor	Canada	KF839517	KF839545	KF839573

Species	Specimen	Locality		Accession Number		
				28S	*Per*	*COI*
	IL_JPN_01(F)	Iwate Medical University	Japan	KF839518	KF839546	KF839574
	IL_JPN_02(F)	Iwate Medical University	Japan	KF839519	KF839547	KF839575
	IL_SWZ_01(F)	Lausanne-Suisse	Switzerland	KF839520	KF839548	
	IL_USA_01(F)	Michigan	United States of America	KF839521	KF839549	
	IL_USA_02(F)	Michigan	United States of America	KF839522	KF839550	KF839576
Lucilia infernalis	In_BRN_01(F)	Parc National de la Kibira	Burundi	KF839523	KF839551	KF839577
	In_RWN_01(F)	Nyungwe Forest Reserve	Rwanda	JN792780	JN792857	JN813094
Lucilia mexicana	Mx_USA_01(F)	New Mexico	United States of America	KF839524	KF839552	KF839578
	Mx_USA_02(F)	New Mexico	United States of America	KF839525		KF839579
Lucilia papuensis	Pa_AUS_01	-	Australia	KF839526		
Lucilia porphyrina	Po_AUS_01	-	Australia	KF839527	KF839553	
Lucilia sericata	S_AUS_01 (M)	Seaford	Australia	JN792746	JN792824	JN792668
	S_FRC_01 (F)	Montferrier-Sur-Lez	France	JN792749	JN792827	JN792671
	S_JPN_01 (F)	Osaka	Japan	JN792754	JN792831	JN792678
	S_NAM_01 (F)	Possession Island	Namibia	JN792758	JN792835	JN792682
	S_SA_CT_07 (F)	Cape Town	South Africa	JN792766	JN792843	JN792690
	S_USA_01 (F)	Michigan	United States of America	JN792778	JN792855	JN792703
Lucilia silvarum	Si_GER_01(F)	Kempen	Germany	KF839528		KF839580
Lucilia thatuna	Th_USA_01(F)	Del Norte Co. California	United States of America	KF839529	KF839554	KF839581
	Th_USA_02(F)	Del Norte Co. California	United States of America	KF839530	KF839555	KF839582

(Stevens et al. 2002, Stevens 2003, Tourle et al. 2009, DeBry et al. 2010, Sonet et al. 2012); *Period* (*Per*), a second nuclear gene that is faster-evolving than *28S* to give better phylogenetic resolution; and *Cytochrome oxidase I* (*COI*), the DNA barcoding gene of choice that has been used in previous studies (Stevens et al. 2002, Stevens 2003, Wallman et al. 2005, Wells et al. 2007, Harvey et al. 2008, Liu et al. 2009, Park et al. 2009, Tourle et al. 2009, DeBry et al. 2010, DeBry et al. 2012, Sonet et al. 2012). A region of approximately 650bp in the Domain 1-2 of the *28S* gene was amplified using the primers 5`-CCCCCTGAATTTAAGCATAT-3` and 5`-TTAGACTCCTTGGTCCGTG-3` (Stevens et al. 2002). A region of approximately 600bp of the *COI* gene was amplified using the primers C1-J1709 (5'-ATTGGGGGGTTTGGAAATTG-3`) and C1-N2353 (5'-GCTCGTGTATCAACGTCTATTCC-3`) (Simon et al. 2006). A region of approximately 730bp of the *Per* gene, was amplified using the primers Per5 (5'-GCCTTCAGATACGGTCAAAC-3') (Warman, pers comm) and Per reverse (5`-CCGAGTGTGGTTTGGAGATT-3`) (designed by the authors). Polymerase chain reaction (PCR) amplification was performed using 1μL of DNA in a 25μL reaction. Amplification times were 94 °C for 5 min denaturation, followed by 36 cycles of 94 °C for 30 seconds, 55 °C for 1 min, 72 °C for 30 seconds and a final extension period at 72 °C for 7 min. PCR products were confirmed by gel electrophoresis stained in ethidium bromide. PCR products were then sequenced using an ABI 3730l Genetic Analyzer (Applied Biosystems) and the primers used in amplification.

Additional DNA sequences of *28S*, *Per* and *COI* were obtained from GenBank (www.ncbi.nlm.nih.gov) (Table 2). Additional *COI* barcode sequences were downloaded from the Barcode of Life Database (BOLD) website for all available *Lucilia*, *Hemipyrellia* and *Hypopygiopsis* species and for *Paralucilia paraensis* (Mello, 1972) and *Chrysomya chloropyga* (Wiedemann, 1818) which were included as additional outgroups. Duplicate sequences from the same studies were removed and a total of 207 sequences were included in the analysis. The sequences were aligned and edited using the BioEdit v7.0.9 software (Hall 1999).

Morphological data

The states of the 14 morphological characters defined by Stevens and Wall (1996) were obtained from Aubertin (1931, 1933), Stevens and Wall (1996) and Whitworth (2010) for all of the *Lucilia* and *Hemipyrellia* species for which sequences were available (Table 3). Museum specimens were inspected where possible to complete the character state matrix. *Calliphora vicina* was included as an outgroup.

Phylogenetic analysis

Separate Bayesian inference analyses were performed on each gene in MrBayes (Huelsenbeck and Ronquist 2001) using the best-fitting nucleotide substitution model

Table 2. GenBank sequences included in this study.

Species	Locality		Accession Number		
			28S	***Per***	***COI***
C. vicina	Bristol	UK	AJ300131		AJ417702
D. fasciata	-	Hawaii			AY074902
D. lucilioides	-	Hawaii			AY074903
D. robusta	-	Hawaii			AY074898
H. ligurriens	-	China			DQ345092
H. ligurriens	-	Taiwan			AY097334
H. ligurriens	-	Taiwan			DQ453493
H. pulchra	-	China			DQ345091
L. adiosoemartoi	-	Indonesia			AY074901
L. ampullacea	Langford	UK	AJ300137		
L. ampullacea	Bristol	UK			DQ453487
L. ampullacea	-	Korea			EU925394
L. bazini	-	Taiwan			AY346450
L. bazini	-	China			DQ345082
L. caesar	Langford	UK	AJ300138		AY417703
L. caesar	Bristol	UK			DQ453488
L. caesar	-	Korea			EU880196
L. cluvia	New Orleans	USA	AJ551440		DQ453490
L. cluvia	Volusia Co. Florida	USA			JQ942371
L. coeruleiviridis	New York	USA			FJ650558
L. cuprina	-	China			DQ345087
L. cuprina	Honolulu	Hawaii			AJ417704
L. cuprina	Oahu	Hawaii			DQ453496
L. cuprina	-	Taiwan			AY097335
L. cuprina	-	Thailand			EU418577
L. cuprina	Tororo	Uganda			AJ417711
L. cuprina	Townsville	Australia	AJ417709		AJ417710
L. cuprina	Waianae	Hawaii			AJ417705
L. cuprina	Wallaceville	New Zealand		Y19108.1	
L. cuprina	Noordhoek	South Africa	EU626549		
L. cuprina	Cincinnati	USA	FJ650542		
L. eximia	-	Brazil			DQ453491
L. hainanensis	-	Taiwan			AY346451
L. hainanensis	-	China			DQ345084
L. illustris	Langford	UK	AJ300136		AJ551445
L. illustris	-	Korea			EU880204
L. illustris	-	China			DQ345090
L. illustris	-	India			DQ200168
L. mexicana	San Francisco	USA	AJ551441		DQ453492
L. mexicana	California	USA			FJ650563
L. mexicana	California	USA			FJ650562
L. papuensis	-	China			DQ345085

Species	Locality		Accession Number		
			28S	***Per***	***COI***
L. porphyrina	-	Taiwan			AY097336
L. porphyrina	-	Japan			AY074900
L. porphyrina	-	China			DQ345089
L. richardsi	Usk	-	AJ551142		
L. sericata	Perth	Australia			AB112833
L. sericata	Nerja	Spain			AJ417716
L. sericata	Kingsbury	UK			AJ417713
L. sericata	Hilerod	Denmark	AJ300140		EF531193
L. sericata	Harare	Zimbabwe			AJ417717
L. sericata	-	China			DQ345086
L. sericata	Langford	UK	AJ300139		
L. sericata	Los Angeles	USA	AJ300141		
L. silvarum	Durham	UK	AJ551443		
L. silvarum	-	USA			FJ650564
L. silvarum	Linn Co., OR	USA			JQ942455
L. taiyuanensis	-	China			DQ345088
L. thatuna	San Francisco	USA	AJ551444		DQ453489
L. thatuna	Del Norte Co., California	USA			JQ942464

(GTR+G in all cases) from jModelTest (Posada 2008). One cold and three hot chains were run for 5 000 000 generations, sampling every 1 000 generations with burn-in of 1 000 samples (20%). Incongruence length difference (ILD) tests (Farris et al. 1994) were run in PAUP*4b10 (Swofford 2003) to quantify the differences in topology between trees for *28S*, *COI* and *Per*. Analyses were then conducted on two combined data sets (nuclear *28S* and *Per*; and total *28S*, *Per* and *COI*), each partitioned by gene, with the parameters as above.

A network analysis for the *COI* data was created using the NeighborNet algorithm in SplitsTree4 (Huson and Bryant 2008) and the uncorrected P-distance method.

The *COI* barcode sequences (~700 bp long, between base numbers 1490 and 2198) retrieved from on-line databases were aligned along with our new sequences (~640 bp long, between base numbers 1709 and 2353) for a region approximately 800 bp long in which every sequence overlapped the others by at least 490 bp. Bayesian inference analysis was performed in MrBayes (Huelsenbeck and Ronquist 2001) using the best-fitting nucleotide substitution model (GTR+G) from jModelTest (Posada 2008).

Maximum parsimony analysis of the morphological data (Table 3) using Fitch parsimony was performed in Paup*4b10 (Swofford 2003). Statistical support for nodes was assessed by bootstrapping with 100 replicates retaining a maximum of 10 000 trees. Strict consensus and 50% majority rule trees were produced from the analysis.

The zoogeographic distributions of species in the Luciliinae (Table 4) were mapped onto the trees.

Table 3. Binary coding of 14 morphological characters for the genera *Lucilia* and *Hemipyrellia*. 1 – Colour of the basicostal scale (0 = black/brown, 1 = white/cream); 2 – Number of postsutural acrostichal bristles (0 = two pairs, 1 = three pairs); 3 – Eye separation in the male (0 = distance of greater than the width of the third antennal segment, 1 = less than the width of the third antennal segment); 4 – Number of anterio-dorsal bristles on the mid tibia (0 = one, 1 = two); 5 – Colour of the palpi (0 = yellow/orange, 1 = black/brown); 6 – Subcostal sclerite (0 = bristles absent, 1 = bristles present); 7 – Colour of the squamae (0 = uniform white/cream, 1 = partially or totally brown); 8 – Wings (00 = hyaline, 01 = lightly infuscated, 11 = heavily infuscated); 9 – Eye separation in the female (0 = distance of greater than one quarter of the width of the head, 1 = less than one quarter of the width of the head); 10 – Colour of antennae (0 = uniformly dark, 1 = non-uniform); 11 – Male hypopygium (00 = inconspicuous, 01 = conspicuous, 11 = highly conspicuous); 12 – Colour of abdomen and thorax (0 = predominantly brassy green/green, 1 = predominantly purple/blue/black); 13 – Colour of the legs (00 = dark brown, 01 = brown/black, 11 = black); 14 – Lower squamal lobe (0 = setae absent, 1 = setae present). (Stevens and Wall 1996).

Species	Character number													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Calliphora vicina	1	1	1	1	0	0	1	00	0	0	11	1	01	1
Hemipyrellia fernandica	0	0	0	0	1	1	0	00	1	0	00	0	11	0
Hemipyrellia ligurriens	0	0	0	0	0	1	0	00	1	1	01	0	11	0
Hemipyrellia pulchra	0	0	0	1	0	?	0	00	0	1	00	0	11	0
Lucilia ampullacea	0	0	1	0	0	1	0	00	0	0	00	0	01	0
Lucilia bufonivora	0	0	0	0	1	0	0	00	0	0	01	0	11	0
Lucilia coeruleiviridis	1	0	1	0	0	0	0	00	1	1	00	0	00	0
Lucilia caesar	0	0	1	0	0	1	0	00	0	0	11	0	01	0
Lucilia cluvia	1	0	0	0	0	0	0	00	1	0	00	0	00	0
Lucilia cuprina	1	1	0	0	0	0	0	00	0	0	01	0	11	0
Lucilia eximia	0	0	1	0	0	0	1	00	0	1	00	0	00	0
Lucilia fayeae	0	0	1	0	0	0	1	01	0	0	00	1	00	0
Lucilia illustris	0	0	1	0	0	1	0	00	0	0	01	0	11	0
Lucilia infernalis	0	1	1	0	0	1	1	11	0	1	00	1	01	0
Lucilia mexicana	0	0	1	0	0	0	1	01	0	1	00	0	11	0
Lucilia papuensis	0	0	1	1	0	1	1	01	0	1	00	0	11	0
Lucilia porphyrina	0	0	1	0	0	1	1	01	1	0	00	1	00	0
Lucilia richardsi	1	1	0	1	1	0	0	00	0	0	00	0	11	0

Species	Character number													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Lucilia sericata	1	1	0	0	0	0	0	00	0	0	00	0	11	0
Lucilia silvarum	0	1	0	0	1	0	0	00	0	0	01	0	11	0
Lucilia thatuna	1	1	1	0	0	0	0	00	1	0	00	0	11	0

Table 4. Zoogeographic distribution of species of Luciliinae included in this study. Symbols in brackets represent anthropogenic introductions.

Species	Region						
	Hawaii	Afrotropical	Australasian	Oriental	Palaearctic	Nearctic	Neotropical
Dysctritomyia spp.	X						
Hypopygiopsis spp.			X	X			
Hemipyrellia spp.		X	X	X			
H. fernandica		X					
L. infernalis		X					
L. cuprina		X	X	X	(X)	X	
L. sericata		(X)	(X)	X	X	X	(X)
L. silvarum					X	X	
L. thatuna						X	
L. adiosoemartoi				X			
L. bazini				X			
L. hainanensis				X			
L. taiyuanensis				X			
L. papuensis			X	X			
L. porphyrina			X	X	X		
L. ampullacea				X	X		
L. caesar				X	X		
L. illustris				X	X	X	
L. cluvia					X	X	
L. coeruleiviridis						X	
L. mexicana						X	
L. fayeae							X
L. eximia							X

Results

Molecular data

Sequencing of the *28S*, *Per* and *COI* genes resulted in 1932 bp being aligned – 656 bp for *28S*, 700 bp for *Per* and 576 bp for *COI*. A total of 46 specimens were sequenced for *28S*, 41 specimens for *Per* and 39 specimens for *COI*. These sequences were submitted to GenBank (Table 1).

The ILD test for *28S* and *Per* showed these two genes to be highly congruent (p = 1.00) and the datasets were therefore concatenated for the analyses. The ILD test for *28S*, *Per* and *COI* showed the combination of these genes to be incongruent (p =

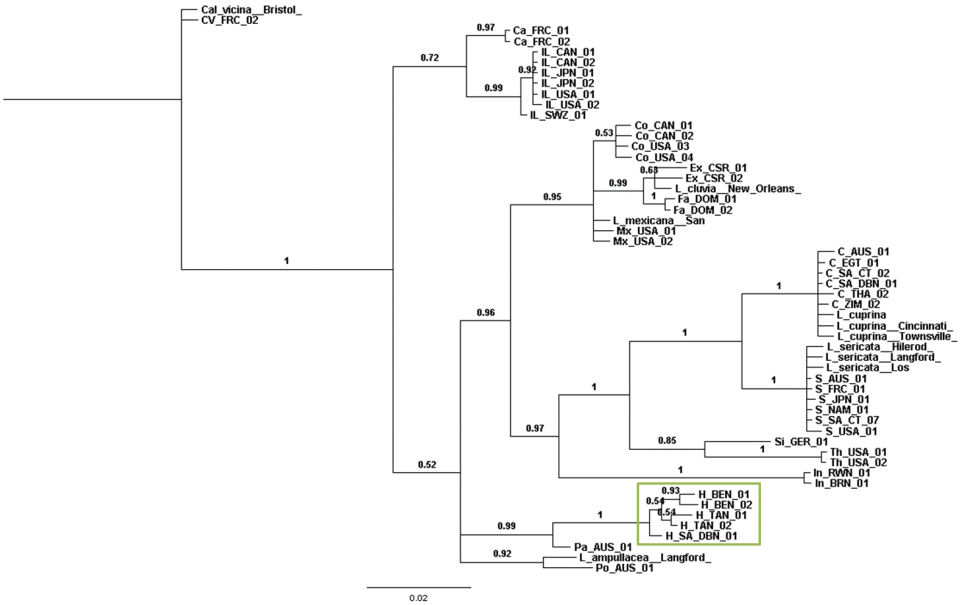

Figure 1. Bayesian inference tree constructed from concatenated nuclear genes *28S* + *Per*. Posterior probabilities are indicated on nodes. Green box = *Hemipyrellia fernandica*. C = *L. cuprina*, Ca = *L. caesar*, Co = *L. coeruleiviridis*, CV = *Calliphora vicina*, Ex = *L. eximia*, Fa = *L. fayeae*, H = *Hemipyrellia fernandica*, IL = *L. illustris*, In = *L. infernalis*, Mx = *L. mexicana*, Pa = *L. papuensis*, Po = *L. porphyrina*, S = *L. sericata*, Si = *L. silvarum*, Th = *L. thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town.

0.03). Despite the incongruence between the nuclear (*28S* and *Per*) and mitochondrial (*COI*) data, these data sets were also concatenated and an analysis run on the total molecular evidence.

The Bayesian inference tree (Fig. 1) for the nuclear genes (*28S* and *Per*) clearly showed that *L. sericata* and *L. cuprina* are sister clades with 100% support. *Lucilia thatuna* Shannon, 1926 and *L. silvarum* Meigen, 1826 form a sister clade to the *L. sericata* + *L. cuprina* clade. The specimens of *H. fernandica* all grouped together and were sister to *L. papuensis* Macquart, 1843. The *Hemipyrellia* clade sat within the *Lucilia* clade (Fig. 1).

In the Bayesian inference tree for the mitochondrial gene (*COI*) (Fig. 2), *L. cuprina* was paraphyletic with respect to *L. sericata*. The *L. cuprina* + *L. sericata* clade was poorly resolved with respect to the *L. silvarum* + *L. taiyuanensis* Chu, 1975 clade. The *H. fernandica* sequences grouped with those of *H. ligurriens* and *H. pulchra* from GenBank and this clade was sister to *Lucilia infernalis* Villeneuve, 1914. This *Hemipyrellia* + *L. infernalis* clade sat within the *Lucilia* clade on the tree. Two specimens from Taiwan assigned to *H. ligurriens* grouped with the *L. cuprina* specimens. The three

Figure 2. Bayesian inference tree constructed from mitochondrial gene *COI*. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia* sp. Blue box = *Dysctritomyia* sp. C = *L. cuprina*, Ca = *L. caesar*, Co = *L. coeruleiviridis*, CV = *Calliphora vicina*, Ex = *L. eximia*, Fa = *L. fayeae*, H = *Hemipyrellia fernandica* IL = *L. illustris*, In = *L. infernalis*, Mx = *L. mexicana*, S = *L. sericata*, Si = *L. silvarum*, Th = *L. thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town.

Dyscritomyia sequences included in the analysis grouped together monophyletically outside *Lucilia*.

The Bayesian inference tree for the incongruent concatenated total evidence molecular dataset (*28S*, *Per* and *COI*) (Fig. 3) showed *L. sericata* and *L. cuprina* to be sister clades with strong support. The *H. fernandica* sequences sat within *Lucilia*, and the rest of the tree was topologically similar to the gene trees.

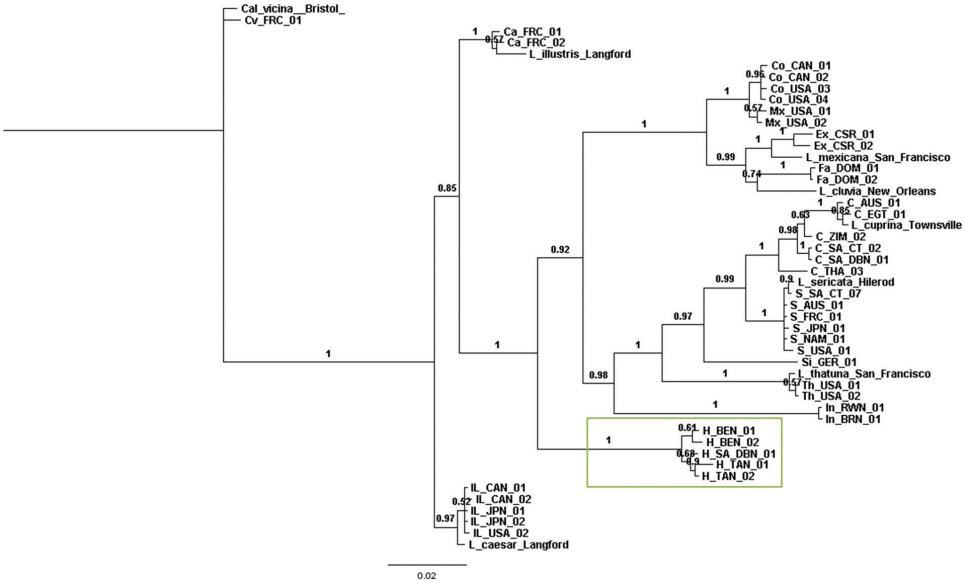

Figure 3. Bayesian inference tree constructed from the concatenated nuclear (*28S* & *Per*) and mitochondrial (*COI*) genes. Posterior probabilities indicated on nodes. Green box = *Hemipyrellia fernandica*. C = *L. cuprina*, Ca = *L. caesar*, Co = *L. coeruleiviridis*, CV = *Calliphora vicina*, Ex = *L. eximia*, Fa = *L. fayeae*, H = *Hemipyrellia fernandica* IL = *L. illustris*, In = *L. infernalis*, Mx = *L. mexicana*, S = *L. sericata*, Si = *L. silvarum*, Th = *L. thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominican Republic, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town.

The NeighborNet analysis (Fig. 4) clearly showed seven distinct major splits. The New World species (*L. coeruleiviridis*, *L. cluvia* Walker, 1849, *L. eximia* Wiedemann, 1819, *L. mexicana* and *L. fayeae* Whitworth, 2010) grouped together; *L. caesar*, *L. illustris*, *L. porphyrina* Walker, 1856, *L. ampullacea* Villeneuve, 1922, *L. adiosoemartoi*, *L. papuensis* Macquart, 1843, *L. bazini* Séguy, 1934 and *L. hainanensis* Fan, 1965 formed a group; *L. infernalis* was isolated, as was *H. fernandica*; the bulk of the *Lucilia* species that are primary facultative parasites (*L. sericata*, *L. cuprina*, *L. silvarum* and *L. thatuna*) grouped together; and *Calliphora vicina* and the *Dyscritomyia* species as the outgroups formed separate but neighbouring splits.

Bayesian inference analysis of the *COI* barcode data set generated a tree (Fig. 5) with very strong posterior probabilities for most clades except for the *L. sericata* + *L. cuprina* + *L. taiyuanensis* (p = 0.61) and *L. caesar* + *L. illustris* (p = 0.58) clades. The *Hemipyrellia* species all formed a distinct clade within *Lucilia* with 100% support. One of the *Hypopygiopsis infumata* (Bigot, 1877) sequences forms a clade with *L. hainanensis* + *L. papuensis* + *L. bazini* and the other sequence groups with the *Hemipyrellia* sequences. *Paralucilia paraensis* sat outside *Lucilia* with *Chrysomya chloropyga*, confirming its classification as a chrysomyine.

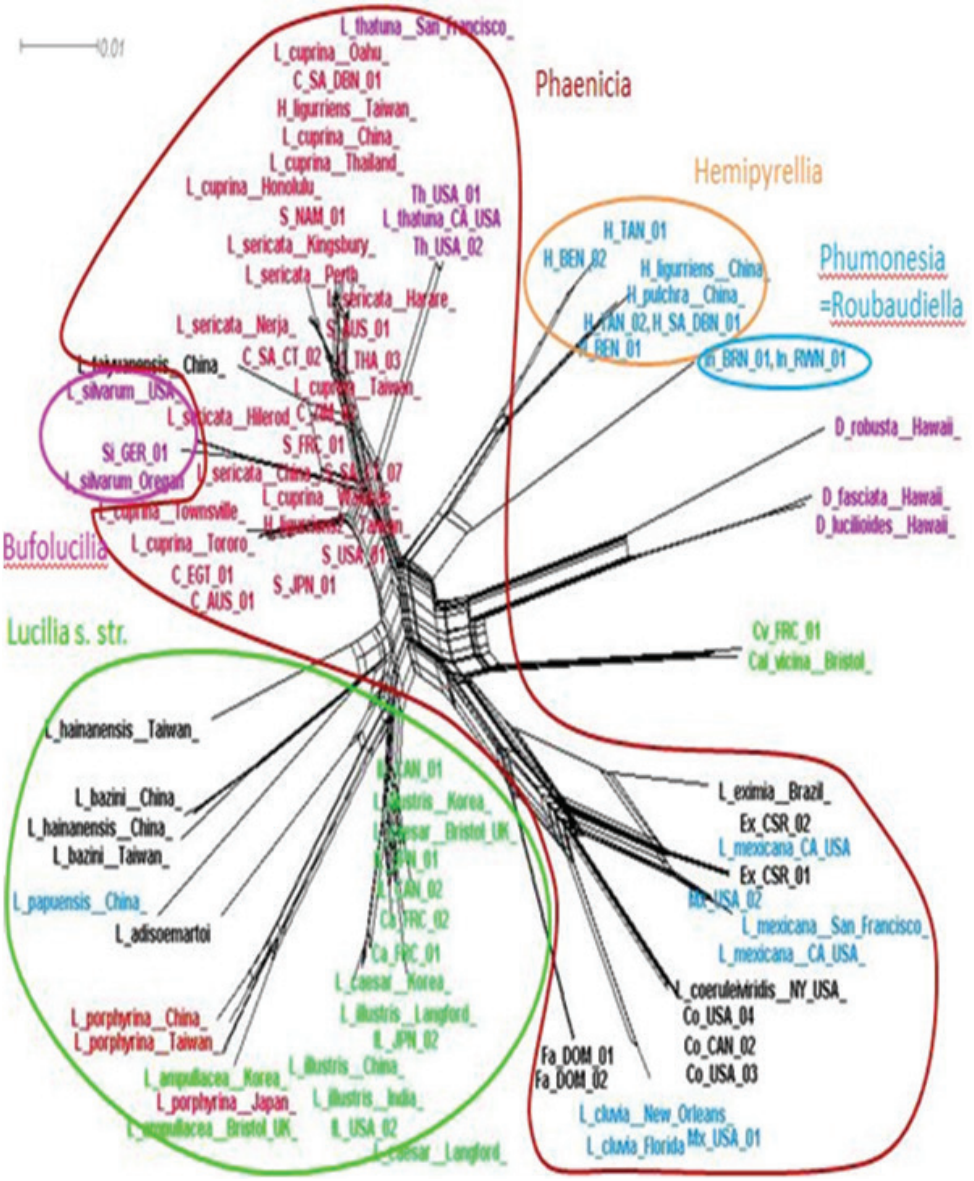

Figure 4. NeighborNet network diagram constructed from *COI* data showing parasitic behaviour (coloured text) and previous sub-generic status of *Lucilia* according to Hall (1948) (ellipses). Text colours: Red = primary facultative parasite, green = secondary facultative parasite, purple = parasite (unknown if primary or secondary), blue = saprophage, black = unknown parasitic behaviour. C = *cuprina*, Ca = *caesar*, Co = *coeruleiviridis*, CV = *Calliphora vicina*, Ex = *eximia*, Fa = *fayeae*, H = *Hemipyrellia fernandica*, IL = *illustris*, In = *infernalis*, Mx = *mexicana*, S = *sericata*, Si = *silvarum*, Th = *thatuna*, AUS = Australia, BRN = Burundi, CAN = Canada, CSR = Costa Rica, DOM = Dominica, FRC = France, GER = Germany, JPN = Japan, NAM = Namibia, EGT = Egypt, RWN = Rwanda, SWZ = Switzerland, SA = South Africa, TAN = Tanzania, THA = Thailand, USA = United States of America, ZIM = Zimbabwe. DBN = Durban, CT = Cape Town.

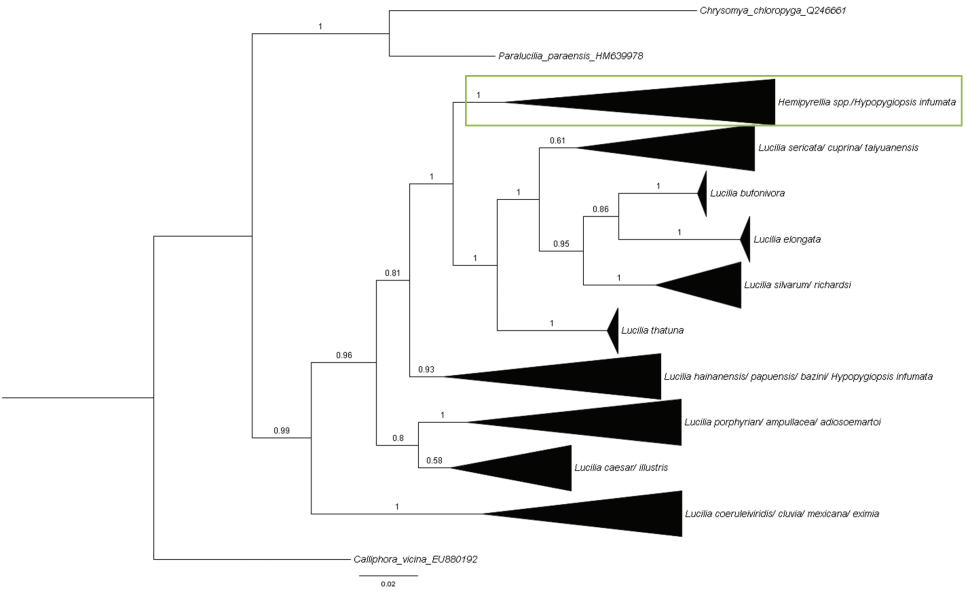

Figure 5. Bayesian inference tree constructed using *COI* barcode sequences. Posterior probabilities indicated on nodes. Support within the collapsed nodes is variable. Green box = *Hemipyrellia* sp.

Morphological data

The strict consensus parsimony tree for the morphological characters was largely uninformative, forming only two clades, with the majority of the species being unresolved (tree not shown). The 50% majority rule consensus tree (Fig. 6) grouped *L. sericata*, *L. cuprina*, *L. silvarum*, *L. bufonivora* and *L. thatuna* together. *Lucilia coeruleiviridis* and *L. cluvia* grouped together in all of the trees. The *Hemipyrellia* species formed a clade within *Lucilia*, and *L. caesar* and *L. illustris* grouped together.

Discussion

The majority rule consensus tree of the morphological characters (Fig. 6) was largely incongruent with the molecular phylogenetic trees (Figs 1–3, 5). The only clade that was congruent contains *L. sericata* + *L. cuprina* + *L. richardsi* + *L. silvarum* + *L. bufonivora* + *L. thatuna*. In the *COI* Bayesian inference tree this clade included *L. elongata* too (Fig. 5). This is partly due to disparities in taxon sampling and possibly partly a result of the limited character set available for the morphological parsimony analysis. It is ideal to have at least three times more characters than species in this type of analysis (Stevens and Wall 1996), whereas the matrix has 21 species and 17 character states, which limits the conclusions about general trends that can be drawn from these morphological data. This discussion will therefore focus on the results of the molecular analyses.

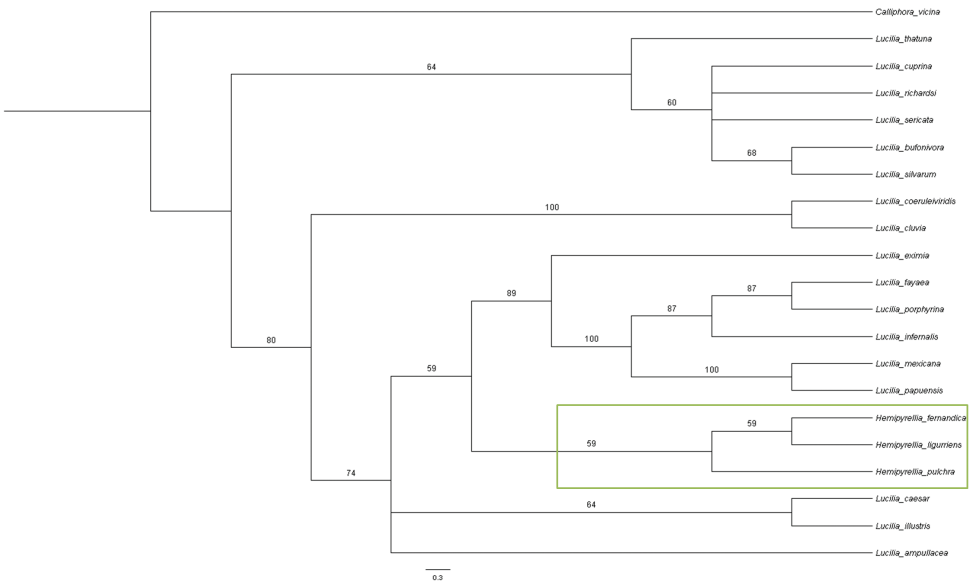

Figure 6. Majority rule consensus tree for 21 species of *Lucilia* and *Hemipyrellia* constructed from morphological characters listed in Table 3. Green box = *Hemipyrellia* sp.

Relationship of *L. sericata* and *L. cuprina*

Although only about half of the *Lucilia* species listed as valid by Aubertin (1933) were included in this study, these results strongly suggest that *L. sericata* and *L. cuprina* are indeed sister species. All of the Bayesian inference analyses (Figs 1–3) indicated this with strong support from the nuclear genes (*28S* and *Per*) and total evidence (*28S* + *Per* + *COI*) trees and weaker support from the *COI* gene alone. *Lucilia cuprina* was paraphyletic (Fig. 2) with respect to *L. sericata* in the mitochondrial gene (*COI*) tree, as shown previously (using the same sequences but weaker auxiliary taxon sampling) to be the result of introgressive hybridisation between these two species (Williams and Villet 2013). In another study (McDonagh and Stevens 2011), the nuclear gene elongation factor-1 alpha (*EF-1α*) did not recover *L. sericata* and *L. cuprina* as sister-species, but the clade containing *L. sericata* was poorly resolved and thus the conclusion was weakly supported, but the *28S* and *COI* gene trees both recovered *L. sericata* and *L. cuprina* as sister species with strong support (McDonagh and Stevens 2011).

Molecular identification of *Lucilia* species

It has already been established that *L. sericata* and *L. cuprina* show a case of ancient introgression, and that they still interbreed (Williams and Villet 2013). This is a widely

acknowledged problem for identification using partial *COI* sequences alone (Rubinoff et al. 2006, Nelson et al. 2007, Roe and Sperling 2007, Williams et al. 2008, Tantawi et al. 2010, Williams and Villet 2013). Other problematic species pairs occur in the genus (DeBry et al. 2012, Sonet et al. 2012), and it is important to recognise the cause(s) and to document genes that are more useful for identification in these contexts.

In the Bayesian inference trees based on mitochondrial (*COI*) (Fig. 2) and total evidence (*28S*, *Per* and *COI*) (Fig. 3), *L. mexicana* was paraphyletic with respect to *L. coeruleiviridis*. This has been observed in the continental United States of America (DeBry et al. 2012), where these two species were found to share a mitochondrial haplotype. The *L. mexicana* specimens with this *L. coeruleiviridis* haplotype appear to be limited to a geographic area including Texas and New Mexico (DeBry et al. 2012). This study independently confirms this pattern, since our new sequences of *L. mexicana* from New Mexico grouped with *L. coeruleiviridis*, and the GenBank specimens of *L. mexicana* from California formed a distinct clade (Figs 2-3). This suggests introgression between *L. mexicana* and *L. coeruleiviridis*. The nuclear genes separated *L. coeruleiviridis* and *L. mexicana*, although *L. mexicana* was not resolved in this analysis (Fig 1). In the Bayesian inference tree based on the *Per* gene alone (tree not shown), these two species are recovered as sister clades with 100% support, which suggests that nuclear genes will separate these two species as they do for *L. sericata* and *L. cuprina* (Williams and Villet 2013).

Lucilia caesar and *L. illustris* also share haplotypes (Sonet et al. 2012). In the *COI* tree (Fig. 2), *L. caesar* specimens from France and Korea and one specimen of *L. illustris* from the UK were not resolved, but the remainder of the *L. caesar* and *L. illustris* specimens formed a mixed clade with 100% support. These two species can therefore not be unambiguously identified using only *COI*. The nuclear genes in this study (Fig. 1) separated these two species but used only two specimens of *L. caesar* from France and seven specimens of *L. illustris* from Japan, Switzerland, Canada and the United States of America. Including specimens from other countries may give a different result as was seen in a previous study (Sonet et al. 2012) where *L. caesar* and *L. illustris* could not be reliably identified using either mitochondrial or nuclear genes as the intraspecific and interspecific genetic distances were very low. This might result from hybridisation or incomplete lineage sorting (Sonet et al. 2012).

These three species pairs highlight the need for using more than one gene to identify species, as has been suggested in previous studies (Rubinoff et al. 2006, Nelson et al. 2007, Roe and Sperling 2007, Williams et al. 2008, Tantawi et al. 2010, Williams and Villet 2013). It also highlights a problem in using *COI* as a universal 'barcoding' gene (Rubinoff et al. 2006, Roe and Sperling 2007, Whitworth et al. 2007, Sonet et al. 2012, van Nieukerken et al. 2012, Jordaens et al. 2013), especially in a forensic context. While cases of ancient introgression remain genetically identifiable (DeBry et al. 2012, Williams and Villet 2013), cases of incomplete lineage sorting may be intractable, and morphological identification may be the best solution, especially if the identifications need to go to court.

Diversification of Luciliinae

The Luciliinae showed two strong patterns underlying their diversification: biogeographical radiation and the diversification of parasitism.

The analyses (summarised in Fig. 4) showed geographically distinct clusters of species from the New World (*L. eximia* + *L. mexicana* + *L. coeruleiviridis* + *L. cluvia* + *L. fayeae*), the Oriental region (*L. hainanensis* + *L. bazini* + *L. papuensis* + *L. adiosoemartoi* Kurahashi, 1988), and Eurasia (*L. porphyrina* + *L. ampullacea*). *Hemipyrellia* formed a monophyletic Old World lineage (Aubertin 1931). *Lucilia infernalis* is found only in Africa (Aubertin 1933) and the sequences from Rwanda and Burundi formed a separate group. One component of phylogenetic diversification within *Lucilia* is therefore certainly biogeographical.

Lucilia sericata, *L. cuprina*, *L. thatuna* and *L. silvarum* form a clade of facultatively parasitic species, with *L. sericata* and *L. cuprina* being primary facultative parasites. This group is geographically diverse, with only *L. thatuna* being restricted to one region, the United States of America. Likewise, *L. caesar* and *L. illustris* form a clade that represents secondary facultative parasites. *Lucilia illustris* is Holarctic, while *L. caesar* is restricted to the Palaearctic (DeBry et al. 2012). *Dyscritomyia* is endemic to the Hawaiian Islands (Wells et al. 2002) and phylogenetically coherent. Its members are attracted to carrion and are suspected of breeding in carrion and parasitizing snails (Hardy 1981).

Many *Lucilia* species are myiasis-causing (Zumpt 1965), with *L. cuprina* being the most recognised and often referred to as the sheep-strike blowfly (Hepburn 1943, Ullyett 1945, Vogt and Woodburn 1979, Heath and Bishop 2006). Other species of *Lucilia* known to be facultative parasites include *L. sericata*, *L. silvarum*, *L. thatuna*, *L. richardsi*, *L. porphyrina*, *L. illustris*, *L. caesar*, and *L. ampullacea*; the only obligately parasitic species in the genus are *L. bufonivora* and possibly *L. elongata* (Aubertin 1933, Hall 1948, Zumpt 1965, Rognes 1991, McDonagh and Stevens 2011). There are also saprophagous species within *Lucilia*, including *L. mexicana*, *L. cluvia*, *L. papuensis* and *L. infernalis* (Hall 1948, Zumpt 1965). None of these different parasitic behaviours are limited to any particular geographical area (Fig. 4). This implies that diversification of breeding behaviours has also been a component of phylogenetic diversification within *Lucilia*, independent of biogeography.

Taxonomy of Luciliinae

Lucilia Robineau-Desvoidy, 1830 (type species: *Lucilia caesar* (Linnaeus, 1758) has a complex nomenclatural history that is integrally related to its biogeographical and dietary radiation. Several authors including Bigot, van der Wulp, Brauer and Bergenstamm, Girschner, Hough, Kramer, Shannon and Malloch (Aubertin 1933) contributed to the ultimate development of this genus. Early studies of the European *Lucilia*

were conducted by Stein (1924), Richards (1926), Collin (1926) and Séguy (1928) and Shannon published on the North and South Amercican *Lucilia* (1926) (Aubertin 1933). Aubertin (1933) published the most comprehensive review of the genus and recognised 27 species. This genus is widely spread across with world. The adults of this genus feed on nectar, carrion and decomposing material and the females are oviparous (Aubertin 1933). The larvae of this genus develop on decomposing animal material. Several species have developed specialised parasitic behaviour such as *L. cuprina*, which lays its eggs on living sheep and the larvae feed on the live animals, causing myiasis. *Lucilia bufonivora* is a parasite of toads.

Phaenicia Robineau-Desvoidy, 1863 (type species: *Phaenicia concinna* Robineau-Desvoidy, 1863 = *Musca sericata* Meigen, 1826) has a history of varied usage. Hall (1948) divided *Lucilia* into several separate genera including *Bufolucilia*, *Phaenicia* and *Lucilia sensu stricto*. Hall's (1948) separation of species into the genera *Phaenicia* and *Lucilia* was primarily based on the presence or absence of bristles on the subcostal sclerite and the character of the ocellar triangle. In contrast, Malloch (1926) used the yellow colour of the basicostal scale and the presence of three postsutural acrostichal bristles to define his concept of *Phaenicia*. The use of *Phaenicia* has persisted in North American literature (Stevens and Wall 1996, Byrd and Castner 2010), but is not generally used in other parts of the world as it is seen as a junior synonym of *Lucilia* (Zumpt 1965).

In the network analysis (Fig. 4), the species that would be assigned to *Phaenicia* based on Hall's (1948) criteria can clearly be seen to be part of two distant clades. These species occur in both the Old and New Worlds, showing vast geographic ranges. The group includes species that are primary facultative parasites and species that are saprophages. Hall's (1948) usage of *Lucilia s.str.* refers only to *Lucilia illustris* (and *L. caesar* for clarity between the two) as he focused only on Nearctic blowflies. The remaining species that would fall into this clade based on his diagnostic criteria grouped with *L. caesar* and *L. illustris* in our analyses (Fig. 4), and includes species that are primary and secondary facultative parasites as well as species that are saprophagous.

Bufolucilia Townsend, 1919 (type species: *Lucilia bufonivora*) includes the species *bufonivora*, *silvarum* and (by monophyly) *elongata*, which are found in Europe and North America. *Bufolucilia* forms a part of the clade that includes most of the facultatively parasitic *Lucilia* species (Fig. 4). There is no obvious reason to separate *Lucilia* into (sub)genera based on the parasitic behaviour of the species because primary and secondary facultatively parasitic and saprophagous species are spread throughout the genus (Fig. 4). Recognising *Bufolucilia* also makes *Phaenicia* paraphyletic (Fig. 4).

Phumonesia Villeneuve, 1914 and ***Roubaudiella*** Séguy, 1925 (type species: *Phumonesia infernalis* Villeneuve, 1914 = *Roubaudiella caerulea* Robineau-Desvoidy, 1863) are monotypic genera founded on the same species, and therefore objective synonyms. The only species shows affinities with *Hemipyrellia* in some analyses (Fig. 2, 4), and is always embedded inside *Lucilia*, leaving no reason to recognise a separate genus.

Similarly, *Francilia* Shannon, 1924, and *Acrophagella* Ringdahl, 1942, are objective synonyms because they are based on the same species. Several other genus-group

taxa have been erected within the Luciliinae, including *Caesariceps* Rodendorf, 1926, *Dasylucilia* Rodendorf, 1926, *Luciliella* Malloch, 1926 and *Viridinsula* Shannon, 1926. Their status needs assessment, and the results presented here suggest that morphological analyses alone will not be sufficient. Phylogenetic studies including a selection of both nuclear and mitochondrial genes are recommended.

Hemipyrellia Townsend, 1918 (type species: *Lucilia fernandica* Macquart, 1855) was erected as a genus by Townsend (1918) and revised by Aubertin (1931). It had previously been suggested that *Hemipyrellia* was a synonym of *Lucilia* (Shannon 1926). *Hemipyrellia* is restricted to the Old World and the species are saprophagous. The results of this study place *Hemipyrellia* within *Lucilia* for both nuclear and mitochondrial analyses with 100% support (Figs 1 and 2), the *COI* barcode Bayesian tree (Fig. 5) with very strong support, and the morphological majority rule consensus tree (Fig. 6) with weak (56%) support.

In two studies of Australian blowflies, *Hemipyrellia* was found to be a sister-group to *Lucilia* (Wallman et al. 2005, Nelson et al. 2012), but these studies included only species of *Lucilia* that occur in Australia, thus *Hemipyrellia* may be a sister-clade to Australian *Lucilia* as an artefact of taxon sampling. Similarly, another study (Singh and Wells 2013) found *Hemipyrellia* to be sister-group to *Lucilia*, but this was based on one specimen of *Lucilia sericata* and one specimen of *Hemipyrellia fernandica.* Several other studies have sequenced *Hemipyrellia* specimens and found them to lie within *Lucilia* (Wells et al. 2007, Park et al. 2009, Liu et al. 2011, McDonagh and Stevens 2011). Two specimens of *H. ligurriens* from Taiwan (Fig. 2) group within the *L. cuprina* clade. This is probably a misidentification because the specimens of *H. ligurriens* and *H. pulchra*, both from China, group with *H. fernandica* sequenced in this study. Assuming that the other *Hemipyrellia* specimens are not all misidentified, these previous studies together with the results of this study provide strong support for the synonymy of *Hemipyrellia* and *Lucilia*.

Dyscritomyia Grimshaw, 1901 (type species: *Prosthetochaeta robusta* Grimshaw, 1901) contains 35 nominal species that are all found exclusively on the Hawaiian Islands (James 1981). The biology of *Dyscritomyia* differs from the other Luciliinae in that at least some species are viviparous and produce only one larva at a time that is retained in the uterus for the first two instar stages. Little is known about their parasitic behaviour but it is assumed that *Dyscritomyia* species are facultatively parasitic saprophages (Hardy 1981). *Dyscritomyia* was included in the *COI* Bayesian inference analysis and was recovered as a separate clade to *Lucilia* (Fig. 2). In previous studies, *Dyscritomyia* was recovered within *Lucilia* when analysing the *COI* and *EF-1a* genes (Wells et al. 2007, McDonagh and Stevens 2001) but it was recovered as a sister clade to *Lucilia* when analysing the *28S* gene (McDonagh and Stevens 2011). *Dyscritomyia* was also recovered as a sister group to *Lucilia* in a study of the *COI* and *COII* genes (Wells et al. 2002). The current study used only a 576 bp region of the total *COI* gene from the sequences available on GenBank that were used in the study of Wells et al. (2002), but still recovered *Dyscritomyia* as a sister clade to *Lucilia*. It therefore does not appear that the length of the *COI* sequence affects the analysis significantly.

This study used 20 species of *Lucilia* in the *COI* analysis while the previous studies used six and 13 species, respectively (Wells et al. 2002, McDonagh and Stevens 2011). The position of *Dyscritomyia* relative to *Lucilia* may be determined by the taxon sampling of *Lucilia*, as mentioned regarding *Hemipyrellia*. This highlights the need for a more comprehensive study of this genus and inclusion of as many *Dyscritomyia* and *Lucilia* species as possible to confirm the taxonomic relationship between *Dyscritomyia* and *Lucilia*.

Hypopygiopsis Townsend, 1916 (type species: *Hypopygiopsis splendens* Townsend, 1916 = *Hypopygiopsis fumipennis* Walker, 1856) is restricted to the Asian and Australasian regions of the world (Kurahashi 1977). This genus apparently exhibits both oviparous and larviparous behaviour. The larval behaviour includes both facultative parasitism and saprophagy. *Hypopygiopsis* was included in the Bayesian inference analysis of the *COI* barcode dataset. One *Hypopygiopsis infumata* sequence grouped within *Lucilia* (Fig. 5) as part of a clade including *L. hainanensis* + *L. papuensis* + *L. bazini*. On closer examination of the sequences, *Hypopygiopsis infumata* was identical to the *Lucilia bazini* sequence from China. The *L. hainanensis* sequence from China that groups with these two sequences differs by only one base pair. This places doubt on the identification of these sequences and prevents any meaningful inferences being drawn. The second *Hypopygiopsis infumata* sequence groups with *Hemipyrellia*. There are only five sequences of *Hypopygiopsis* publically available and therefore the limited number of sequences constrains the credibility of this result and it is recommended that more sequences of this genus are examined to clarify if this genus should also be synonymised with *Lucilia*.

Conclusion

Lucilia sericata and *L. cuprina* are indeed sister-species. *Lucilia mexicana* is confirmed to be paraphyletic with respect to *L. coeruleiviridis*, possibly as a result of hybridisation and introgression. *Lucilia caesar* and *L. illustris* are both paraphyletic and further studies with different genes are needed to determine if these two species can be identified using molecular methods. *Hemipyrellia* should be synonymised with *Lucilia* because this genus sits within *Lucilia* in all of the analyses conducted in this study. *Dyscritomyia* requires further studies to confirm its phylogenetic positioning with regard to *Lucilia* because taxon sampling appears to have an impact on the analysis. The limited number of sequences available for *Hypopygiopsis* and the apparent misidentification of sequences prevent any conclusions being drawn about its relationship to *Lucilia*. In this study we have identified at least three cases of misidentified sequences from GenBank, which is a well-known problem (Bridge et al. 2003, Harris 2003, Nilsson et al. 2006, Valkiūnas et al. 2008). There is no geographic pattern to the distribution of the different parasitic behaviours within the Luciliinae and no reason to sub-divide *Lucilia* into genera or sub-genera based on either geographic location or parasitic behaviour.

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RESEARCH ARTICLE

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Revision of the Japanese species of *Epicephala* Meyrick with descriptions of seven new species (Lepidoptera, Gracillariidae)

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Abstract

Epicephala moths are involved in obligate mutualisms with their Phyllanthaceae hosts, in which the female moths assure pollination and, in return, their progeny develop by consuming the seeds. Ecological, molecular and geographical data suggest that the genus includes several hundred species, but the majority remains to be formally described. Here we revise the Japanese species of *Epicephala* Meyrick, 1880. In addition to two previously named species, seven species are newly described: *E. anthophilia* **sp. n.**, *E. lanceolatella* **sp. n.**, *E. perplexa* **sp. n.**, *E. obovatella* **sp. n.**, *E. corruptrix* **sp. n.**, *E. parasitica* **sp. n.** and *E. nudilingua* **sp. n.** The first four are species involved in obligate pollination mutualism, while the fifth is a pollinating seed parasite and the last two are derived non-pollinating seed parasites of herbaceous *Phyllanthus*. Each of the nine Japanese *Epicephela* species is specialized to a single plant species in the genera *Glochidion*, *Breynia* or *Phyllanthus*, except for *E. obovatella* and *E. corruptrix* that each utilizes two closely related *Glochidion* species. Considerable variations are found in pollination and oviposition behaviors among species, which are reflected in their proboscis and ovipositor morphologies, respectively. Molecular phylogeny indicated that there have been repeated transitions in oviposition mode during the diversification of *Epicephala*, which were accompanied by changes in ovipositor morphology, as suggested by a correlation analysis. Keys to species are provided.

Keywords

Active pollination behavior, *Breynia*, DNA barcode, *Glochidion*, Gracillariidae, Japan, obligate pollination mutualism, *Phyllanthus*

Introduction

The genus *Epicephala* Meyrick, 1880 (Gracillariidae) has recently become an important taxon in ecology and evolutionary biology because of their mutualisms with plants in the genera *Glochidion*, *Breynia* and *Phyllanthus* (Phyllanthaceae) (Kato et al. 2003; Kawakita and Kato 2009; Kawakita 2010). The females of *Epicephala* possess specialized sensilla-bearing proboscises and use them to actively collect, transport and deposit pollen on host flowers to ensure food for their seed-feeding larvae (Kato et al. 2003; Kawakita and Kato 2006; Kawakita et al. 2015). *Epicephala* moths are the only documented pollinators for many of these hosts, making this interaction an obligate mutualism. A number of *Epicephala* species have secondarily lost the pollination behavior and became parasitic (Kawakita and Kato 2009; Kawakita et al. 2015), which is often accompanied by the loss of the specialized sensilla on the proboscis (Kawakita et al. 2015).

Glochidion, *Breynia* and *Phyllanthus* belong to the well-defined tribe Phyllantheae, which contains over 1,200 species globally (Hoffmann et al. 2006). Of these, ca. 500 species are thought to depend exclusively on *Epicephala* for pollination (Kawakita and Kato 2009). Because there is high level of species-specificity between the plants and the moths (Kawakita and Kato 2006; Kawakita et al. 2010), a comparable number of *Epicephala* species likely exist; however, the genus currently consists of only 53 named species (De Prins and De Prins 2005, 2014; Li and Yang 2015; Li et al. 2015). There is clearly a need to rapidly advance the taxonomy of the genus, especially because most published ecological and evolutionary studies on this mutualism treated *Epicephala* with arbitrary and variable names (Kawakita and Kato 2004a,b, 2009; Kawakita et al. 2004, 2010, 2015; Okamoto et al. 2007; Hembry et al. 2012, 2013; Goto et al. 2010; Svensson et al. 2010), making comparisons among studies problematic (but see Hu et al. 2011; Zang et al. 2012a; Li and Yang 2015; Li et al. 2015 for recent taxonomic advancement of Chinese *Epicephala*).

Aside from the remarkable pollination behavior, *Epicephala* is noteworthy among other genera of Gracillariidae in being seed parasitic and having a well-developed ovipositor (Kato et al. 2003). Gracillariid moths are predominantly leaf miners; the seed-feeding habit is otherwise only known in *Conopomorpha* Meyrick, 1885, which contains species that attack seeds of tropical fruit trees such as lychee and longan (both Sapindaceae) and cacao (Malvaceae). The recently described *Conopomorpha flueggella* Li, 2011, which feeds on the seeds of *Flueggea suffruticosa* (Phyllanthaceae) (Hu et al. 2011), is distantly related to other members of *Conopomorpha* but closely related to *Epicephala* (Kawakita et al. 2010). Thus, the evolution of seed feeding and the colonization of Phyllanthaeae likely occurred in the common ancestor of *Epicephala* and *Conopomorpha flueggella*, followed by the evolution of pollination behavior, sensilla-bearing proboscis and ovipositor in *Epicephala*. Ovipositors are used by female *Epicephala* to deposit eggs internally in floral tissue (Kato et al. 2003; Kawakita et al. 2015), unlike other members of Gracillariidae that oviposit externally. Internal oviposition is secondarily lost in *Epicephala vitisidaea* Li, Wang & Zhang, 2012 and *E. mirivalvata* Li, Wang & Zhang, 2012 that lay eggs in the narrow space between the

tepals and ovary (Kawakita and Kato 2004; Zang et al. 2012b), although functional ovipositors are retained in both species.

The purpose of this paper is to provide descriptions of the *Epicephala* species occurring in Japan, where most published ecological studies have been conducted. The names used in published studies to refer to each species described here are also given to facilitate the interpretation of published results.

Methods

A total of 496 adult pinned specimens were used for this study, from which 132 genital dissections were made. Descriptions focused on the adult stage because adults provide a wealth of morphological traits useful for diagnosis and because immature stages of *Epicephala* are poorly known. For genital dissections, the whole abdomen was removed and incubated for 2–4 h in 10% KOH, and residual scales and soft parts were removed in 70% ethanol. Genitalia were then stained in fuchsin acid for 30 min to 2 h, dehydrated in a series of 70–100 % ethanol and mounted in Euparal (Waldeck GmbH & Co. KG, Division Chroma, Münster, Germany) on a glass slide. To study the sensilla on the proboscis, the entire head was removed, incubated in KOH as above and mounted in Euparal on a glass slide without staining. Observation and measurements were made under an Olympus BX53F microscope at 10–40× with the aid of a micrometer scale.

Images of adults were captured using the Olympus E-330 camera mounted on Olympus SZX10 dissection microscope, and those of genitalia and mouthparts were captured using the Canon EOS Kiss X5 camera mounted on the Olympus BX53F microscope. Images were taken at various depths and subsequently stacked using the CombineZP software (www.hadleyweb.pwp.blueyonder.co.uk). All images were then edited with Adobe Photoshop CC into final figures.

To infer the phylogenetic positions of the Japanese species within *Epicephala*, we assembled published nucleotide sequence data for the cytochrome oxidase subunit 1 (COI), arginine kinase (ArgK) and elongation factor 1-alpha (EF1α) genes available in GenBank for 52 *Epicephala* species (accession numbers are provided in Suppl. material 1), and used them to reconstruct the phylogeny of the genus. Sequence data are already available for the nine *Epicephala* species treated in this study (Kawakita and Kato 2006, 2009), but they have not been analyzed together with those of other *Epicephala* for which data are available. Because a large number of *Epicephala* sequences are presently available in GenBank, only one sequence per species per locus was sampled for the analysis. The COI, ArgK and EF1α gene partitions consisted of 582 bp, 723 bp and 522 bp, respectively. Phylogeny was constructed using the maximum-likelihood method in Treefinder (Jobb 2011) with substitution models proposed by the program (GTR+G, J3+G and J2+G for COI, ArgK and EF1α, respectively). Robustness of the tree was validated by a bootstrap analysis in Treefinder with 1,000 replicates. *Conopomorpha flueggella* was included in the analysis, and *Stomphastis labyrinthica* (Meyrick, 1918), *Melanocercops ficuvorella* (Yazaki, 1926) and *Cuphodes diospyrosella* (Issiki, 1957) served as outgroups (Kawakita et al. 2010).

It was observed that *Epicephala* species that oviposit through the ovary wall possess a characteristic angular ovipositor tip, therefore it was tested whether there was a correlation between oviposition site and ovipositor morphology using the Pagel's (1994) correlation method as implemented in the Mesquite software (Maddison and Maddison 2015). Because data on oviposition site and ovipositor morphology are also available for *E. lativalvaris* Li, Wang & Zhang, 2012 (Zang et al. 2012a,b), these were included in the analysis. Oviposition site was categorized as either (1) oviposition through ovary wall or (2) oviposition in stylar tissue or external. Ovipositor morphology was categorized as either (1) angular or (2) non-angular. Using the maximum-likelihood phylogeny obtained as described above, we tested whether correlated evolution is more likely than the null hypothesis of independent evolution using Mesquite.

The level of intra- and interspecific divergences in the COI barcoding region (same as the above 582-bp fragment) was also quantified by analyzing all *Epicephala* barcode sequences presently available in GenBank. For each of the nine *Epicephala* species, the maximum pairwise distance was calculated within species and the minimal distance to other species. We also reconstructed a maximum-likelihood phylogeny in Treefinder to test for the monophyly of each species, following the method described above for the multi-gene data set. The GTR+G substitution model was chosen for the analysis.

All type materials have been deposited in the Zoological Collection of the Kyoto University Museum (KYO). Unless otherwise stated, specimens were collected by the primary author. All botanical names follow Govaerts et al. (2000).

Keys to the Japanese species of *Epicephala*

Males

1	Aedeagus with cornutus extending well beyond apex of aedeagus	***nudilingua***
–	Aedeagus without cornutus extending well beyond apex of aedeagus	**2**
2	Valva with a long spine 1/2 length of cucullus at base	***parasitica***
–	Valva without long spine at base	**3**
3	Sacculus with a single well developed spine at apex	***corruptix***
–	Sacculus without solo spine at apex	**4**
4	Sacculus with a row of spines running parallel to ventral margin	***perplexa***
–	Sacculus without a row of spines	**5**
5	Aedeagus with spiniform cornuti on dorsal and ventral sides	***vitisidaea***
–	Aedeagus without spiniform cornuti	**6**
6	Sacculus as long as or slightly shorter than cucullus	**7**
–	Sacculus distinctly shorter than cucullus	**8**
7	Sacculus acute apically; projection on dorsal margin hook-like	***lanceolatella***
–	Sacculus rounded apically; projection on dorsal margin round	***bipollenella***
8	Dorsal margin of sacculus with projection bearing spines	***obovatella***
–	Sacculus without spine-bearing projection	***anthophilia***

Females

1 Seventh sternite and tergite fused laterally.....***parasitica***
– Seventh sternite connected to seventh tergite by intersegmental membrane **2**
2 Lamella postvaginalis bilobed distally for > 1/2 of its length **3**
– Lamella postvaginalis not bilobed, or if bilobed, each lobe no longer than 1/2 of lamella postvaginalis itself.....**5**
3 Lobe of lamella postvaginalis round, club-shaped..... ***obovatella***
– Lobe of lamella postvaginalis finger-shaped.....**4**
4 Lobes of lamella postvaginalis dilated laterally; signa present.....***anthophilia***
– Lobes of lamella postvaginalis straight; signa absent.....***nudilingua***
5 Lamella postvaginalis heavily sclerotized and strongly curved.....***perplexa***
– Lamella postvaginalis not heavily sclerotized or strongly curved.....**6**
6 Ovipositor apically bilobed and not dentate..... ***vitisidaea***
– Ovipositor apically dentate and not bilobed.....**7**
7 Ovipositor angular at apex..... ***corruptrix***
– Ovipositor rounded at apex **8**
8 Lamella postvaginalis > 1/2 width of seventh sternite..... ***lanceolatella***
– Lamella postvaginalis < 1/2 width of seventh sternite..... ***bipollenella***

Species descriptions

***Epicephala anthophilia* sp. n.**
http://zoobank.org/C379F786-7FA0-44DF-A07F-C17EE05E8A65
Figs 1–7

Epicephala sp. 1 (Kato et al. 2003); *Epicephala* sp. (*acuminatum*) (Kawakita et al. 2004); Clade 2 (Kawakita and Kato 2006); *Epicephala* sp. ex *G. acuminatum* (Kawakita and Kato 2009; Kawakita et al. 2015); *Epicephala* sp. 2 (*G. acuminatum*) (Kawakita et al. 2010).

Diagnosis. This species is morphologically similar to *E. eriocarpa* Li, Wang & Zhang, 2012 but differs from the latter in having a longer and apically acute sacculus, lamella postvaginalis with distal arms stretched outwardly, and shorter ductus bursae relative to antrum.

Description. *Wingspan*: 9.2–11.0 mm.

Head: With numerous white scales on dorsal surface. Labial palpus with dark brown scales. Antenna brown, about 1.2× as long as forewing. Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Thorax: White dorsally. Forewing brown with narrow white band on dorsum from base to 2/3 of entire length; three pairs of white bands beginning at costal and dorsal

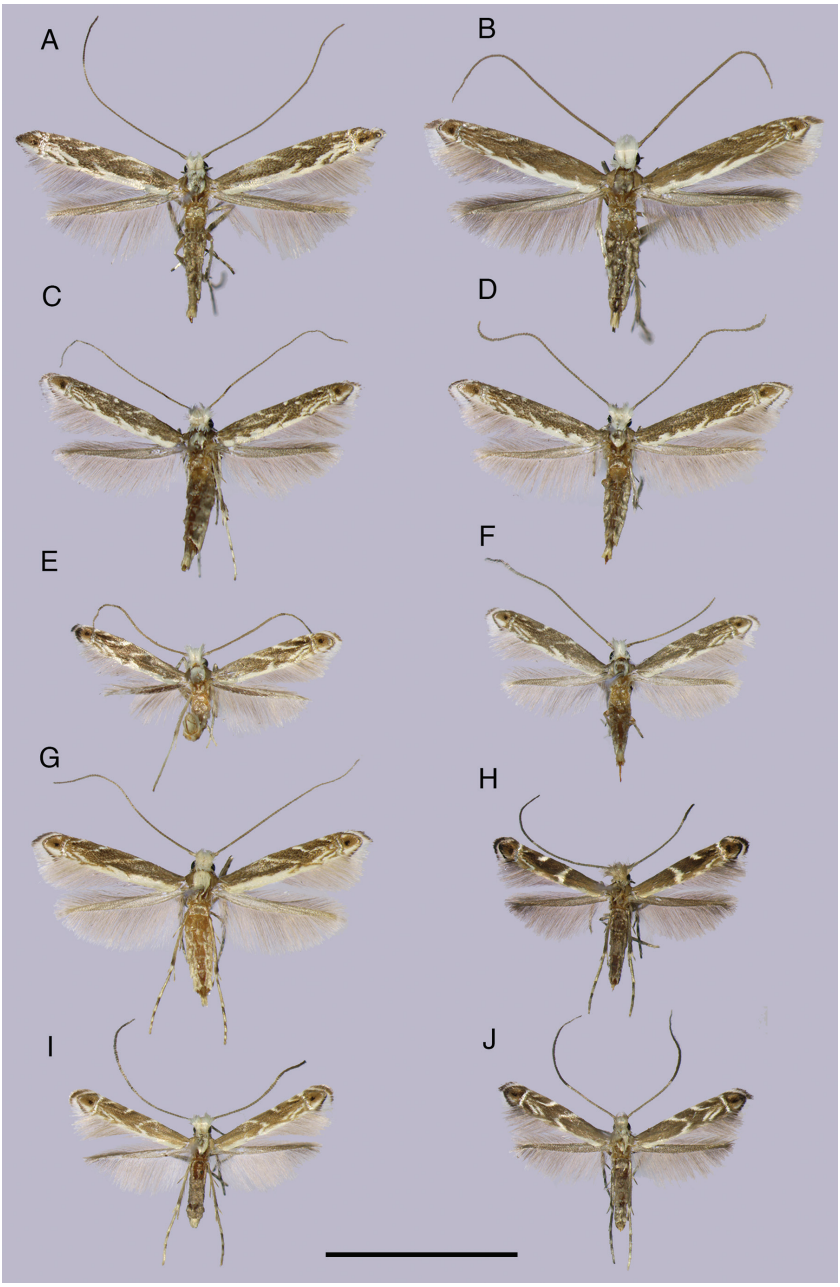

Figure 1. Representative specimens of the nine *Epicephala* species in Japan. Wing pattern of *E. parasitica* is sexually dimorphic, so specimens of both sexes are shown for this species. **A** *E. anthophilia* (Amami Island, Kagoshima, ♀, holotype) **B** *E. bipollenella* (Henoko, Okinawa, ♀) **C** *E. lanceolatella* (Cape Hedo, Okinawa, ♀, holotype) **D** *E. perplexa* (Cape Hedo, Okinawa, ♀, holotype) **E** *E. obovatella* (Tomogashima, Wakayama, ♂, paratype) **F** *E. corruptrix* (Takae, Okinawa, ♀, holotype) **G** *E. vitisidaea* (Yona, Okinawa, ♀) **H** *E. parasitica* (Yonaguni Island, Okinawa, ♀, holotype) **I** *E. parasitica* (Hateruma Island, Okinawa, ♂) **J** *E. nudilingua* (Watarase-yusuichi, Tochigi, ♀, holotype). Scale bar: 5 mm.

margins near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white spot or band near costa and dorsum; distal end fringed with narrow white band; cilia grayish brown. Hindwing brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen rounded triangular. Cucullus rectangular oblong, apex rounded, inner surface covered with numerous hairs; dorsal margin longer than ventral margin. Sacculus 0.7× length of cucullus, wider than cucullus near base but abruptly tapering at mid-length, acute apically; inner surface of dorso-distal portion with minute sclerotized teeth. Vinculum U-shaped; saccus slender, 5/6 length of vinculum. Aedeagus straight; lateral surface with a pair of sclerotized longitudinal ridges near mid-length, fringed by a few spines.

Female genitalia: Lamella postvaginalis H-shaped, about 0.5× length of seventh abdominal segment, 4× as broad as ostium bursae; distal arms longer than basal arms and stretched outwardly. Antrum long, as long as lamella postvaginalis. Ductus bursae 1.6× length of antrum, with longitudinal parallel ridges for its entire length. Corpus bursae oval, as long as ductus bursae; signum triangular, at 1/3 from base. Apophyses posteriores about 1.5× length of apophyses anteriores. Ovipositor dentate laterally, rounded apically.

Material examined. 16♂, 15♀. Holotype ♀ – **JAPAN: Kagoshima Prefecture:** Amami Island, Tatsugo, Nagakumo-toge (28.447828, 129.589449), 280 m, collected on female flower of *Glochidion acuminatum* in the act of pollination and oviposition, 10.v.2015 (KYO). The specimen possesses *Glochidion* pollen on proboscis. Paratypes – same locality as holotype, 10.xii.2007, collected as larvae in fruits of *Glochidion acuminatum* and reared to adults, 6♂, 7♀ (KYO). Other specimens – **JAPAN: Kagoshima Prefecture:** Amami Island, Tatsugo, Nagakumo-toge, 4.xi.2004, 3♂; Amami Island, Asado, 8.xii.2009, 4♂, 5♀ (R. Goto); Amami Island, Setsuko, 4.xi.2004, 1♂; Amami Island, Yakugachi, 19.xii.2005, 2♂, 1♀; Amami Island, Yakugachi, 13.xii.1997, 1♀ (M. Kato).

DNA barcodes. AY221964, AY221965, AY525718, DQ298944–DQ298956.

Known host and adult behavior. Known only from *Glochidion acuminatum*. Pollination behavior present (Fig. 8A). Oviposition from apical stylar pit, in stylar tissue (Fig. 8B). Larva feeds on seeds.

Distribution. Found in a few islands with high elevation in the Ryukyu Archipelago (Amami Island and Okinawa Island; Fig. 9A). The host plant *Glochidion acuminatum* is distributed throughout Southeast Asia from southern Japan to India, so this species is likely to be found in other parts of the host plant's range.

Etymology. The name *anthophilia* (an adjective) is derived from the Latin *antho-* (= flower) and *-philia* (= love, affection), commemorating that the flower-visiting behavior of *Epicephala* was first found in this species (Kato et al. 2003).

Remarks. The flight period of this species is restricted to a 3–4 week period in May in Amami Island, corresponding to the flowering period of its host plant *G. acuminatum*. The egg undergoes a prolonged dormancy in the flower for up to five months,

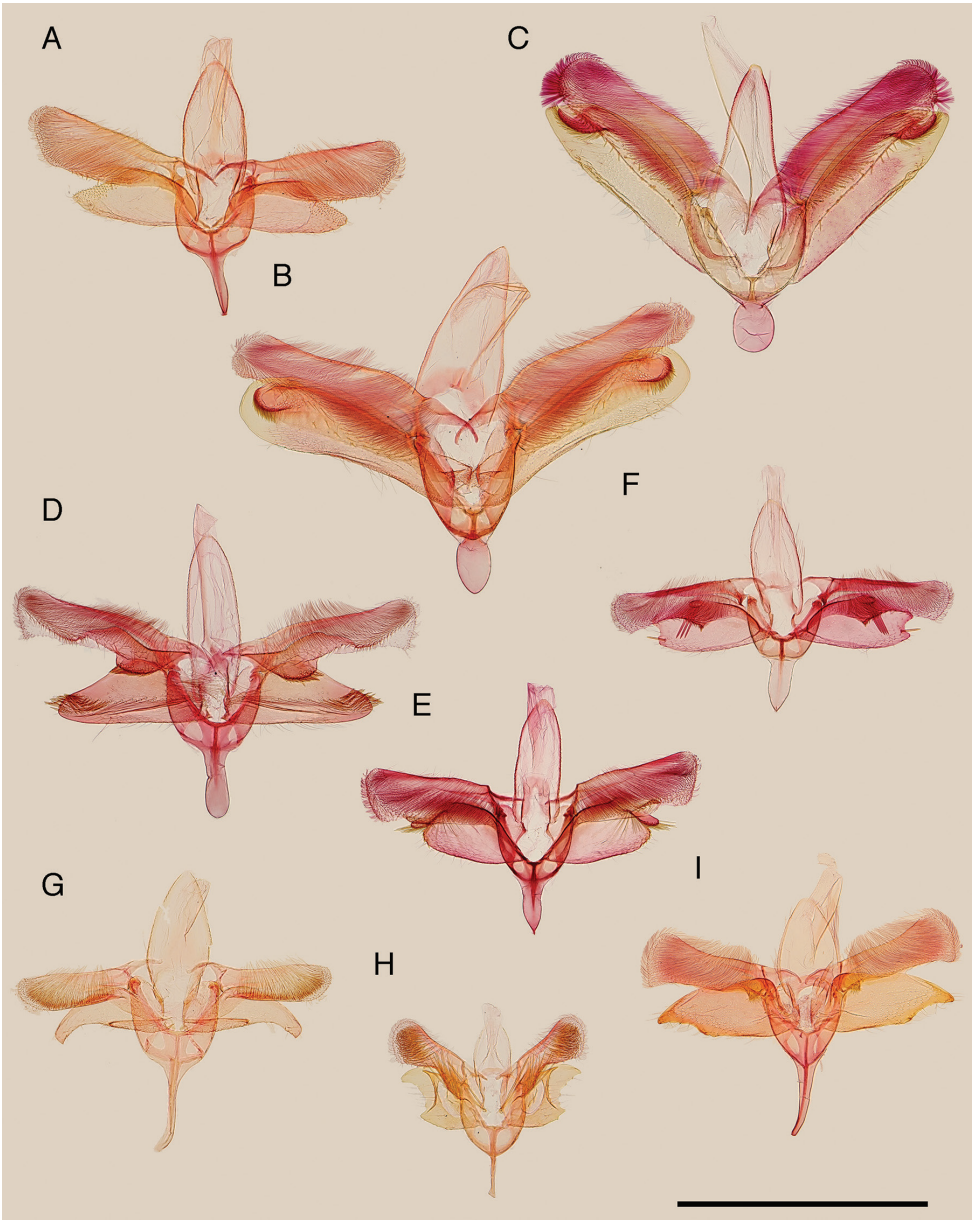

Figure 2. Valva of the Japanese *Epicephala* species. **A** *E. anthophilia* (paratype, slide No. AK249) **B** *E. bipollenella* (slide No. AK258) **C** *E. lanceolatella* (non-type, slide No. AK270) **D** *E. perplexa* (paratype, slide No. AK272) **E** *E. obovatella* (non-type, slide No. AK245) **F** *E. corruptrix* (paratype, slide No. AK260) **G** *E. vitisidaea* (slide No. AK234); **H**, *E. parasitica* (non-type, slide No. AK290) **I** *E. nudilingua* (paratype, slide No. AK292). Scale bar: 1 mm.

and the larva hatches and develops as the fruit begins to mature in late October (Goto et al. 2010). The moth overwinters as pre-pupa. *Epicephala anthophilia* is presently the only known univoltine species in the genus.

Epicephala bipollenella Li, Wang & Hu, 2012

Figs 1–7

Epicephala sp. 2 (Kato et al. 2003); *Epicephala* sp. (*zeylanicum*) (Kawakita et al. 2004); Clade 6 (Kawakita and Kato 2006); *Epicephala* sp. ex *G. zeylanicum* (Kawakita and Kato 2009); *Epicephala* sp. 5 (*G. zeylanicum*) (Kawakita et al. 2010).

Diagnosis. This species is distinctive among other species of *Epicephala* in that the anterior margin and midline of the seventh sternite of females have strong sclerotized wrinkles. The species is similar to *E. lanceolatella* but can be distinguished from the latter by the apically rounded sacculus, stronger wrinkles on seventh sternite of females and broader lamella postvaginalis.

Description. Description as in Zhang et al. (2012a), except the following.

Head: Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Male genitalia: Aedeagus slightly curved downwardly; dorsal surface with a sclerotized longitudinal ridge beginning medially at base and curving left toward apex.

Material examined. 64♂, 51♀. **JAPAN: Kagoshima Prefecture:** Amami Island, Akakina, 3.vii.1999, 1♂, 1♀; Amami Island, Akakina, 2.x.2002, 1♂; Amami Island, Akakina, 23.xii.2004, 2♂, 1♀; Amami Island, Taira, 24.vi.2008, 2♂, 1♀; **Okinawa Prefecture:** Okinawa Island, Henoko, 13.vi.2004, 41♂, 32♀; Okinawa Island, Henoko, 15.vi.2015, 7♂, 9♀; Okinawa Island, Taiho, 13.vi.2004, 2♂, 1♀; Okinawa Island, Higashi, 13.vi.2004, 3♂, 4♀; Ishigaki Island, Omoto, 25.ix.2005, 1♂, 1♀; Iriomote Island, Funaura, 29.ix.2004, 3♂, 1♀; Iriomote Island, Sonai, 9.ix.2008, 1♂.

DNA barcodes. AY221966–AY221971, AY525733, DQ299033–DQ299039.

Known host and adult behavior. Known only from *Glochidion zeylanicum*. Pollination behavior present. Oviposition from apical stylar pit, in stylar tissue (Fig. 8C). Larva feeds on seeds.

Distribution. Occurs widely throughout the Ryukyu Archipelago, Japan (Fig. 9B). Known also from China (Zhang et al. 2012a).

Remarks. Zhang et al. (2012a) suggest that this species pollinates two *Glochidion* species (*G. zeylanicum* and *G. hirsutum*, hence the name *bipollenella*). However, *G. hirsutum* is a name used to refer to hairy individuals of *G. zeylanicum*, which occur in low frequency in some populations. Similar co-occurrence of glabrous and pubescent individuals is common in *Glochidion* (A. Kawakita, personal observation). This species is therefore better viewed as a specialist of *G. zeylanicum*, at least in populations thus far studied.

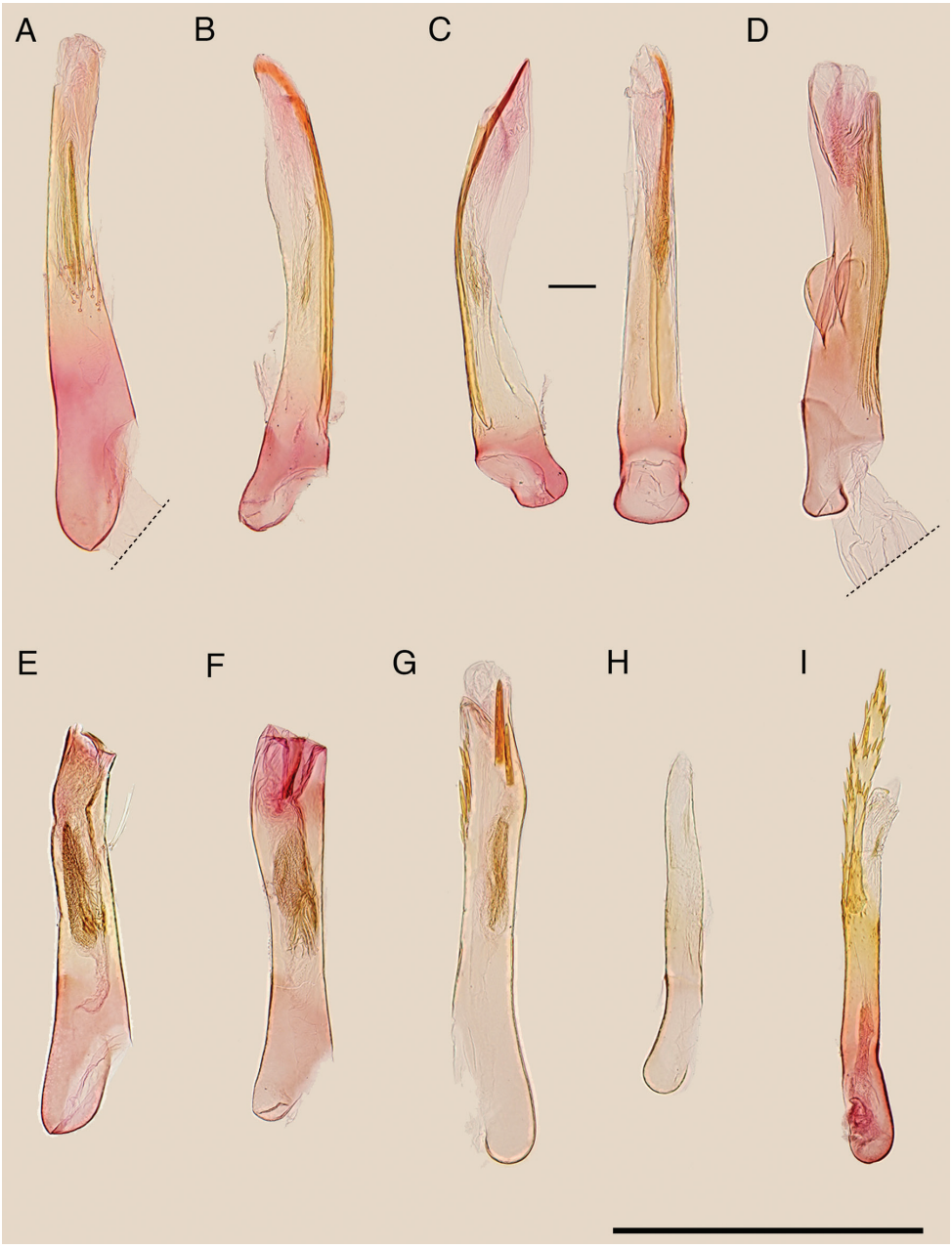

Figure 3. Aedeagus of the Japanese *Epicephala* species. **A** *E. anthophilia* (paratype, slide No. AK249), lateral view **B** *E. bipollenella* (slide No. AK258), lateral view **C** *E. lanceolatella*, lateral (left; non-type, slide No. AK270) and dorsal (right; non-type, slide No. AK271) view **D** *E. perplexa* (paratype, slide No. AK272), lateral view **E** *E. obovatella* (non-type, slide No. AK245), lateral view **F** *E. corruptrix* (paratype, slide No. AK260), lateral view **G** *E. vitisidaea* (slide No. AK234), lateral view **H** *E. parasitica* (non-type, slide No. AK290), lateral view **I** *E. nudilingua* (paratype, slide No. AK292), ventral view. Scale bar: 0.5 mm.

***Epicephala lanceolatella* sp. n.**
http://zoobank.org/E9626266-3849-4EB0-A73B-8A03E6CB45DC
Figs 1–7

Epicephala sp. (*lanceolatum*) (Kawakita et al. 2004); Clade 5 (Kawakita and Kato 2006); *Epicephala* sp. 6 (*G. lanceolatum*) (Kawakita et al. 2010).

Diagnosis. This species is very similar to *E. bipollenella* but can be distinguished from the latter by the apically acute sacculus, the more curved distal appendages on sacculus and broader lamella postvaginalis.

Description. *Wingspan*: 8.8–10.3 mm.

Head: With numerous white scales on dorsal surface. Labial palpus dark brown. Antenna brown, about 1.2× as long as forewing. Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Thorax: White dorsally. Forewing brown with narrow white band on dorsum from base to 2/3 of entire length; three narrow white bands beginning at dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; white spots scattered on costal half; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white band near dorsum; distal end fringed with narrow white band; cilia grayish brown. Hindwing brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen elongated triangular. Cucullus rounded rectangular; inner surface covered with numerous hairs. Sacculus as long and wide as cucullus, acute apically; dorsal margin rounded medially, attached with a plate possessing short spines sparsely on inner surface and terminating distally as inward hook-like projection with dense spines on dorso-ventral surface. Vinculum V-shaped; saccus oval, about 2/5 length of vinculum. Aedeagus slightly curved downwardly; dorsal surface with a sclerotized longitudinal ridge beginning medially at base and curving left toward apex.

Female genitalia: Lamella postvaginalis rounded triangular, bilobed at apex, as long as seventh sternite, 0.7× width of seventh sternite. Antrum short, with a pair of sclerotized parallel ridges. Ductus bursae as long as lamella postvaginalis, with longitudinal parallel ridges for its entire length. Corpus bursae elongate oval; signum absent. Apophyses posteriores 1.6× length of apophyses anteriores. Ovipositor dentate laterally, rounded apically.

Material examined. 36♂, 33♀. Holotype ♀ – **JAPAN: Okinawa Prefecture:** Okinawa Island, Kunigami, Cape Hedo (26.860200, 128.257979), 30 m, collected as larva in fruit of *Glochidion lanceolatum* and reared to adult, 15.vi.2015 (KYO). Paratypes – same data as holotype, 2♂ (KYO); same locality as holotype, 13.vi.2004, 11♂, 12♀ (KYO); Other specimens – **JAPAN: Kagoshima Prefecture:** Amami Island, Setsuko, 19.v.2005, 3♂; Amami Island, Naon, 24.vi.2008; **Okinawa Prefecture:** Ishigaki Island, Omoto, 30.ix.2004, 7♂, 112♀; Iriomote Island, Funaura, 5.x.2003, 3♀; Yonaguni Island, Mantabaru, 20.ix.2004, 9♂, 5♀.

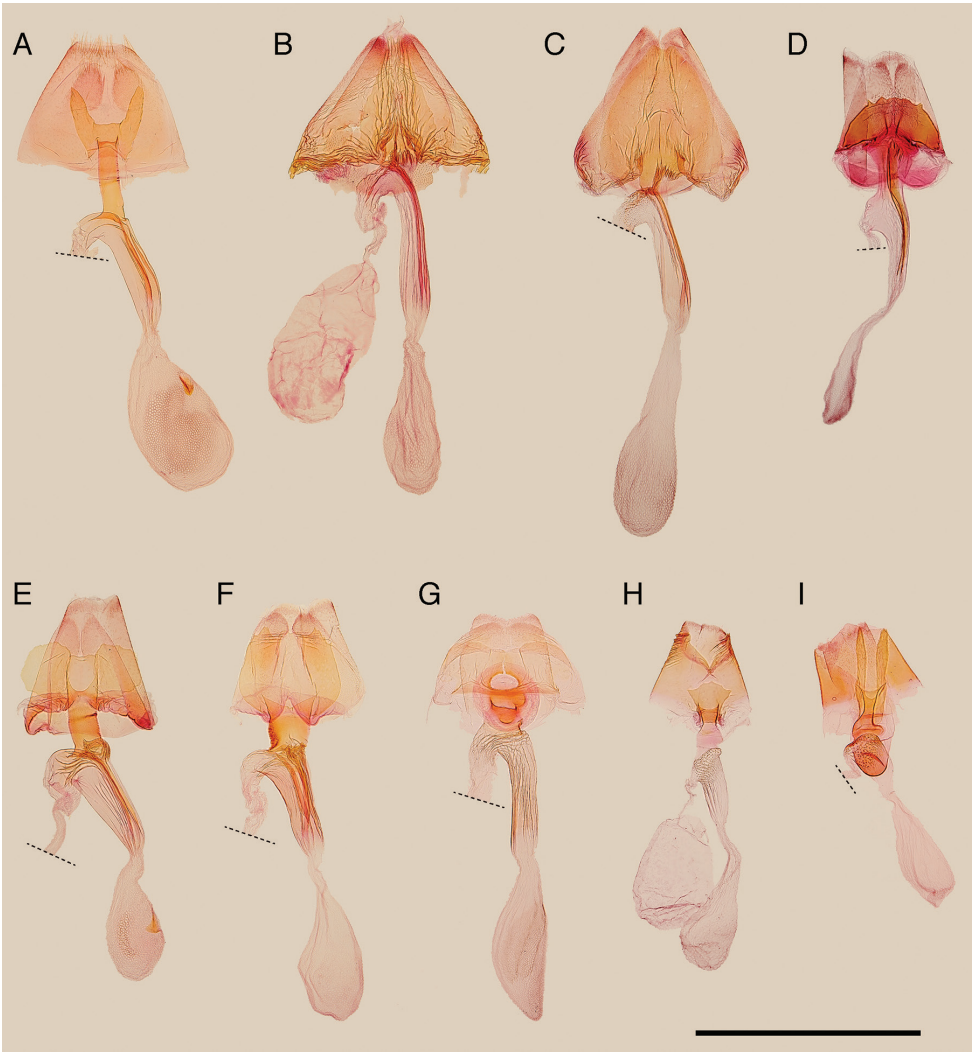

Figure 4. Seventh abdominal segment and corpus and ductus bursae of the Japanese *Epicephala* species. **A**, *E. anthophilia* (paratype, slide No. AK250) **B** *E. bipollenella* (slide No. AK281) **C** *E. lanceolatella* (non-type, slide No. AK251) **D** *E. perplexa* (paratype, slide No. AK253) **E** *E. obovatella* (non-type, slide No. AK246) **F** *E. corruptrix* (paratype, slide No. AK262) **G** *E. vitisidaea* (slide No. AK239) **H** *E. parasitica* (non-type, slide No. AK293) **I** *E. nudilingua* (paratype, slide No. AK296). Scale bar: 1 mm.

DNA barcodes. AY525727, DQ298957–DQ298961, DQ298965, DQ298966, DQ298968, DQ298972, DQ298973, DQ298977, DQ298981–DQ298983, DQ298986–DQ298988, DQ298990–DQ298995.

Known host and adult behavior. Known only from *Glochidion lanceolatum*. Pollination behavior present. Oviposition from apical stylar pit, in stylar tissue (Fig. 8D). Larva feeds on seeds.

Distribution. Ryukyu Archipelago, Japan (Amami Island, Okinawa Island, Ishigaki Island, Iriomote Island and Yonaguni Island; Fig. 9C).

Etymology. The name *lanceolatella* (an adjective) derives from the species name of the host plant *G. lanceolatum*.

***Epicephala perplexa* sp. n.**

http://zoobank.org/131546B9-E33D-4D3F-99AA-838AC256CF9F

Figs 1–7

Clade 3 (Kawakita and Kato 2006); *Epicephala* sp. ex *G. lanceolatum* (Kawakita and Kato 2009; Kawakita et al. 2015); *Epicephala* sp. 3 (*G. lanceolatum*) (Kawakita et al. 2010).

Diagnosis. This species is unlike any other *Epicephala* species in having outward projection on basal cucullus bearing dense spines, row of spines spanning the entire sacculus and rigidly sclerotized and ventrally curved lamella postvaginalis.

Description. *Wingspan*: 8.3–10.0 mm.

Head: With numerous white scales on dorsal surface. Labial palpus dark brown. Antenna brown, about 1.2× as long as forewing. Female proboscis with a large number of trichoid sensilla; sensilla as long as width of proboscis, denser toward base.

Thorax: White dorsally. Forewing brown with narrow white band on dorsum from base to 2/3 of entire length; three narrow white bands beginning at dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; dull white spots scattered on costal half; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white band near dorsum; distal end fringed with narrow white band; cilia grayish brown. Hindwing brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen elongated rounded triangular. Cucullus rounded rectangular at distal half, projected outwardly at basal half with dense spines on outer surface of the projection; ventral margin of basal half folded inwardly; inner surface covered with numerous hairs. Sacculus rounded triangular, 2/3 length of cucullus, with row of long spines on ventral inner surface running parallel to ventral margin and continuing to a cluster of spines at apex. Vinculum U-shaped; saccus oblong, as long as vinculum. Aedeagus slightly curved dorsally at middle, with a pair of half-moon-shaped projections ventrally near mid-length, dorsally with parallel longitudinal ridges for entire length, dilated slightly at apex.

Female genitalia: Lamella postvaginalis rigid, crescent-shaped, curved ventrally, with a pair of teeth on posterior margin, as broad as and 0.5× length of seventh sternite. Antrum 1.2× length of lamella postvaginalis, with a pair of sclerotized parallel ridges continuing to ductus bursae. Ductus bursae as long as antrum, with longitudinal parallel ridges to 2/3 of its length. Corpus bursae elongate oval; signum absent.

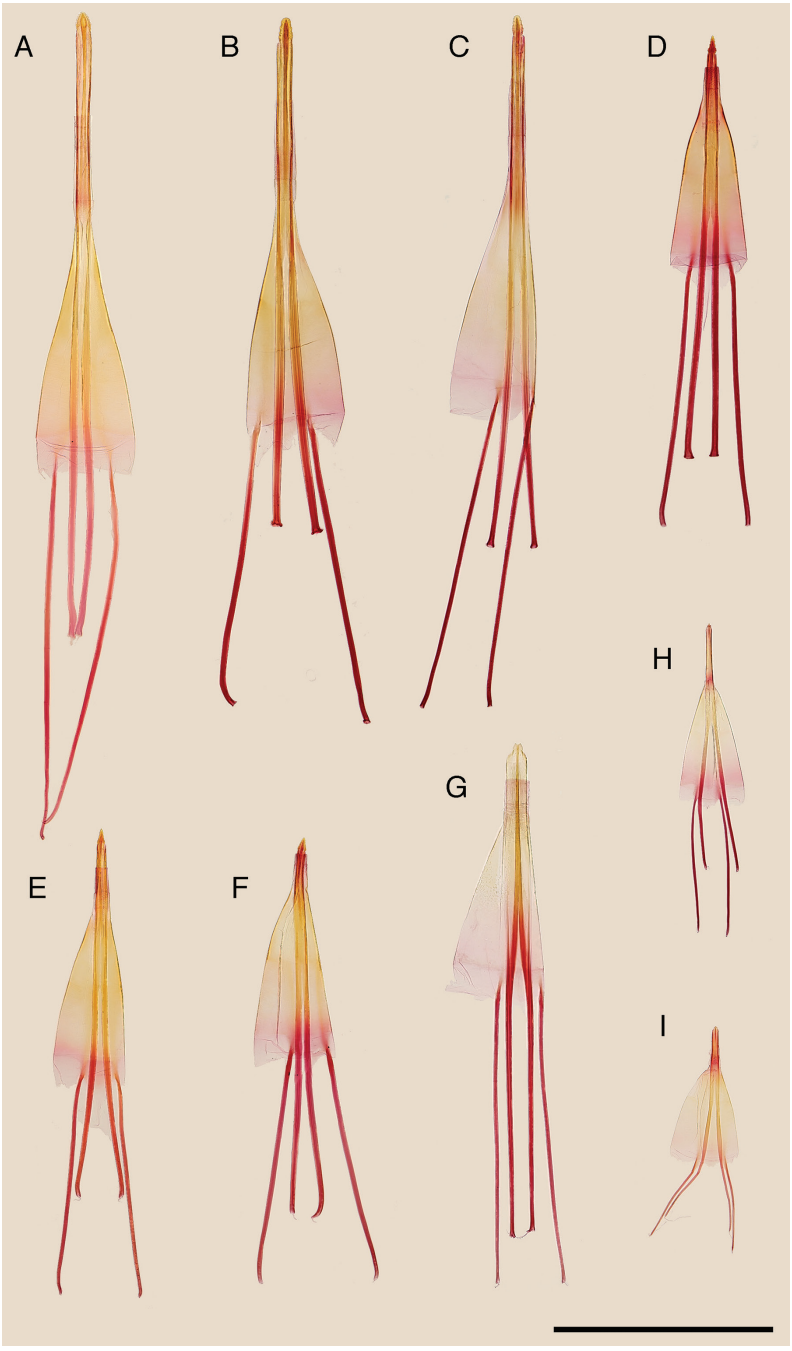

Figure 5. Apophyses and eighth abdominal segment of the Japanese *Epicephala* species. **A** *E. anthophilia* (paratype, slide No. AK250) **B** *E. bipollenella* (slide No. AK281) **C** *E. lanceolatella* (non-type, slide No. AK251) **D** *E. perplexa* (paratype, slide No. AK253) **E** *E. obovatella* (non-type, slide No. AK246) **F** *E. corruptrix* (paratype, slide No. AK262) **G** *E. vitisidaea* (slide No. AK239) **H** *E. parasitica* (non-type, slide No. AK239) **I** *E. nudilingua* (paratype, slide No. AK296). Scale bar: 1 mm.

Apophyses posteriores 1.4× length of apophyses anteriores. Ovipositor dentate laterally, angular at apex.

Material examined. 29♂, 20♀. Holotype ♀ – **JAPAN: Okinawa Prefecture:** Okinawa Island, Kunigami, Cape Hedo (26.860200, 128.257979), 30 m, collected as larva in fruit of *Glochidion lanceolatum* and reared to adult, 15.vi.2015 (KYO). Paratypes – same data as holotype, 10♂ (KYO); same locality as holotype, 13.vi.2004, 3♂, 2♀ (KYO); Other specimens – **JAPAN: Kagoshima Prefecture:** Amami Island, Asado, 30.x.2011, 4♂, 2♀; **Okinawa Prefecture:** Ishigaki Island, Omoto, 30.ix.2004, 11♂, 10♀; Iriomote Island, Funaura, 5.x.2003, 1♂, 3♀; Yonaguni Island, Mantabaru, 20.ix.2004, 2♀.

DNA barcodes. AY221977–AY221979, AY850003, DQ298962–DQ298964, DQ298967, DQ298969–DQ298971, DQ298974–DQ298976, DQ298978–DQ298980, DQ298984, DQ298985, DQ298989, DQ298996–DQ298998.

Known host and adult behavior. Known only from *Glochidion lanceolatum*. Pollination behavior present. Oviposition through ovary wall, in space between the wall and ovule (Fig. 8E). Larva feeds on seeds.

Distribution. Ryukyu Archipelago, Japan (Amami Island, Okinawa Island, Ishigaki Island, Iriomote Island and Yonaguni Island; Fig. 9C). Co-occurs with *E. lanceolatella*.

Etymology. The name *perplexa* is the female form of the Latin adjective *perplexus* (= cryptic), because this species remained hidden until a detailed study on species specificity was performed (Kawakita and Kato 2006).

Remarks. This species occurs in full sympatry with *E. lanceolatella*. The two species may even emerge from the same single fruit. Known ecological difference between the two species is limited to the oviposition behavior, but the difference in the level of sensilla development on the proboscis (Fig. 7) may indicate that this species delivers less pollination benefit than *E. lanceolatella*. Whereas the close relatives of *E. lanceolatella* use plants having close affinity to *G. lanceolatum*, *E. perplexa* is distantly related to these *Epicephala* species (Fig. 10). Thus, the original pollinator of *G. laceolatum* has likely been *E. lanceolatella*, and *E. perplexa* has shifted onto *G. laceolatum* more recently.

***Epicephala obovatella* sp. n.**

http://zoobank.org/EB79ACCF-FDF0-47C3-8D3F-C23DBFFE6F8A
Figs 1–7

Epicephala sp. 3 (Kato et al. 2003); *Epicephala* sp. (*obovatum*) (Kawakita et al. 2004); *Epicephala* sp. (*rubrum*) (Kawakita et al. 2004); Clade 1 (Kawakita and Kato 2006); *Epicephala* sp. ex *G. obovatum* (Kawakita and Kato 2009; Kawakita et al. 2015); *Epicephala* sp. 1 (*G. obovatum*) (Kawakita et al. 2010).

Diagnosis. This species is similar to *E. camurella* Li, 2015 in having dented cucullus, distal projection on sacculus with dense spines, bilobed lamella postvaginalis, smooth

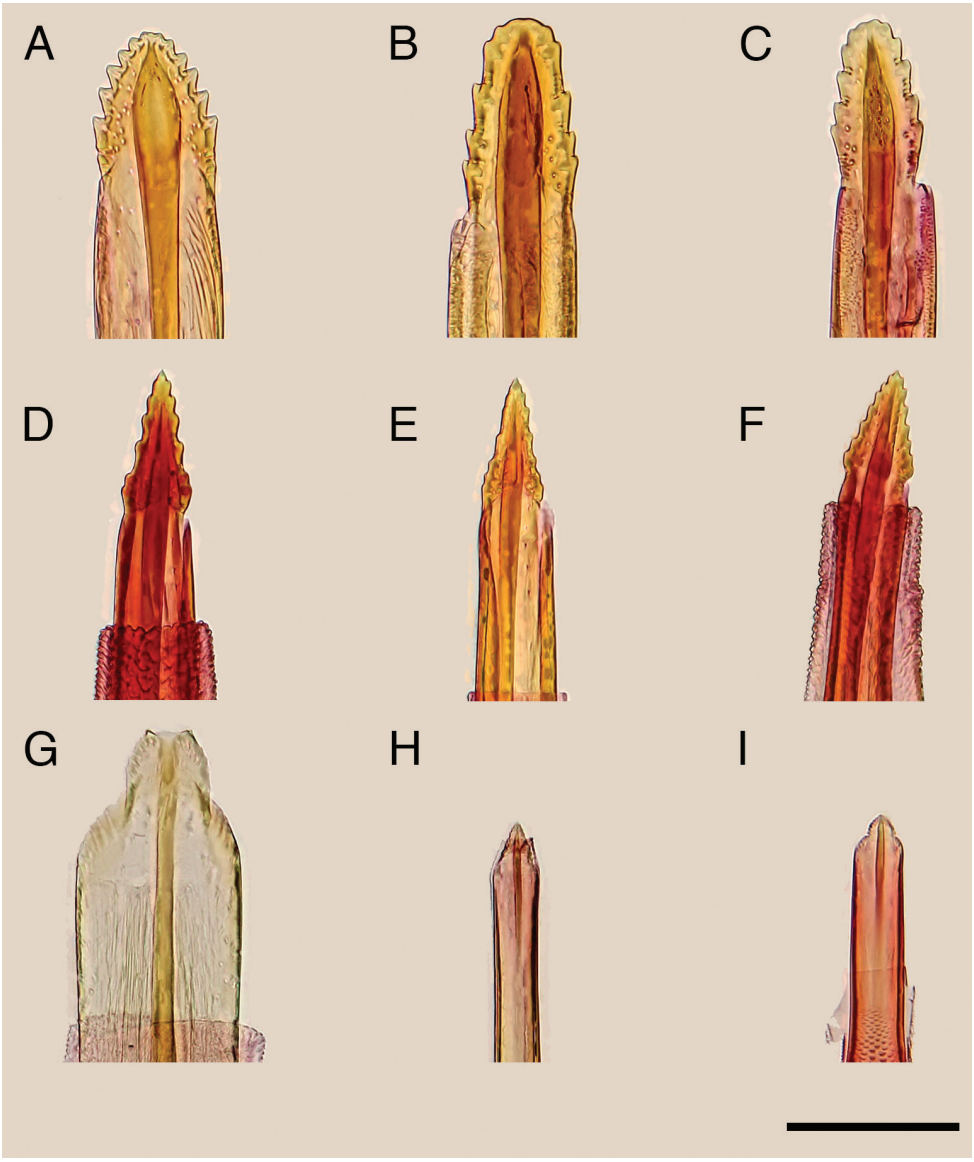

Figure 6. Ovipositor of the Japanese *Epicephala* species. **A**, *E. anthophilia* (paratype, slide No. AK250) **B** *E. bipollenella* (slide No. AK281) **C** *E. lanceolatella* (non-type, slide No. AK251) **D** *E. perplexa* (paratype, slide No. AK253) **E** *E. obovatella* (non-type, slide No. AK246) **F** *E. corruptrix* (paratype, slide No. AK262) **G** *E. vitisidaea* (slide No. AK239) **H** *E. parasitica* (non-type, slide No. AK239) **I** *E. nudilingua* (paratype, slide No. AK296). Scale bar 0.1 mm.

antrum and triangular signa. However, the former clearly differs from the latter in distal projection on sacculus being finger-shaped and each lobe on lamella postvaginalis being club-shaped.

Description. *Wingspan*: 7.5–11.0 mm.

Head: With numerous white scales on dorsal surface. Labial palpus dark brown. Antenna brown, about 1.2× as long as forewing. Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Thorax: White dorsally. Forewing brown with narrow white band on dorsum from base to 2/3 of entire length; three pairs of narrow white bands beginning at costal and dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white band near dorsum; distal end fringed with narrow white band; cilia grayish brown. Hindwing brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen rectangular, acute at apex. Cucullus rounded rectangular, dented at ventral margin at 3/4 of its length; inner surface covered with numerous hairs. Sacculus rounded trapezoid, 0.6× length of cucullus; dorsal margin attached with a narrow plate terminating distally as an inward finger-like projection with a row of spines dorso-ventrally. Vinculum U-shaped; saccus oblong, acute at apex, 0.8× length of vinculum. Aedeagus straight; cornutus absent.

Female genitalia: Lamella postvaginalis strongly bilobed; each lobe club-like, dilated outward, about 0.5× length and width as seventh sternite. Antrum broad, 0.2× width of seventh sternite, as long as lamella postvaginalis, smooth on surface. Ductus bursae 1.8× length of lamella postvaginalis, with bundle of longitudinal parallel ridges for its entire length. Corpus bursae oval, as long as buctus bursae; signum triangular, located medially. Apophyses posteriores 1.5× length of apophyses anteriores. Ovipositor dentate laterally, angular at apex.

Material examined. 6♂, 6♀. Holotype ♀ – **JAPAN: Wakayama Prefecture:** Wakayama, Tomogashima (34.280678, 135.000482), 12 m, collected as larva in fruit of *Glochidion obovatum* and reared to adult, 13.viii.2009 (KYO). Paratypes – same data as holotype, 1♂, 1♀ (KYO). Other specimens – **JAPAN: Wakayama Prefecture:** Wakayama, Tomogashima, 10.vii.2003, 1♂; **Miyazaki Prefecture:** Kushima, Cape Toi, 30.x.1999, 1♂, 3♀ (M. Kato); Kushima, Cape Toi, 9.xi.2001, 1♂; **Kagoshima Prefecture:** Yaku Island, Nagata, 11.xi.2001, 1♂; **Okinawa Prefecture:** Kume Island, Ueshiro, 9.viii.2004, 1♂, 1♀.

DNA barcodes. AY221972–AY221976, AY525731, AY525728, DQ299001–DQ299005, DQ299008–DQ299014, DQ299019–DQ299021, DQ299023, DQ299028–DQ299032.

Known host and adult behavior. *Glochidion obovatum* (mainland Japan, Yaku Island and Kume Island) and *G. rubrum* (Yonaguni Island and Taiwan), which are parapatric sister species. Pollination behavior present. The egg is laid through the ovary wall between the wall and ovule (Fig. 8F). Larva feeds on seeds.

Distribution. Occurs throughout the warm temperate to subtropical regions of Japan (Fig. 9D). Recorded also from Taiwan.

Etymology. The name *obovatella* (an adjective) derives from the species name of the primary host plant *G. obovatum*.

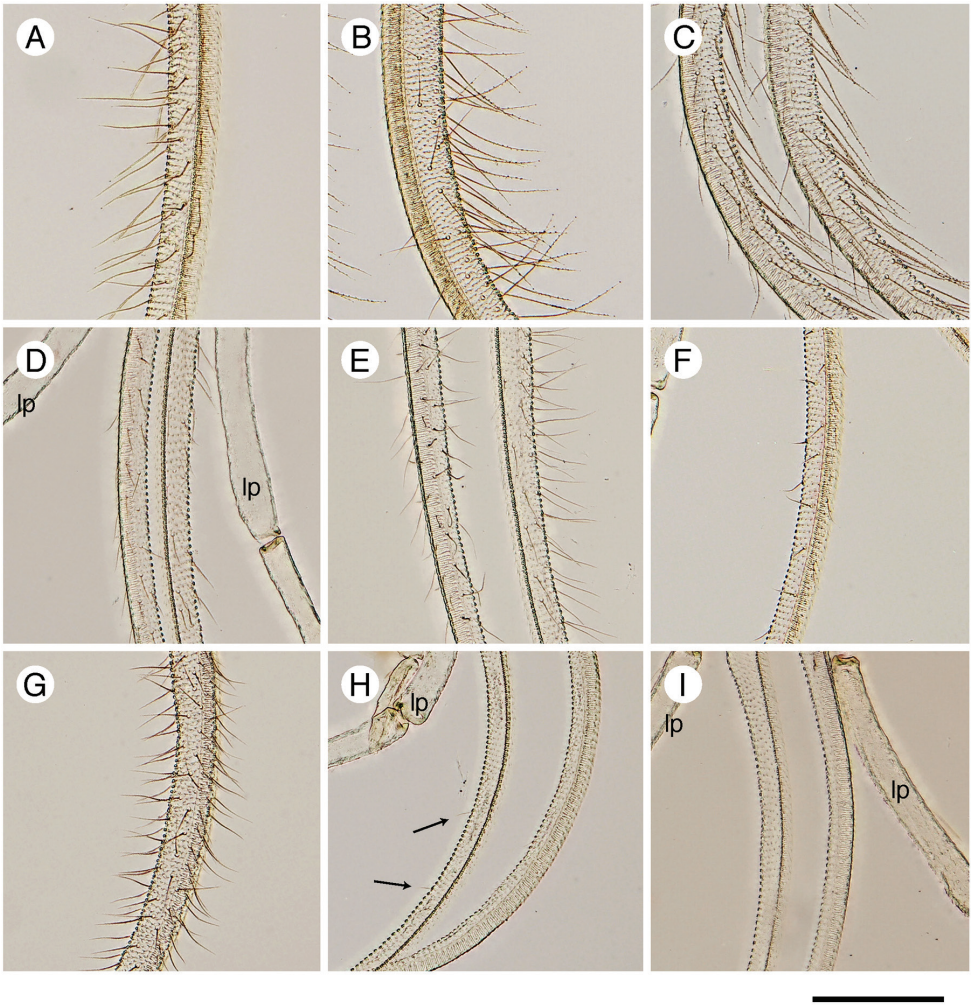

Figure 7. Section of the female proboscis of the Japanese *Epicephala* species. All photographs were taken from non-type specimens. **A** *E. anthophilia* (slide No. AK303) **B** *E. bipollenella* (slide No. AK298) **C** *E. lanceolatella* (slide No. AK300) **D** *E. perplexa* (slide No. AK301) **E** *E. obovatella* (slide No. AK307) **F** *E. corruptrix* (slide No. AK304) **G** *E. vitisidaea* (slide No. AK297) **H** *E. parasitica* (slide No. AK308), arrows indicate rudimentary sensilla **I** *E. nudilingua* (slide No. AK309). lp, labial palp. Scale bar: 0.1 mm.

Remarks. The Taiwanese population of this species is genetically divergent from the Japanese population (>4% divergence in COI; Table 1 and Suppl. material 2). However, they are morphologically indistinguishable (Kawakita and Kato 2006), so we tentatively consider the Taiwanese population as *E. obovatella*.

***Epicephala corruptrix* sp. n.**
http://zoobank.org/4C11534C-7511-44AA-9F94-30951F9CA0A3
Figs 1–7

Clade 4 (Kawakita and Kato 2006); *Epicephala* sp. ex *G. rubrum* (Kawakita and Kato 2009; Kawakita et al. 2015); *Epicephala* sp. 4 (*G. rubrum*) (Kawakita et al. 2010).

Diagnosis. The male genitalia of this species are distinctive and have no parallel in other known species; sacculus possesses a thick spine on apex and a cluster of long spines on dorsal projection. The large, round lamella postvaginalis also distinguishes this species from other known *Epicephala*.

Description. *Wingspan*: 7.2–8.8 mm.

Head: With numerous white scales on dorsal surface. Labial palpus dark brown. Antenna brown, about 1.2× as long as forewing. Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Thorax: White dorsally. Forewing brown with narrow white band on dorsum from base to 2/3 of entire length; two pairs of narrow white bands beginning at costal and dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; dorso-distal band accompanied by another parallel band of same size on distal position; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white band near dorsum; distal end fringed with narrow white band; cilia grayish brown. Hindwing brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen oblong, acute at apex. Cucullus rectangular oblong; ventral margin with acute tip at mid-length terminating with short spine; inner surface covered with numerous hairs. Sacculus ovoid; apex sharply concave, with a long spine on ventral half; basal part of dorsal margin attached with a narrow plate terminating distally as an inward, finger-like projection with 3 or 4 long spines directing dorso-ventrally. Vinculum U-shaped; saccus broad, 0.3× width of vinculum, as long as vinculum, oblong and acute at apex. Aedeagus straight, slightly dilated toward apex; cornutus absent.

Female genitalia: Lamella postvaginalis long and broad, 0.8× width and length of seventh sternite, coarsely dentate at distal end. Antrum broad, 0.4× width and length of lamella postvaginalis, smooth on surface. Ductus bursae as long as seventh sternite, with cluster of longitudinal parallel ridges for its entire length. Corpus bursae oval to elongate oval, as long as buctus bursae; signum absent. Apophyses posteriores 1.5× length of apophyses anteriores. Ovipositor dentate laterally, angular at apex.

Material examined. 22♂, 5♀. Holotype ♀ – **JAPAN: Okinawa Prefecture:** Okinawa Island, Takae (26.652878, 128.248178), 100 m, collected as larva in galled flower of *Glochidion obovatum* and reared to adult, 5.vi.2015 (KYO). Paratypes – same data as holotype, 10♂, 2♀ (KYO). Other specimens – **JAPAN: Kagoshima Prefecture:** Amami Island, Akakina, 4.xi.2004, 5♂, 1♀; Amami Island, Kise, 15.iv.2006, 3♂, 1♀; Amami Island, Kasari, 18.v.2015, 1♂; Tokunoshima Island, Mikyo, 2.xi.2004, 1♂; Okinawa Prefecture: Iriomote Island, Ohara, 14.ii.1998, 2♂ (M. Kato).

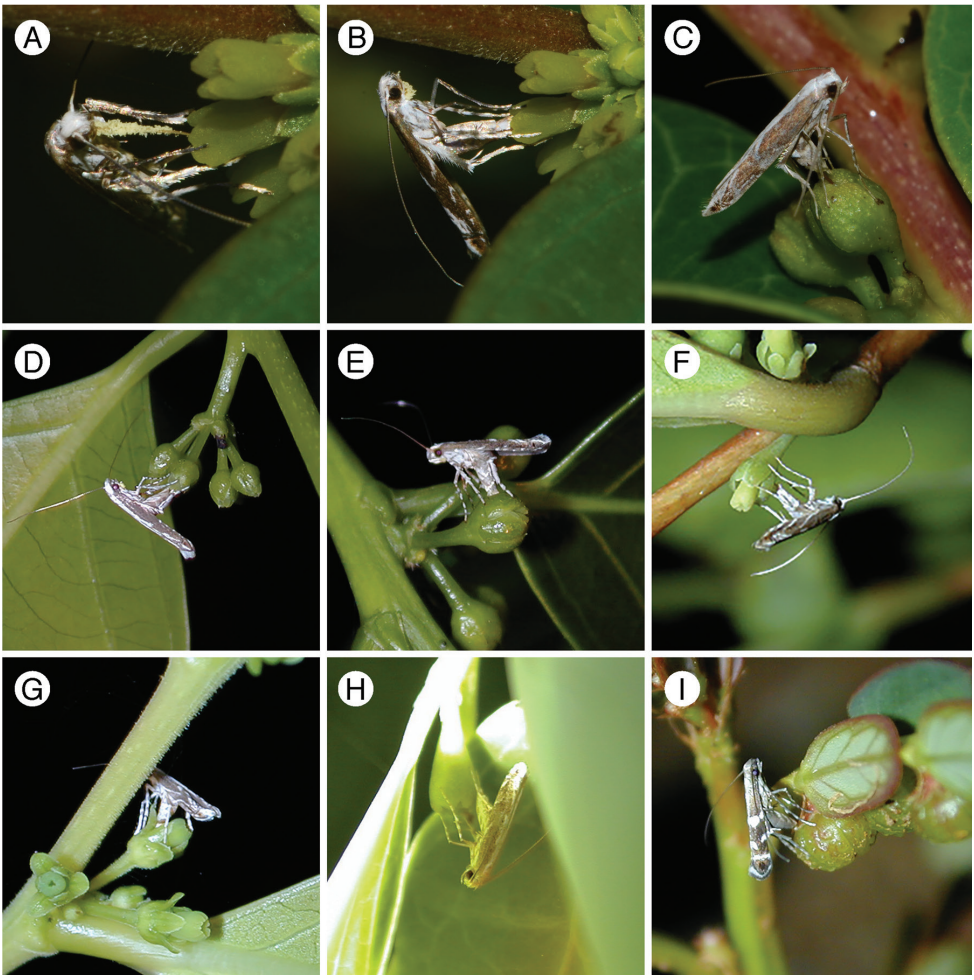

Figure 8. Pollination and oviposition behavior of the Japanese *Epicephala* species. **A** *E. anthophilia* female actively depositing pollen on *Glochidion acuminatum* female flower **B** *E. anthophilia* ovipositing through stylar pit of *G. acuminatum* flower **C** *E. bipollenella* ovipositing through stylar pit of *G. zeylanicum* flower **D** *E. lanceolatella* ovipositing through stylar pit of *G. lanceolatum* flower **E** *E. perplexa* ovipositing through lateral ovary wall of *G. lanceolatum* flower **F** *E. obovatella* ovipositing through lateral ovary wall of *G. obovatum* flower **G** *E. corruptrix* ovipositing through ovary wall of *G. rubrum* flower **H** *E. vitisidaea* ovipositing in the interspace between ovary and tepal **I** *E. parasitica* ovipositing in young fruit of *Phyllanthus lepidocarpus*.

DNA barcodes. DQ298999, DQ299000, DQ299006, DQ299007, DQ299015–DQ299018, DQ299022, DQ299024–DQ299027.

Known host and adult behavior. *Glochidion obovatum* (Amami Island, Tokuno Island and Okinawa Island) and *G. rubrum* (Ishigaki Island and Iriomote Island). The egg is laid through the ovary wall between the wall and ovule (Fig. 8G). Pollination

behavior is present, but in the resulting fruit, the larva galls one of the locules, inducing abnormal development of the ovules (Fig. 11). Such fruits usually do not contain normally developed seeds (Fig. 11).

Distribution. Restricted to several islands in the Ryukyu Archipelago (Amami Island, Tokuno Island, Okinawa Island, Ishigaki Island and Iriomote Island; Fig. 9D).

Etymology. The species name *corruptrix* (a noun in apposition) was inherited from *Tegeticula corruptrix*, a derived parasitic species of yucca moth (Pellmyr et al. 1999). *Epicephala corruptrix* has a potential to corrupt the mutualistic relationship with its host because the species induces gall formation in pollinated flowers which then hardly produce seeds (Fig. 11).

Remarks. This species shares the same host plant with *E. obovatella*, but *E. obovatella* has not been collected from any of the locations where *E. corruptrix* occurs (Fig. 9). Because of the limited mutualistic potential of this species, reproduction of the host plants (*G. obovatum and G. rubrum*) is likely to be severely limited on islands where *E. corruptrix* occurs, in comparison to locations where *E. obovatella* is present.

Epicephala vitisidaea Li, Wang & Zhang, 2012

Figs 1–7

Epicephala sp. ex *B. vitis-idaea* (Kawakita and Kato 2009); *Epicephala* sp. 10 (*Breynia*) (Kawakita et al. 2010).

Diagnosis. Lamella antevaginalis of this species forms a sclerotized complete circle around ostium, a character that cannot be found in any other species of *Epicephala*. Cornuti of short thick spines occurring dorsally and ventrally on distal portion of aedeagus are also distinctive of this species.

Description. Description as in Zhang et al. (2012a), except the following.

Head: Female proboscis with a large number of trichoid sensilla; sensilla 1.5× as long as width of proboscis, denser toward base.

Male genitalia: Cornuti on aedeagus occurring dorsally and ventrally; dorsal cornuti consisting of 4–6 short spines, shorter than 0.7× width of aedeagus; ventral cornuti a pair of thick and long spines, longer than width of aedeagus.

Material examined. 50♂, 31♀. **JAPAN: Kagoshima Prefecture:** Amami Island, Setsuko, 29.ix.2002, 9♂, 1♀; Amami Island, Akakina, 15.v.2003, 5♂, 5♀; Amami Island, Kasari, 18.v.2015, 3♂; Tokuno Island, Amagi, 2.xi.2004, 1♂, 1♀; Okinoerabu Island, Uchijiro, 4.xi.2004, 1♂; **Okinawa Prefecture:** Okinawa Island, Oura, 9.ix.2002, 2♂, 3♀; Okinawa Island, Yona, 15.vi.2015, 1♂, 6♀; Miyako Island, Mt. Nobaru, 24.ix.2004, 18♂, 6♀; Irabu Island, Makiyama, 23.ix.2004, 1♂, 1♀; Ishigaki Island, Mt. Banna, 15.x.2002, 3♂, 1♀; Iriomote Island, 13.x.2002, 6♂, 7♀.

DNA barcodes. FJ235380.

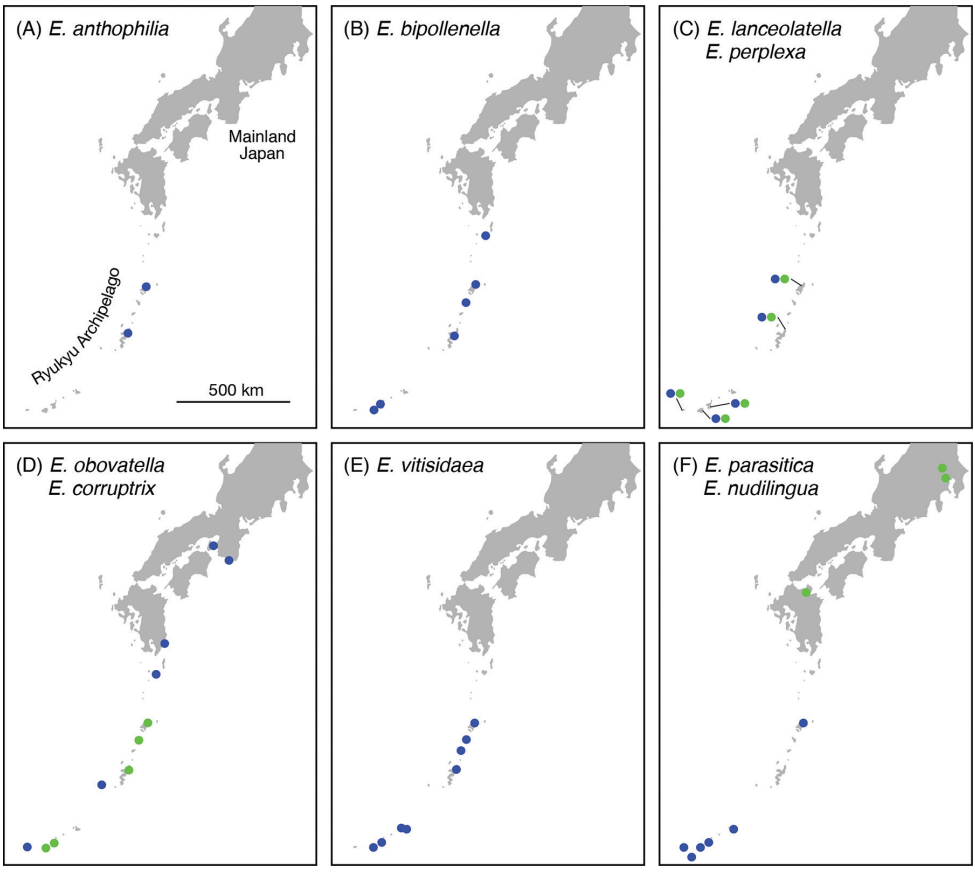

Figure 9. Distribution of the *Epicephala* species in Japan. **A** *E. anthophilia* **B** *E. bipollenella* **C** *E. lanceolatella* (blue) and *E. perplexa* (green) **D** *E. obovatella* (blue) and *E. corruptrix* (green) **E** *E. vitisidaea* **F** *E. parasitica* (blue) and *E. nudilingua* (green). Information based on this study and Kawakita and Kato (2006).

Known host and adult behavior. Known only from *Breynia vitis-idaea*. The adult is the pollinator of the host plant (Kawakita and Kato 2004b). Eggs are placed in the interspace between the tepal and ovary (Fig. 8H), so the ovipositor does not penetrate floral tissue. Larva feeds on seeds.

Distribution. In Japan occurs widely in the Ryukyu Archipelago (Fig. 9E). Known also from China (Zhang et al. 2012a).

Epicephala parasitica sp. n.

http://zoobank.org/7BF0C66B-183D-4042-AE98-F059EA1EA93D

Figs 1–7

Epicephala sp. ex *P. lepidocarpus* (Kawakita and Kato 2009; Kawakita et al. 2015); *Epicephala* sp. 7 (*Phyllanthus*) (Kawakita et al. 2010).

Diagnosis. Sexually dimorphic color pattern and fused seventh sternite and tergite are thus far unknown in any species of *Epicephala*, making this species highly distinctive within the genus. Overall small size, row of thick spines on ventral margin of cucullus, long spine at cucullus base and numerous short spines on inner cucullus add to the uniqueness of this species in the genus.

Description. *Wingspan*: 5.7–7.5 mm.

Head: Females with numerous grayish brown scales on dorsal surface; males with numerous white scales. Labial palpus dark brown to black in females, dark brown in males. Antenna dark brown in females, grayish brown in males, about 1.2× as long as forewing. Trichoid sensilla on female proboscis rudimentary, shorter than width of proboscis, less than 30 per galea.

Thorax: Brown dorsally in females, white in males. Forewing of females dark brown with narrow white band on dorsum from base to 1/4 of entire length, medially with narrow white band extending from costa to dorsum; a pair of narrow white bands beginning at costal and dorsal margin near 2/3 of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 brown with black dot centrally; distal end fringed with narrow white band and terminating with narrow black band; cilia dark brown. Hindwing of females dark brown, 0.8× length of forewing; cilia dark brown. Forewing of males brown with narrow white band on dorsum from base to 2/3 of entire length; three pairs of narrow white bands beginning at costal and dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 orange-brown with black dot centrally, franked by short white band near dorsum; distal end fringed with narrow white band and terminating with narrow brown band; cilia grayish brown. Hindwing of males brown, 0.8× length of forewing; cilia grayish brown.

Male genitalia: Tegumen rounded triangular. Cucullus rectangular oblong; ventral margin medially concave; basal 1/3 of cucullus fringed with long spines on ventral margin; spines longer than width of cucullus; another distinctly long spine occurring at ventral base of cucullus, 1/2 length of cucullus; distal half of cucullus with numerous short spines on inner surface and few hairs. Sacculus broad, 2× width of cucullus, 0.7× length of cucullus, distinctly concave at apex; concave portion of apex fringed with setae; inner wall of sacculus abruptly projecting inward and curved toward dorso-caudal direction, pointed apically; ventral edge of projection fringed with setae. Vinculum U-shaped; saccus thin and rod-shaped, 0.6× length of vinculum. Aedeagus straight; cornutus absent.

Female genitalia: Seventh sternite completely fused to seventh tergite to form a cylindrical segment. Caudal end of seventh sternite with row of parallel latitudinal ridges. Lamella postvaginalis trapezoid, dilated toward apex, small, 0.3× width and length of seventh sternite, slightly convex and weakly dentate on caudal margin. Antrum smooth, 0.2× width and 0.5× length of seventh sternite. Ductus bursae as long as seventh sternite, with short lateral sac at base; surface of sac and franking portion of

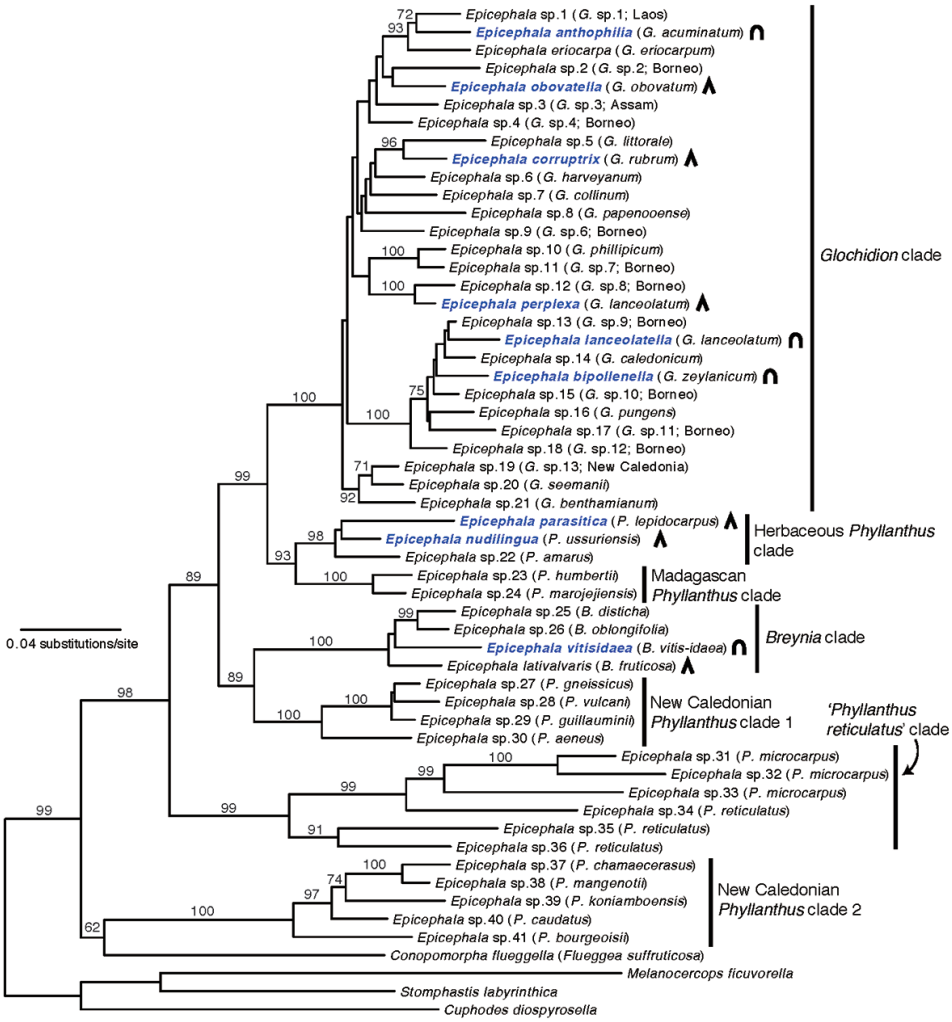

Figure 10. Maximum-likelihood phylogeny of *Epicephala* species based on sequences of the COI, ArgK and EF1α genes. Numbers above nodes are maximum-likelihood bootstrap support values based on 1,000 replications. The Japanese *Epicephala* species are marked in blue. Symbols right to species names donate ovipositor morphology: inverted U-shape, rounded apically; inverted V-shape, acute apically.

ductus bursae with numerous teeth on surface. Corpus bursae elongate oval, as long as combined antrum and ductus bursae; signum absent. Apophyses posteriores 1.7× length of apophyses anteriores. Ovipositor dentate laterally, angular at apex.

Material examined. 46♂, 40♀. Holotype ♀ – **JAPAN: Okinawa Prefecture:** Yonaguni Island, Sonai (24.468434, 123.002118), 50 m, collected as larvae in fruit of *Phyllanthus lepidocarpus* and reared to adult, 16.xii.2012 (KYO). Paratypes – same data as holotype, 2♂, 5♀ (KYO). Other specimens – **JAPAN: Kagoshima Prefecture:** Amami Island, Setsuko, 17.xi.2002, 2♂, 2♀; **Okinawa Prefecture:** Miyako Island,

Mt. Nobaru, 24.ix.2004, 5♂, 3♀; Ishigaki Island, Omoto, 30.ix.2004, 17♂, 11♀; Iriomote Island, Funaura, 5.x.2003, 13♂, 10♀; Iriomote Island, near the mouth of Urauchi River, 29.ix.2004, 6♂, 5♀; Hateruma Island, 17.xii.2012, 1♂, 3♀.

DNA barcodes. FJ235386.

Known host and adult behavior. Known only from *Phyllanthus lepidocarpus*. Pollination behavior absent. Oviposition in immature fruit, through ovary wall (Fig. 8I). Larva feeds on seeds.

Distribution. Widely distributed in the Ryukyu Archipelago, Japan (Fig. 9F). The host plant *Phyllanthus lepidocarpus* is a common weed along roadsides and in cultivated land. Although *P. lepidocarpus* also occurs in mainland Japan, *E. parasitica* has only been found in the Ryukyu Archipelago.

Etymology. The name *parasitica* is the female form of the Latin adjective *parasiticus* (= parasitic), in reference to the parasitic nature of the species.

Remarks. This and the following species (*E. nudilingua*) belong to a derived clade of *Epicephala* specialized to herbaceous species of *Phyllanthus* (Fig. 10). Pollination behavior has not been observed in any of the species in this clade, so they are pure parasites that derived from a pollinating ancestor (Kawakita and Kato 2009).

***Epicephala nudilingua* sp. n.**

http://zoobank.org/462F7BC1-3195-449A-A7D7-5846ADFC724D

Figs 1–7

Epicephala sp. ex *P. ussuriensis* (Kawakita and Kato 2009; Kawakita et al. 2015).

Diagnosis. Aside from *E. parasitica*, this species is smaller than any other known species of *Epicephala*. Exaggerated cornutus, spiracle on seventh tergite, bilobed lamella postvaginalis and heavily sclerotized and curved antrum, clearly distinguish this species from other known *Epicephala*.

Description. Wingspan: 7.0–8.3 mm.

Head: With numerous gray scales on dorsal surface. Labial palpus dark brown. Antenna dark brown, about 1.2× as long as forewing. Trichoid sensilla on female proboscis absent.

Thorax: Grayish white dorsally. Forewing dark brown with narrow white band on dorsum from base to 1/3 of entire length; three pairs of narrow white bands beginning at costal and dorsal margin near 1/2 to 3/4 length of wing and extending obliquely toward wing apex, terminating before reaching mid-width of wing; a narrow silver band with metallic reflection extending from costa to dorsum at 5/6 length; distal 1/6 brown with black dot centrally, franked by narrow white band near dorsum; distal end fringed with narrow white band and terminating with narrow dark brown band; cilia grayish dark brown. Hindwing dark brown, 0.8× length of forewing; cilia grayish dark brown.

Male genitalia: Tegumen rounded triangular. Cucullus rectangular oblong, dilated at apex, covered with numerous hairs on inner surface; ventral base with small out-

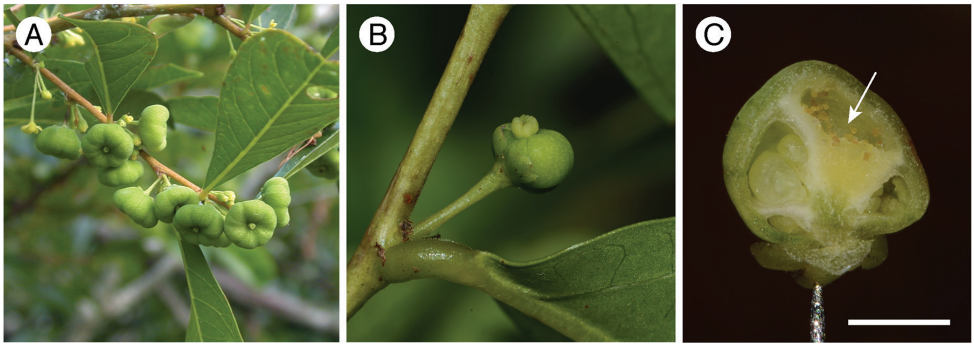

Figure 11. Fruits and galls produced by *Epicephala* species on *Glochidion obovatum*. **A** Fruit produced after pollination by *E. obovatella* (Tomogashima, Wakayama) **B** Gall induced on female flower by *E. corruptrix* (Takae, Okinawa) **C** Cross section of the gall induced by *E. corruptrix*. Arrow indicates the galled locule with feeding trace of *Epicephala* larva. Note that the irregularly developed ovules of the galled locule have merged indistinguishablly to septa. Scale bar 2 mm.

Table 1. Maximum pairwise intraspecific and minimum interspecific divergences in the COI barcoding region for the nine Japanese *Epicephala* species.

Species	Number of DNA barcodes available in database	Maximum divergence within species (%)	Minimum divergence from other species (%)
E. anthophilia	16	0.34	4.30
E. bipollenella	14	0	5.01
E. lanceolatella	24	0.17	4.66
E. perplexa	23	0.52	4.61
E. obovatella	28	4.12	4.30
E. corruptrix	13	0.52	4.12
E. vitisidaea	1	—	6.19
E. parasitica	1	—	6.53
E. nudilingua	1	—	5.33

ward projection; surface of projection with numerous thin spines. Sacculus elongate triangular, acute at apex, 1.6× width of cucullus at base, 0.9× length of cucullus; distal portion of ventral margin slightly concave. Vinculum V-shaped; saccus thin and tapering, as long as vinculum. Aedeagus straight; cornutus large, emerging from 2/3 length of aedeagus and extending beyond apex of aedeagus for 0.3× length of aedeagus, 0.5× as thick as aedeagus, with thick spines sparsely on surface.

Female genitalia: Seventh tergite with a pair of spiracle anteriorly. Lamella postvaginalis deeply bilobed, 2× as broad as ostium bursae, as long as seventh sternite; each lobe finger-shaped, extending straight toward caudal end. Antrum heavily sclerotized, smooth on surface, abruptly curved ventrally and posteriorly to continue to ductus bursae. Ductus bursae curving abruptly anteriorly, gradually tapering to continue to corpus bursae; basal 1/3 with numerous sclerotized teeth on surface. Corpus bursae elongate oval, as long as combined antrum and ductus bursae; signum absent. Apo-

physes posteriores 1.7× length of apophyses anteriores. Ovipositor dentate laterally, weakly angular at apex.

Material examined. 19♂, 7♀. Holotype ♀ – **JAPAN: Tochigi Prefecture:** Fujioka, Watarase-yusuichi (36.226554, 139.671697), 20 m, collected as larva in fruit of *Phyllanthus ussuriensis* and reared to adult, 22.ix.2012 (KYO). Paratypes – same data as holotype, 13♂, 4♀ (KYO). Other specimens – **JAPAN: Tokyo Prefecture:** Machida, Minamiotani, 29.x.2004, 6♂, 1♀; **Oita Prefecture:** Bungo-Takada, Shinei, 8.ix.2015, 1♀ (T. Hirano).

DNA barcodes. FJ235387.

Known host and adult behavior. Known only from *Phyllanthus ussuriensis*. Oviposition behavior has not been observed in the wild. Floral dissection suggests that the egg is laid in young fruit through the ovary wall between the wall and ovule. Larva feeds on seeds.

Distribution. Known only from three populations in Tochigi, Tokyo and Oita Prefecture, Japan (Fig. 9F). The host plant *P. ussuriensis* is widespread in the temperate regions of Japan and other parts of East Asia, so the species is likely to be found elsewhere. The plant was common at damp habitats in flood plains before 1980s but is now uncommon and locally threatened in Japan.

Etymology. The name *nudilingua* (a noun in apposition) derives from the Latin *nudus* (= naked) and *lingua* (= tongue) in reference the hairless proboscis of the female, which is a derived condition in *Epicephala*.

Phylogenetic results

Phylogenetic analysis of the 53 *Epicephala* species resulted in a fairly resolved phylogeny (Fig. 10), consistent with previous phylogenetic analyses of *Epicephala* (Kawakita and Kato 2009; Kawakita et al. 2010, 2015; Hembry et al. 2013). *Conopomorpha fluueggella* was recovered as sister to one of the two clades comprising the New Caledonian *Epicephala* species. The Japanese species belong to either one of the following three well-supported clades: the clade consisting of species associated with *Glochidion*, the clade restricted to species found on *Breynia*, and the clade containing all known species attacking herbaceous *Phyllanthus*.

Analysis of correlated evolution between oviposition site and ovipositor morphology in Mesquite indicated that the two traits exhibit greater correlation than expected under the null hypothesis of independent evolution ($P < 0.001$).

Maximum pairwise divergence in DNA barcode within species was generally low (< 1%) with the exception of *E. obovatella* that exhibited moderate divergence (4.12%) between populations in Japan and Taiwan (Table 1; Kawakita and Kato 2006). Nevertheless, all *Epicephala* species for which multiple DNA barcodes were available were strongly recovered as respectively monophyletic groups in the ML phylogeny (Suppl. material 2). Minimum distances to heterospecifics were 4.12–6.53% for the nine *Epicephala* species studied (Table 1).

Discussion

The nine species of *Epicephala* in Japan were clearly distinguishable to each other based on the morphology of both male and female genitalia, but they are usually very difficult to identify based on wing pattern. The extent of genital morphological variation of *Epicephala* is remarkable (also see Zang et al. 2012a; Li and Yang 2015; Li et al. 2015), especially when it is compared to that of other comparably large genera of Gracillariidae (e.g., *Caloptilia*) where there is very low variation in genital morphology but far greater variation in wing pattern (Kumata 1982). The morphology of the female genitalia is as diagnostic in differentiating species as that of the male genitalia, which is also uncommon in Gracillariidae. The level of interspecific genetic variation in *Epicephala* (Table 1) is not necessarily higher or even lower than those of other gracillariid genera (*Cuphodes*, 6.6–15.7%; *Diphtheroptila*, 7.3–11.6%; *Caloptilia*, 6.6–10.7%; Kawakita et al. 2010), which may indicate that genital traits in fact evolve faster in *Epicephala* than in other genera. The reason for this pattern is unknown, but there may be differences among lineages of Gracillariidae in the mechanism of reproductive isolation that can account for the observed pattern. Studies aimed at assessing the roles of genital morphology and wing pattern variation in reproductive isolation may be interesting.

The analysis of correlated evolution between oviposition site and ovipositor morphology suggested a clear linkage between these traits. Based on the phylogeny (Fig. 10), there have been repeated transitions in oviposition site during the diversification of *Epicephala*, which may indicate that shifts in oviposition site are adaptive. Because some *Glochidion* plants selectively abort flowers with heavy egg load and abortion is likely based on the extent of mechanical damage to flowers (Goto et al. 2010; personal communication of R. Goto, University of Michigan), external oviposition in *E. vitisidaea* and others may have evolved to circumvent the abortion response in their host plants. By contrast, the adaptive significance of apical and lateral ovipositions is less clear. However, the observation that *E. lanceolatella* and *E. perplexa*, which co-occur on the same *G. lanceolatum* host, display apical and lateral ovipositions, respectively, suggests that these oviposition strategies likely represent distinct niches. Detailed study on the evolution of oviposition strategy in *Epicephala* may reveal previously unknown aspect of the coevolution between the moths and their hosts.

Another finding of this study that deserves further pursuit is the galling potential of *E. corruptrix* (Fig. 11). Because *E. corruptrix* possesses the pollination behavior (Kawakita and Kato 2006), it is not a pure parasite. However, the benefit they confer to the plants is likely to be very small, especially when compared with *E. obovatella* that uses the same *G. obovatum* and *G. rubrum* hosts in different locations (Fig. 9). Because our sampling is still limited, a study is needed to verify whether or not *E. corruptrix* and *E. obovatella* co-occur in any location and to compare their contributions to their host plant's reproduction. *Epicephala corruptrix* will be a good model to study how shifts in the cost/benefit balance occur in mutualisms and potentially drive the collapse of the interaction.

With the seven new species described here, the genus *Epicephala* now consists of 60 described species (Li and Yang 2015; Li et al. 2015). However, the number is far lower

than the few hundred species estimated from ecological, molecular and biogeographical data (Kawakita 2010). Because the present study only focused on species occurring in Japan, which is near the northern end of the distribution range of *Epicephala*, taxonomic studies encompassing broader biogeographic regions are clearly needed. For example, the molecular phylogeny of *Epicephala* (Fig. 10) suggests that there are clades confined to Madagascar or New Caledonia where none of the described *Epicephala* species occur. These regions are known for hotspots of *Phyllanthus* diversity and thus potentially have large numbers of undescribed *Epicephala* species. There is also a high diversity of *Phyllanthus* in the New World where *Epicephala* has not been recorded previously, but recent observations suggest that *Epicephala* is also widespread in the Neotropics (A. Kawakita and M. Kato, unpublished data). Accelerating the taxonomy of *Epicephala* at a global scale is therefore highly important in facilitating the ecological and evolutionary studies of this model group.

Acknowledgements

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Supplementary material 1

GenBank accession numbers

Authors: Atsushi Kawakita, Makoto Kato

Data type: Table

Explanation note: GenBank accession numbers of the sequences used in the multigene phylogenetic analysis.

Supplementary material 2

COI phylogeny of *Epicephala*

Authors: Atsushi Kawakita, Makoto Kato

Data type: Figure

Explanation note: Maximum-likelihood phylogeny of *Epicephala* moths based on 582 base pairs of the mitochondrial COI gene.

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RESEARCH ARTICLE

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Cobitis takenoi sp. n. (Cypriniformes, Cobitidae): a new spined loach from Honshu Island, Japan

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Abstract

A new species of spined loach, *Cobitis takenoi* **sp. n.**, is described based on the holotype and ten paratypes collected from Tango District, Honshu Island, Japan. The new species is distinguished by a combination of the following character states: 1) the lamina circularis at the base of the pectoral fin in adult male having a simple roundish plate form; 2) a narrowing of the upper segments of the first branched ray of the pectoral fin; 3) a short maxillary barbel whose length equals diameter of the eye; 4) 14 prepelvic myotomes, and 5) L3 and L5 well developed, forming longitudinal obvious stripes in males during the spawning season.

Keywords

Cobitoidei, Tango tetraploid form of *Cobitis striata*, *Cobitis* sp. 5, freshwater fish

Introduction

The genus *Cobitis* Linnaeus, 1758 (Cypriniformes: Cobitidae) includes small, slender-bodied benthic freshwater fishes. The genus is characterised by the following features: the suborbital spine is erectile; the mouth is small and inferior with three pairs of barbels; body pigmentation is organised in one dorsal and four lateral longitudinal lines or rows of blotches; and the presence of the lamina circularis at the base of the pectoral fin in adult males (Nalbant 1963, Kottelat and Freyhof 2007, Kim 2009). Approximately 80 species of the genus have been identified in Eurasia and northwestern

Africa (Kottelat 2012, Nakajima 2012, Chen and Chen 2013, Chen et al. 2013, Buj et al. 2014, Erkakan and Özdemir 2014, Chen et al. 2015, Mousavi-Sabet et al. 2015, Nakajima and Suzawa 2016). Nine species within *Cobitis*, namely 1) *C. biwae* Jordan & Snyder, 1901, 2) *C. striata* Ikeda, 1936, 3) *C. matsubarae* Okada & Ikeda, 1939, 4) *C. takatsuensis* Mizuno, 1970, 5) *C. shikokuensis* Suzawa, 2006, 6) *C. magnostriata* Nakajima, 2012, 7) *C. minamorii* Nakajima, 2012, 8) *C. kaibarai* Nakajima, 2012 and 9) *C. sakahoko* Nakajima & Suzawa, 2015, and six subspecies, namely 1) *C. minamorii tokaiensis* Nakajima, 2012, 2) *C. m. oumieneis* Nakajima, 2012, 3) *C. m. yodoensis* Nakajima, 2012, 4) *C. m. saninensis* Nakajima, 2012, 5) *C. striata fuchigamii* Nakajima, 2012, and 6) *C. s. hakataensis* Nakajima, 2012 have been described in Japan (Nakajima 2012, Hosoya 2013, Nakajima and Suzawa 2016).

Previously, Takeno et al. (2010) reported a *Cobitis* species from Tango District, Honshu Island, Japan, which they tentatively named as a 'Tango tetraploid form' of *Cobitis striata*. This species had clearly distinctive differences in body colouration patterns and mitochondrial DNA sequences as compared to other Japanese species of spined loach. Therefore, they concluded that the species was an unknown new species (Takeno et al. 2010). However, to date, this spined loach remained undescribed. In the current paper, I describe it as a new species on the basis of 11 type specimens.

Materials and methods

I examined 11 specimens collected from a small river in Tango District, Kyoto Prefecture, Honshu Island, Japan (Figs 1, 2). There is a risk of this new species being commercially overfished for the ornamental fish market (Takeno et al. 2010, Kitagawa 2015). Therefore, the precise locality of the population is not revealed in the current paper so as to protect the species. All specimens were fixed in 10% formalin and preserved in 70% ethanol. The methods used for counting and measurement of body morphological features followed Kottelat and Freyhof (2007) and Nakajima (2012). All measurements performed using a digital calliper and were recorded to the nearest 0.1 mm. The last two branched rays articulating on the last complex pterygiophore of the dorsal and anal fins were counted as one ray. The prepelvic myotome number (PMN) was defined as the number of muscle segments between the base of the pectoral fin and the origin of the pelvic fin (Nakajima 2012). The right pectoral fin of holotype and some paratypes was resected and was made transparent by placing it in 4% KOH for 24 h. After staining with alizarin red S + 1% KOH for 24 h, the lamina circularis and the upper segments of the first branched ray of the pectoral fin (USP) were observed and sketched using a stereomicroscope. The dorsal and lateral colour patterns were organised in five longitudinal lines of pigmentation, which were abbreviated as lines L1 to L5 according to the scheme of Takeda and Fujie (1945) (see also Nakajima 2012). The black spots at the caudal-fin base and the markings of the dorsal and caudal fins are additionally described.

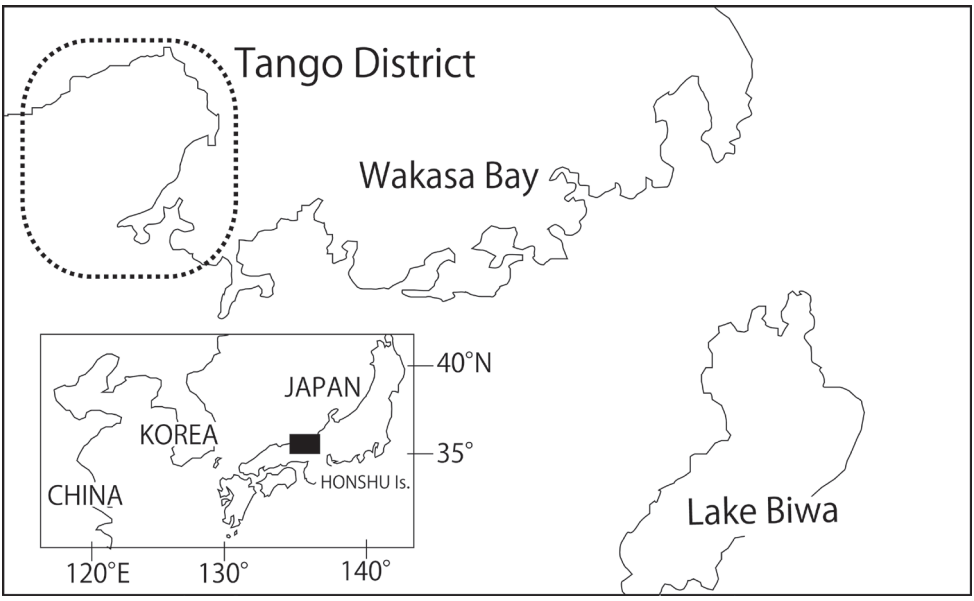

Figure 1. Map showing the collection area of the type series of *Cobitis takenoi* sp. n.

Figure 2. Habitat of *Cobitis takenoi* sp. n.

The type series were deposited in the following collections: KPM – the Kanagawa Prefectural Museum of Natural History, Odawara, Kanagawa, Japan; TKPM – the Tokushima Prefectural Museum, Tokushima, Japan; KUN – the Faculty of Agriculture, Kinki University, Nara, Japan and JNC – private collection of the author, Japan.

Taxonomy

***Cobitis takenoi* sp. n.**
http://zoobank.org/13895F3E-53D6-4C24-BE3B-E6DBDF7BEF07
Figs 3–5, Table 1

'Tango tetraploid form' of *Cobitis striata*: Takeno et al. 2010: 108, fig. 2; *Cobitis* sp. 5: Nakajima et al. 2012: 92, fig. 3e; *Cobitis* sp.: Hosoya 2013: 331; *Cobitis* sp.: Kawase 2015: 181.

Type materials. Holotype: KPM-NI 31994, 60.4 mm standard length (SL), male, Tango District, Kyoto Prefecture, Honshu Island, Japan; collected by K. Tominaga on 17 Apr. 2010. Paratypes: 10 specimens, all from same locality as the holotype: TKPM-P 7363, 7364, 53.2–67.5 mm SL, male and female, same data as holotype; KPM-NI 31995–31999, 49.4–70.5 mm SL, 3 males and 2 females, collected by J. Nakajima on 12 Nov. 2010; KUN-P 45133, 57.7 mm SL, male, collected by K. Tominaga on 5 Jul. 2014; JNC 188, 189, 58.6–60.6 mm SL, 2 males, same data.

Diagnosis. Maxillary barbel short, more of the same eye diameter; lamina circularis in adult males simple and roundish; USP narrow; PMN 14; line L5 organised in 11–17 oblong or ovoid blotches out of spawning season, and lines L3 and L5 in adult male well-developed longitudinal obvious stripes during spawning season; upper and lower spot at caudal base not connected; tetraploid.

Description. Dorsal-fin rays iii, 7; anal-fin rays iii, 5; pectoral-fin rays i, 7–8; pelvic-fin rays ii, 6; caudal-fin rays 8+8. Body elongate, laterally compressed. Head and snout elongated. Interorbital space narrow, convex. Eye relatively large. Caudal peduncle relatively compressed. Mouth small, inferior, arched with fleshy lips; lower lip divided with 2 well-developed lobes; upper lip with transverse wrinkles on the surface. Barbels, 3 pairs, first on rostorum, second on maxilla, third on maxillomandibula; each barbel well-developed, length of maxillary barbel short, same as the eye diameter; the length of the rostral and maxillary barbels shorter than that of mandibular barbel (Fig. 4a). Lateral line short, reaching the pectoral-fin base. PMN 14. Very small cycloid scales on the trunk. Suborbital spine two-pronged and incurved; length of the outer spine one-third of that of the inner spine (Fig. 4b). First branched ray of the pectoral fin longer than rest (Fig. 4c); pectoral fin in adult males longer than that in females. USP narrow (Figs 4c, d, see also Fig. 6). Lamina circularis at the base of the pectoral fin in adult males simple and roundish (Fig. 4d). Dorsal-fin base equidistant from the base of the caudal fin and the top of the snout. Pelvic-fin origin below the second or third branched dorsal fin ray.

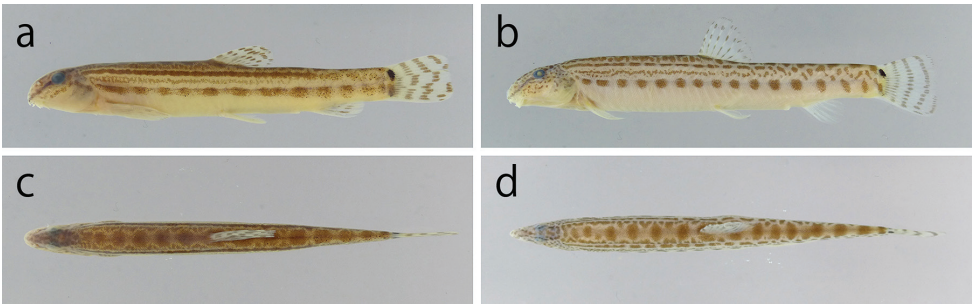

Figure 3. Male (**a**, **c** holotype, KPM-NI 31994, 60.4 mm SL) and female (**b**, **d** paratype, KPM-NI 31999, 70.5 mm SL) specimens of *Cobitis takenoi* sp. n. **a**, **b** Lateral view **c**, **d** dorsal view.

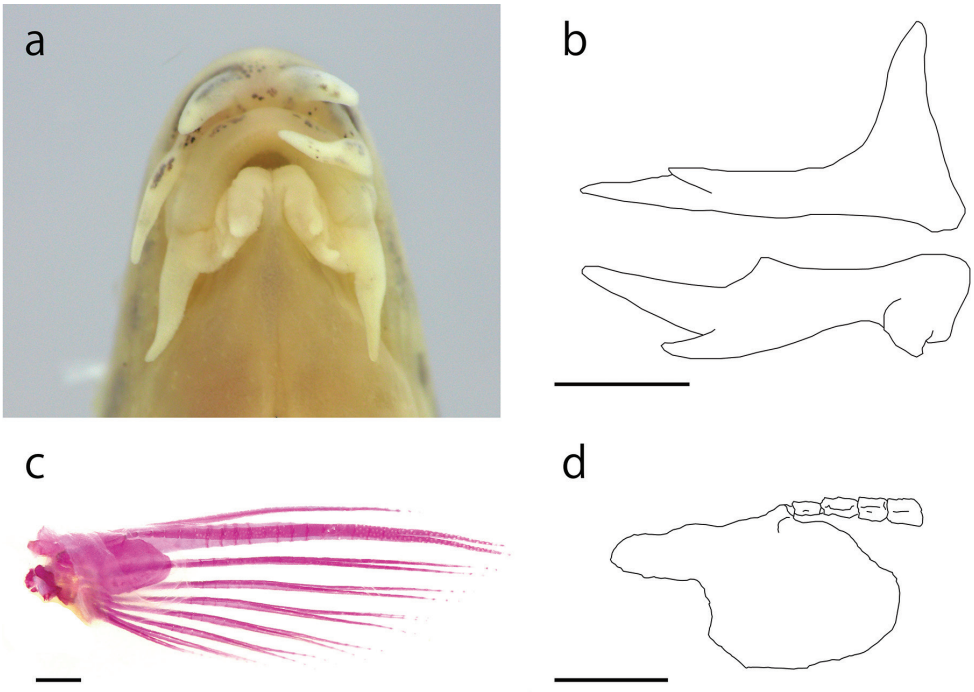

Figure 4. *Cobitis takenoi* sp. n., KPM-NI 31994, holotype. **a** Mouth **b** right suborbital spine, lateral view (upper), and dorsal view (lower) **c** dorsal view of the pectoral fin **d** lamina circularis and upper segments of the first branched soft ray (USP). Scale bars: 1 mm.

Anal fin not reaching the caudal-fin base. Margin of anal and dorsal fins slightly roundish. Caudal fin slightly roundish. Abdominal vertebrae 22 (21–23); caudal vertebrae 20 (19–21); total vertebrae 42 (40–44) (Takeno et al. 2010). Largest recorded specimens 65.5 mm SL in male and 84.9 mm SL in female (Takeno et al. 2010).

Colouration. Body yellowish white with dark brown pigmentation in fresh. A clear streak running from the tip of the snout to the occiput, crossing to the eye. Upper part

Figure 5. Change in colouration of the *Cobitis takenoi* sp. n. adult male (paratype, KUN-P 45133, 57.7 mm SL). **a** Spawning season, 8 July 2014 **b** non-spawning season, 5 December 2014.

of the head covered with amorphous spots; opercle and snout covered with amorphous patterns. Caudal and dorsal fins with 3–4 arcuate bars. Anal fin pigmented along fin rays. Upper spot at caudal base jet-black, size comparable to the eye diameter, lower spot at the caudal base relatively inconspicuous and small; upper and lower spots at the caudal base not connected. ***Male out of spawning season*** (Figs 3a, 5b). Body pigmentation organised in 1 dorsal and 4 lateral lines. Line L1 consisting of a series of 11–16, saddle or oval shaped blotches. Line L2 formed by a longitudinal jugged line or convex semicircular spots or chained small angular blotches, only present on dorsal part of body. Line L3 formed by a sharp longitudinal line or narrow dotted line, reaching to the post-dorsal body, with intermissive posterior part. Line L4 formed by narrow web like line or dots, reaching to dorsal body. Line L5 organised in 11–17 blotches from the upper part of the pectoral fin to the caudal-fin base; blotches roundish, frequently oblong or ovoid. ***Male in the spawning season*** (Fig. 5a). Line L4 not visible or formed by faint longitudinal line. Lines L3 and L5 well developed, forming longitudinal obvious stripes from the upper part of the pectoral-fin base to the caudal-fin base, often intermissive posterior part of L3. ***Female*** (Fig. 3b). Similar to males out of spawning season.

Sexual dimorphism. Males having a roundish lamina circularis at the base of the pectoral fins; females do not. Generally, the body size of females larger than that of males. Lines L3 and L5 of adult males well developed, forming longitudinal obvious stripes during the spawning season; females do not.

Table 1. Counts and morphometric measurements of *Cobitis takenoi* sp. n.

		Holotype	Paratypes	
			7 males	3 females
SL (mm); mean (range)		60.6	55.5 (49.4–58.6)	67.4 (64.2–70.5)
Counts	Dorsal fin	iii, 7	iii, 7	iii, 7
	Anal fin	iii, 5	iii, 5	iii, 5
	Pectoral fin	i, 8	i, 7–8	i, 7–8
	Pelvic fin	ii, 6	ii, 6	ii, 6
	Caudal fin	8+8	8+8	8+8
In % SL; mean (range)	HL	20.0	20.7 (19.8–21.7)	20.2 (19.3–21.5)
	Body depth	15.7	14.4 (13.2–17.6)	13.3 (12.5–13.9)
	Predorsal length	50.2	49.7 (47.6–53.1)	51.1 (49.5–52.6)
	Preanal length	74.6	74.8 (71.2–77.1)	76.0 (73.9–78.8)
	LPP	32.0	31.6 (28.6–33.6)	34.0 (32.2–35.4)
	LPA	25.7	25.2 (24.2–26.9)	25.7 (24.9–27.3)
	DCP	9.7	9.5 (9.0–10.8)	9.3 (8.8–9.6)
In % HL; mean (range)	Snout length	35.9	36.4 (31.6–44.2)	43.0 (42.3–44.2)
	Eye diameter	18.8	19.7 (17.9–21.9)	17.9 (17.7–18.1)
PMN		14	14.0	14.0

SL, standard length; HL, lateral head length; LPP, length of between pectoral-fin base and pelvic-fin origin; LPA, length of between pelvic-fin base and anal-fin origin; DCP, depth of caudal peduncle; PMN, prepelvic myotome number

Ploidy. Tetraploid (Takeno et al. 2010).

Etymology. The specific name is dedicated to Mr. Makoto Takeno, the discoverer of this spined loach.

Distribution. Tango District, Kyoto prefecture, Honshu Island, Japan.

Habitat and biology. This species inhabits sandy-mud bottoms of the middle and lower reaches of rivers (Fig. 2). Life histories are unknown.

mtDNA cyt*b* sequence. AB533231–AB533234 (Takeno et al. 2010).

Japanese name. Tango-suji-shima-dojyô (Nakajima et al. 2012).

Comparison. This new species is distinguished from nine species of *Cobitis* in the Japanese archipelago (*C. biwae*, *C. striata*, *C. matsubarae*, *C. takatsuensis*, *C. shikokuensis*, *C. magnostriata*, *C. minamorii*, *C. kaibarai* and *C. sakahoko*) by a combination of the following character states: a short maxillary barbel equaling in length the eye diameter (vs. longer than the eye diameter in *C. matsubarae*, *C. takatsuensis*, *C. shikokuensis* and *C. sakahoko*); a simple roundish lamina circularis (vs. beak-shaped or narrow in *C. biwae*; quite narrow in *C. takatsuensis* and *C. shikokuensis*; rectangular with a neck in *C. sakahoko*); a narrow USP (vs. broad in *C. matsubarae*, *C. takatsuensis*, *C. shikokuensis*, *C. magnostriata* and *C. sakahoko*); PMN 14 (vs. commonly 12 in *C. minamorii*; commonly 13 in *C. striata* and *C. kaibarai*); a L5 formed of blotches out of spawning

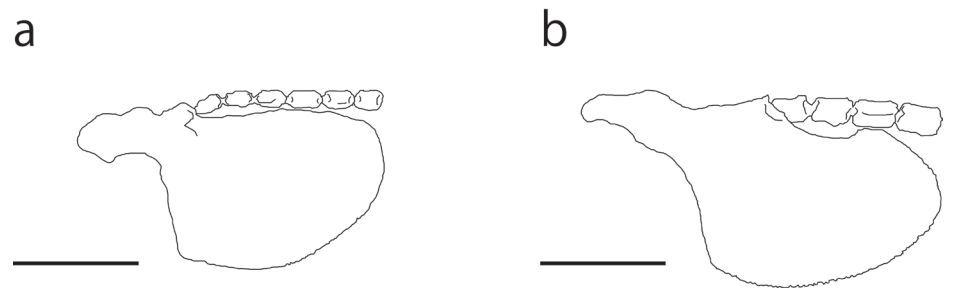

Figure 6. Narrow and broad types of upper segments of the first branched soft ray (USP). **a** Narrow type (*Cobitis kaibarai*) **b** broad type (*Cobitis magnostriata*). Redrawn from Nakajima (2012). Scale bars: 1 mm.

season (vs. stripe-like in and out of spawning season in *C. takatsuensis* and *C. magnostriata*); both spots at caudal base obvious (vs. lower spot inconspicuous in *C. striata* and *C. kaibarai*); and ploidy tetraploid (vs. diploid in *C. striata*, *C. takatsuensis*, *C. shikokuensis*, *C. minamorii* and *C. kaibarai*). These comparative data were summarised from Nakajima and Suzawa (2016).

Remarks. Till date, *C. takenoi* has only been found in one small river system, and the habitat is under threat from river improvement. In addition, some threatened freshwater fishes are captured and sold illegally in Japan (e.g. *Parabotia curtus*, Watanabe et al. 2015), and this new species is similarly at the risk of being commercially overfished for the ornamental fish market (Takeno et al. 2010). Therefore, the species is ranked as a critically endangered species (CR) – as *Cobitis* sp. – in the Japanese Red List (Kitagawa 2015). The distribution pattern, suitable habitat and life history of this species are not well-known. Basic biological investigations are required for its effective conservation.

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A new blue-tailed Monitor lizard (Reptilia, Squamata, *Varanus*) of the *Varanus indicus* group from Mussau Island, Papua New Guinea

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Abstract

We describe a new species of *Varanus* from Mussau Island, north-east of New Guinea. The new species is a member of the *Varanus indicus* species group and is distinguished from all other members by both morphological and molecular genetic characters. It is the third species of *Varanus* reported from the Bismarck Archipelago and the first record of a yellow tongued member of the *Varanus indicus* species group from a remote oceanic island. The herpetofauna of Mussau Island has not been well studied but the discovery of this new species is in accordance with recent findings indicating that the island may harbor several unknown endemic vertebrates. The distribution of the closely related *Varanus finschi* is also discussed in the light of recent fieldwork and a review of old records.

Keywords

Melanesia, Bismarck Archipelago, St. Matthias islands, Varanidae, *Varanus doreanus*, *Varanus finschi*, *Varanus yuwonoi*, mitochondrial phylogeny, biogeography, taxonomy

Introduction

The varanid subgenus *Euprepiosaurus* Fitzinger comprises two species groups: the *V. indicus* and *V. prasinus* species groups. The subgenus is geographically restricted to a large region east of Wallace's line with the Solomon Islands and parts of Micronesia forming the eastern and northern boundaries (Ziegler et al. 2007a, Sweet and Pianka 2007). The systematic arrangement is well-supported by molecular and morphological studies (Ziegler and Böhme 1997, Fitch et al. 2006, Vidal et al. 2012). Several new monitor lizards of the subgenus *Euprepiosaurus* have been discovered from islands in the southwest Pacific since the early 1990s. This increase has mainly been the result of taxonomic studies of museum collections, and the appearance of novel species through the international trade in live animals. Eleven species have been described from the Moluccas and Raja Ampat islands in eastern Indonesia, more often as a result of new specimens arriving through the animal trade rather than resulting from field studies and scientific collections (e.g. Böhme and Ziegler 1997, Harvey and Barker 1998).

Over the same time period, the monitors of Papua New Guinea and the Solomon Islands have received considerably less scientific attention. Papua New Guinea has no legal international live animal trade and it's fauna is less represented in European museum collections. Since 1990 only two new species have been described from Papua New Guinea, both from revisions of colonial era museum collections: *Varanus telenesetes* (Sprackland 1991) (possibly a synonym of *V. bogerti* Mertens [Weijola obs.]), and *Varanus finschi* Böhme, Horn & Ziegler, 1994. As a consequence, the Melanesian islands have been considered less diverse in comparison to the Moluccas (Ziegler et al. 2007a).

As part of a larger survey of the monitors of the Bismarck Archipelago of Papua New Guinea in 2012, VW collected three specimens of a previously unknown blue-tailed species of the *V. indicus* species group from Mussau Island in the St Matthias group. Previously three individual monitor lizards in total had been recorded on two separate occasions from the St Matthias group - a juvenile specimen collected in 1944 (AMNH 85887) and two adult specimens collected during the Noona Dan Expedition in 1961-1962 (ZMUC 4272-4273) (identified as *V. finschi* in Philipp et al. [2007]).

Four species of the *V. indicus* species group (including the new taxon from Mussau) share the occurrence of yellow pigmentation on the tongue (Harvey and Barker 1998). Although taxon sampling in published molecular phylogenies has been limited, these yellow-tongued monitors have consistently formed a basal clade within the *V. indicus* species group (Ast 2001, Welton et al. 2013). *Varanus doreanus* Meyer is widespread on New Guinea, Aru, Biak, Waigeo, Salawati and parts of northern Cape York (Ziegler et al. 2007a). *Varanus finschi* Böhme, Horn & Ziegler, 1994 is likely endemic to New Britain (see discussion). *Varanus yuwonoi* Harvey & Barker is endemic to Halmahera and possibly nearby islands (Weijola 2010).

Molecular genetic and morphological studies of the newly collected material from Mussau Island clearly show the population represents a distinct taxon of yellow-tongued monitor. The concept best applicable to allopatric species is probably the Evolutionary Species Concept (ESC) (Simpson 1951) and more recent integrative ap-

proaches such as the Unified Species Concept (de Queiroz 2007). On account of its distinctive morphology, phylogenetic position and geographically isolated distribution we recognize the Mussau monitor as a unique evolutionary lineage and describe it as a new species herein.

Materials and methods

Taxonomy. We follow the nomenclature of de Lisle (2009) for the taxa treated. The taxonomic identities of *V. cerambonensis* and *V. indicus* (*sensu* Philipp et al. 1999) included in the molecular phylogeny have recently been challenged (see Weijola and Sweet 2015, Weijola 2015) but until a ruling from the ICZN is issued we follow the nomenclature of Philipp et al. (1999).

Morphology. We obtained data for the meristic characters used by Brandenburg (1983) and in later works on the *V. indicus* group (e.g., Ziegler et al. 2007b, Weijola and Sweet 2010). Measurements were taken to the nearest 0.5 mm (head) or 1 mm with a steel tape or calipers. Comparative scale counts for *V. doreanus* and *V. yuwonoi* were taken from the literature (Brandenburg 1983, Harvey and Barker 1998, Ziegler et al. 2007b). Specimens listed in Brandenburg (1983) were identified by VW. We used PAST (Hammer et al. 2001) for Principal Components Analyses (PCA). The variance-covariance matrix was used on the unaltered scalation data including P, Q, S, T, X, XY, m, N and R characters. Definitions of, and abbreviations used for measurements, proportion indices and scale counts are presented in Table 1.

Museum abbreviations used are: ABTC: Australian Biological Tissue Collection (South Australian Museum, Adelaide), AMNH: American Museum of Natural History (New York), AMS: Australian Museum (Sydney), BPBM: Bernice Pauahi Bishop Museum (Honolulu), NMW: Naturhistorische Museum Wien (Wien), QM: Queensland Museum, RMNH: Naturalis museum (Leiden), UMMZ: Museum of Zoology, University of Michigan, ZMA: Zoological Museum of the University of Amsterdam (currently Naturalis), ZMB: Zoologische Museum der Humboldt Universität (Berlin), ZMUC: Zoological Museum, University of Copenhagen, and ZMUT: Zoological Museum, University of Turku.

Molecular genetic methods. A 661 bp fragment of the mitochondrial genome, including the 3' end of the NADH dehydrogenase subunit 4 (*ND4*) gene (710 bp) and the 5' end of $tRNA^{His}$ (64 bp) gene, was amplified and sequenced (hereafter referred as *ND4*) using the forward primer 5'- TGA CTA CCA AAA GCT CAT GTA GAA GC-3' (Forstner et al. 1995) with the reverse primer 5' CAT TAC TTT TTA CTT GGA TTT GCA CCA-3' (Arévalo et al. 1994). A 566 bp fragment of the mitochondrial *16S rRNA* gene was amplified and sequenced using the forward primer: 5' - CGC CTG TTT ATC AAA AAC AT - 3' with the reverse primer: 5' - CCG GTC TGA ACT CAG ATC ACG T – 3' (Palumbi et al. 1991).

The amplification reactions were performed in a final volume of 50ul using the Phusion U Hot Start PCR Master Mix (ThermoFisher Scientific, St. Leon-Rot, Ger-

Table 1. Definitions of, and abbreviations used for measurements, proportion indices and scale counts.

Symbol	Description
Measurements	
SVL	Snout to vent length
F	tail length
TL	total length
E	body length from gular fold to cloaca
D	head-neck length from tip of snout to gular fold
A	head length from snout to anterior dorsal margin of tympanum
B	head width at maximum span of postorbital arch
C	head depth at midpoint of orbit
G	facial length from center of nostril to anterior margin of orbit
H	snout length from tip of snout to center of nostril
I	temporal length from anterior margin of eye to anterior border of tympanic recess
Proportion Indices	
1	relative tail to body length - F/SVL
2	relative position of nostril to eye - G/H
9	relative position of nostril to tip of snout - [A-I]/G
10	relative head length to width - A/B
11	relative head length to height - A/C
Scale Counts	
S	Midbody scale rows
XY	dorsal scale rows from dorsal margin of tympanic recess to anterior margin of hind limbs
T	transverse rows of mid-ventral scales from gular fold to anterior margin of hind limbs
X	transverse rows of dorsal scales from posterior margin of tympanic recess to gular fold
m	scales around neck at anterior margin of gular fold
N	rows of mid-ventral scales from tip of snout to gular fold
P	scales from rictus to rictus across dorsum of head
Q	scales around tail base
R	scales around tail counted at 1/3 of the length from the base
DOR	number of dorsal scalerows from the last occipital scale to a point dorsal to the posterior margin of the cloaca
VEN	Number of mid-ventral scales from the gular fold to the anterior margin of the cloaca

many). The PCR profile for the *ND4* amplification was 9 min at 94 °C (initialization step, one cycle), 30 sec at 94 °C (denaturation step, 35 cycles), 25 sec at 46,5 °C (annealing step, 35 cycles), 35 sec at 72 °C (extension step, 35 cycles) and 2 min at 72 °C (final elongation step, 1 cycle). The corresponding profile for the *16S rRNA* amplification was 9 min at 94 °C (initialization step, one cycle), 30 sec at 94 °C (denaturation step, 35 cycles), 25 sec at 55 °C (annealing step, 35 cycles), 35 sec at 72 °C (extension step, 35 cycles) and 2 min at 72 °C (final elongation step, 1 cycle). A negative control (no template present) was also included in all PCRs. All PCR products were analyzed by gel electrophoresis on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide (Promega, Madison, USA) before they were sequenced.

PCR products were sequenced by the Beckman Coulter Genomics company (Essex, UK). GenBank accession numbers of the new sequences are provided in Table 2.

Phylogenetic analysis. Resulting sequences were aligned by MUSCLE (Edgar 2004) as implemented in GENEIOUS v8.1.4 and concatenated for phylogenetic analysis. Bayes factors were used to assess all possible alternative partitioning strategies for five data subsets: 1st, 2nd and 3rd codon positions, the tRNA and *16S rRNA* in PartitionFinder v1.0.0 (Lanfear et al. 2012). The Akaike Information Criterion (AIC) and Bayes Information Criterion (BIC) were used to assess the best fit partition strategy and nucleotide substitution model for each data subset in the selected partition strategy. Sequences were analysed phylogenetically using Bayesian and maximum likelihood (ML) methods. Bayesian analysis was conducted using MrBayes v3.2.5 (Ronquist and Huelsenbeck 2003). The analysis was run with model parameters unlinked using default priors for ten million generations with two independent runs and two chains sampling every 500 generations. The first 25% of sampled trees were discarded as burn-in and convergence was assessed by examining effective sample sizes (ESS values), split frequencies of clades across runs and likelihood plots through time in TRACER v1.6 (Rambaut and Drummond 2007). Evolutionary trees were constructed with the ML criterion of optimality implemented in the web server version of RAxML (Stamatakis et al. 2008), which uses the GTR+Γ model of nucleotide substitution. The robustness of phylogenetic hypotheses was tested with non-parametric bootstrapping. *Varanus prasinus*, from the sister lineage to the *V. indicus* species group, was used as outgroup.

Net average sequence divergence between lineages (*dA*) was calculated from the *ND4* data only in MEGA v5 (Tamura et al. 2011) as: $dA = dXY - (dX + dY)/2$, where, dXY is the average distance between groups X and Y, and dX and dY are the within-group means. Net average sequence divergence was calculated more broadly for sister species pairs of *Varanus* where more than one sequence was available for each member of the pair from our data and the data of Fitch et al. (2006), Smith et al. (2007), Smissen et al. (2013), Maryan et al. (2014), Doughty et al. (2014) and GenBank accessions for *V. komodoensis*.

Results

***Varanus semotus* Weijola, Donnellan & Lindqvist, sp. n.**
http://zoobank.org/B5D753CF-7C2F-42B4-A7FE-376F0E8FCF6A
Figs 1–3

Holotype. ZMUT Sa176 (field nr. 60) (Figs 1–2) collected by Valter Weijola just north of the village of Nai, 30 September 2012, 2m elev. Mussau Island, St. Matthias group, Papua New Guinea, latitude -1.525, longitude. 149.749.

Paratypes. ZMUT Sa177 (field nr 64), ZMUT Sa178 (field nr 66) collected by Weijola near Nai 4 and 7 October 2012. Mussau Island, Papua New Guinea, latitude -1.525, longitude 149.749, ZMUC 4272 (field number E192) and ZMUC 4273

Table 2. Specimens examined morphologically (*), or sequenced for mtDNA. Voucher registration numbers (#), collection localities and GenBank accession numbers are listed.

Species	Voucher Registration #	Collection Locality	GenBank *ND4, 16S RNA*
V. cerambonensis	WAM R109448	Banda Is., Ind.	KU513445, KU513465-
V. cerambonensis	WAM R109476	Banda Is., Ind.	KU513446, KU513466
*V. doreanus**	AMS R28680	Gamog, Karkar Is. PNG	-
*V. doreanus**	AMS R25686	Gamog, Karkar Is. PNG	-
*V. doreanus**	AMS R25687	Gamog, Karkar Is. PNG	-
*V. doreanus**	AMS R129210	Jama, East Sepik Prov., PNG	-
*V. doreanus**	BPBM 19509	Mt Obree, Northern Prov., PNG	KU513447, KU513467
*V. doreanus**	Naturalis ZMA10190	? Indonesia	-
*V. doreanus**	Naturalis ZMA10193	Sabang, West Papua, Ind.	-
*V. doreanus**	Naturalis ZMA10194a	Noord River, West Papua, Ind.	-
*V. doreanus**	Naturalis ZMA10195	Wendessi, West Papua, Ind.	-
*V. doreanus**	Naturalis ZMA10199	Sermonai River, West Papua, Ind.	-
*V. doreanus**	Naturalis ZMA12125	Hollandia (Jayapura), Papua, Ind.	-
*V. doreanus**	Naturalis RMNH5164	Digoel River, West Papua, Ind.	-
*V. doreanus**	Naturalis RMNH7035	Manokwari	-
*V. doreanus**	Naturalis RMNH21029	Gariau-lake jamoer, West Papua, Ind.	-
*V. doreanus**	Naturalis RMNH21051	Fak Fak, West Papua, Ind.	-
*V. doreanus**	Naturalis RMNH21055b	Manokwari, West Papua, Ind.	-
*V. doreanus**	QM J15363	Cape York, Qld. Aus.	-
*V. doreanus**	QM J18103	Claudie River, Qld, Aus.	-
*V. doreanus**	QM J32020	Pascoe River, Qld. Aus.	-
V. doreanus	UMMZ 227117	Merauke, Papua, Ind.	KU513448, KU513468
*V. finschi**	AMS R5618	Duke of York, East New Britain, PNG	-
*V. finschi**	AMS R129614	Amelei, New Britain, PNG	-
*V. finschi**	ZMUT Sa186	Nodup, New Britain, PNG	KU513443, KU513463
*V. finschi**	ZMUT Sa190	near Kokopo, New Britain, PNG	KU513444, KU513464
V. finschi	MNHN 00 192	Blanche Bay, New Britain, PNG	-
V. finschi	MNHN 00 195	Blanche Bay. New Britain, PNG	-
V. indicus	ZMUT Sa191	Normanby Is., PNG	KU513455, KU513476
V. indicus	ZMUT Sa202	New Britain, PNG	KU513456, KU513477
V. indicus	No voucher, tissue QM A002919	Peach Creek, Qld, Aus.	KU513452, KU513473
V. indicus	WAM R109525	Aru Islands, Ind.	KU513453, KU513474
V. indicus	WAM R109551	Aru Islands, Ind.	KU513454, KU513475
V. indicus	No voucher, tissue ABTC13465	Maningrida, NT, Aus.	DQ525167, KU513469,
V. indicus	AMS R137997	Fergusson Is., PNG	KU513450, KU513471
V. indicus	LSUMZ H10449	Wewak, East Sepik Prov., PNG	KU513451, KU513472
V. jobiensis	AMS R115341	Doido, Chmbu Prov.,PNG	DQ525163, KU513478
V. jobiensis	AMS R116999	Wigote, Sandaun Prov., PNG	KU513457, KU513479
V. melinus	UMMZ 222682	Sula Islands, Ind.	KU513458, KU513480
V. prasinus	AMS R115500, ZFMK 70600	Mt Boobiari, Sandaun Prov., PNG. West Papua, Ind.	DQ525171, EF193687

*V. semotus**	ZMUT Sa176	Mussau Is., PNG	KU513459, KU513482
*V. semotus**	ZMUT Sa177	Mussau Is., PNG	KU513460, KU513483
*V. semotus**	ZMUT Sa178	Mussau Is., PNG	KU513461, KU513484
*V. semotus**	ZMUC 4272	Talumalaus, Mussau Is., PNG	-
*V. semotus**	ZMUC 4273	Talumalaus, Mussau Is., PNG	-
V yuwonoi	UMMZ 225545	Halmahera, Ind.	KU513462, KU513481

(field number E282) collected by the Noona Dan Expedition (presumably by Søren Andersen) on 19 January and 5 February 1962 at Talumalau, Mussau Island, Papua New Guinea.

Other material. AMNH 85887 collected by John Gardiner in 1944, St Matthias Islands, Papua New Guinea.

Etymology. The specific epithet *semotus* is Latin for distant or remote and refers to the isolated occurrence on Mussau, separated by several hundred kilometers from its closest relatives. The term is employed as a masculine adjective.

Diagnosis. *Varanus semotus* sp. n. is distinguished from all other species of *Varanus* by a combination of the following characters. (1) Tongue white/pinkish to pale yellow (white in preservative) occasionally with small patches of dark pigmentation, the yellow pigment concentrated along the mid-dorsal line and the dorsal surface of the tines (Fig. 2). (2) Gular region marbled in black and cream-white. (3) The tail of adult individuals is indistinctly banded on the distal half, with a varying degree of turquoise to bluish pigmentation on the distal 2/3. (4) Juveniles are black with white spots on the head, yellow and orange spots on the dorsum, and have well defined cream colored to pale greenish tail bands (Fig. 3C). (5) The number of dorsal scales, XY, ranges from 149 to 153. (6) The number of midbody scale rows, S, ranges from 152 to 161. (7) The dorsum is black with single- and clustered groups of dispersed yellow/orange scales. (8) There are several complete rows of paryphasmata across the asulcal side of the hemipenis below the lobes. (9) Geographical distribution restricted to Mussau Island.

Comparisons. *Varanus semotus* sp. n. is a member of the *Varanus indicus* species group of the subgenus *Euprepiosaurus* distinguished by the asymmetrical sulcus spermaticus and laterally compressed tail (Ziegler et al. 2007a). Within the *V. indicus* species group it can be distinguished from all species except for *V. doreanus*, *V. finschi* and *V. yuwonoi* by the presence of yellow pigmentation on the tongue. *Varanus semotus* is unlikely to be confused with any other species except for *V. doreanus*, from which it can be difficult to distinguish by external morphology. On average, *V. semotus* has lower XY (149–153 vs. 153–215) and S (152–161 vs. 158–180) scale counts than *V. doreanus. Varanus semotus* exhibit several complete rows of paryphasmata crossing the asulcal side of the hemipenis while this is restricted to the medial part of the trunk and lobes on *V. doreanus* (Fig. 4). In contrast to the morphological similarity of these two species, they show a significant genetic separation: 11.5% mean net sequence divergence (*dA*) (Table 5B). *Varanus semotus* is readily distinguished from *V. finschi* and *V.*

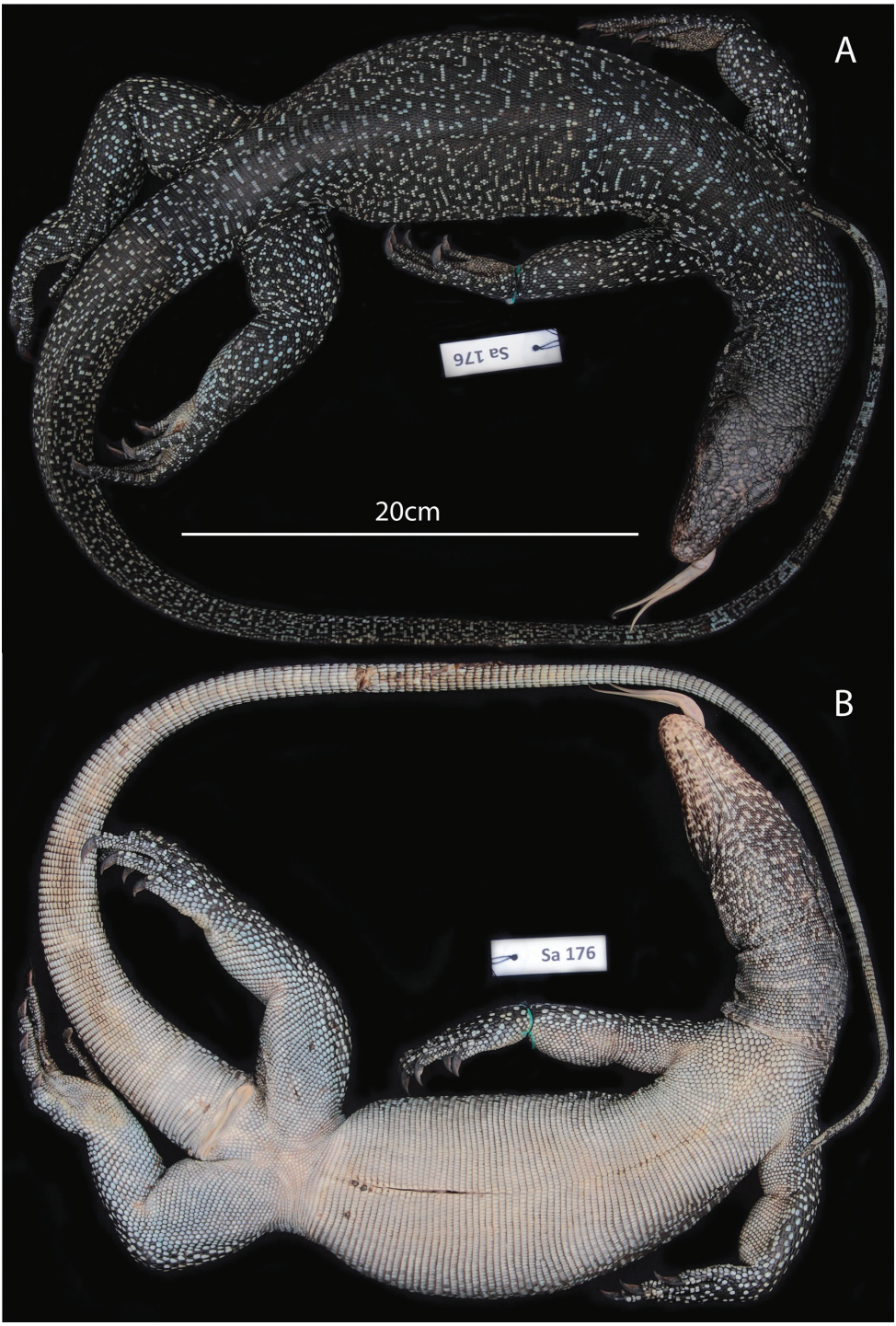

Figure 1. A–D Full body dorsal, ventral, head profile and gular region of the holotype of *V. semotus* -ZMUT Sa176.

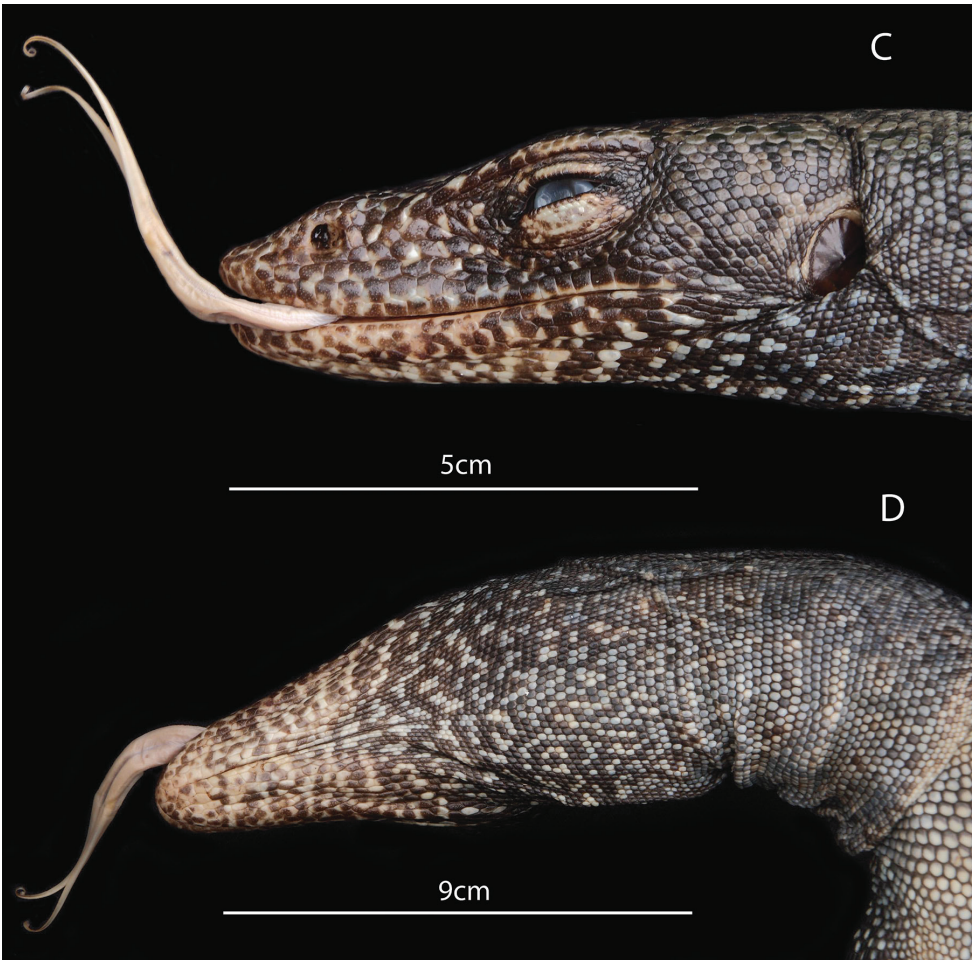

Figure 1. Continued.

yuwonoi, both of which have predominately white to cream colored throats and considerably higher scalecounts (S over 170, XY over 165). Additionally, *Varanus finschi* lacks blue pigmentation on the tailand exhibits transverse rows of yellow ocelli on the dorsum. Furthermore, *V. finschi* and *V. semotus* have a *dA* of 6.4% (Table 5A). *Varanus yuwonoi* has a unique color pattern being predominantly black on the anterior 1/3 of the body, yellow on the lower back and tailbase, and with a blue tail. Furthermore, *V. yuwonoi* and *V. semotus* have a *dA* of 11.6% (Table 5B).

Description of the holotype. A female of a total length of 1010mm (SVL: 390mm, F: 620mm). The specimen is well preserved and has an incision running from below the rib-cage to the lower abdomen. There are unhealed lacerations on the ventral part of the tail at around midlength, possibly from a dog bite. The ground color of the dorsal aspect of the body, tail, head and limbs is black. The tail is long and slender, 1.59 times as long as the body, and 38.75 times as long as it is high (16mm) at midlength. It is rounded at

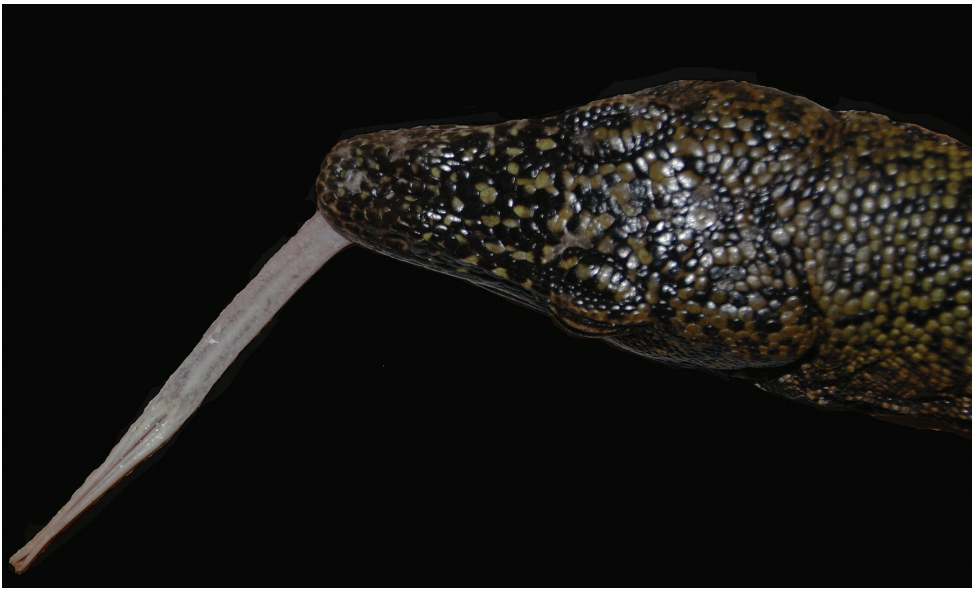

Figure 2. Tongue color of the freshly collected *V. semotus* holotype ZMUT Sa176.

the base, becoming increasingly laterally compressed distally starting at 60mm from the base. Two to five middorsal caudal scale rows form a double ridge extending from 1/8 of its length and distally almost to the tip. There are nine discernible blue crossbands each about 6–9 scale rows wide on the distal half of the tail with intermediate blue markings. The ventral scales are white to cream colored with a narrow line of dark brown pigmentation running along the anterior margin. The gular region is dark brown-black and marbled with yellowish and greyish scales. The nostrils are large and round, positioned closer to the snout than the anterior margin of the eye. Nasal capsules expanded forming a groove on the rostrum. The tongue is whitish (in preservative) with small spots of grey-blue pigmentation along the lateral margins. The teeth are long, sharp and only slightly recurved. The limbs are muscular, claws dark-brown and recurved. The head is dark-brown to black and covered with irregular brown-grey markings.

Nuchal scales are slightly domed to flattened, elongate to polygonal immediately behind the head becoming round to oval towards the shoulders and with 1–10 scale pits. Gular scales flattened, round to irregularly polygonal, equipped with 1–5 pits and sometimes bordered by incomplete rows of granules. Mental scales irregular in shape from rectangular to polygonal and elongate. Dorsal scales slightly elongated, rounded or polygonal and with a low central keel. Most are surrounded by an incomplete row of granular scales and with one or two pits located near the posterior end.

Laterodorsal scales are smaller, round, slightly domed, surrounded by granules and with one to three pits. Middorsal caudal scales rectangular, elongate, with a single pit at the posterior end, and lack granules. Mid-ventral caudals twice as long as mid-dorsal caudal scales, elongate and keeled.

Figure 3. A–C Images of live *V. semotus* at Nai on Mussau Island. **A** an adult in its habitat at the outskirts of Nai **B** an adult basking on the trunk of a palm tree (photos by VW), and **C** a juvenile (photo by Quetzal Dwyer).

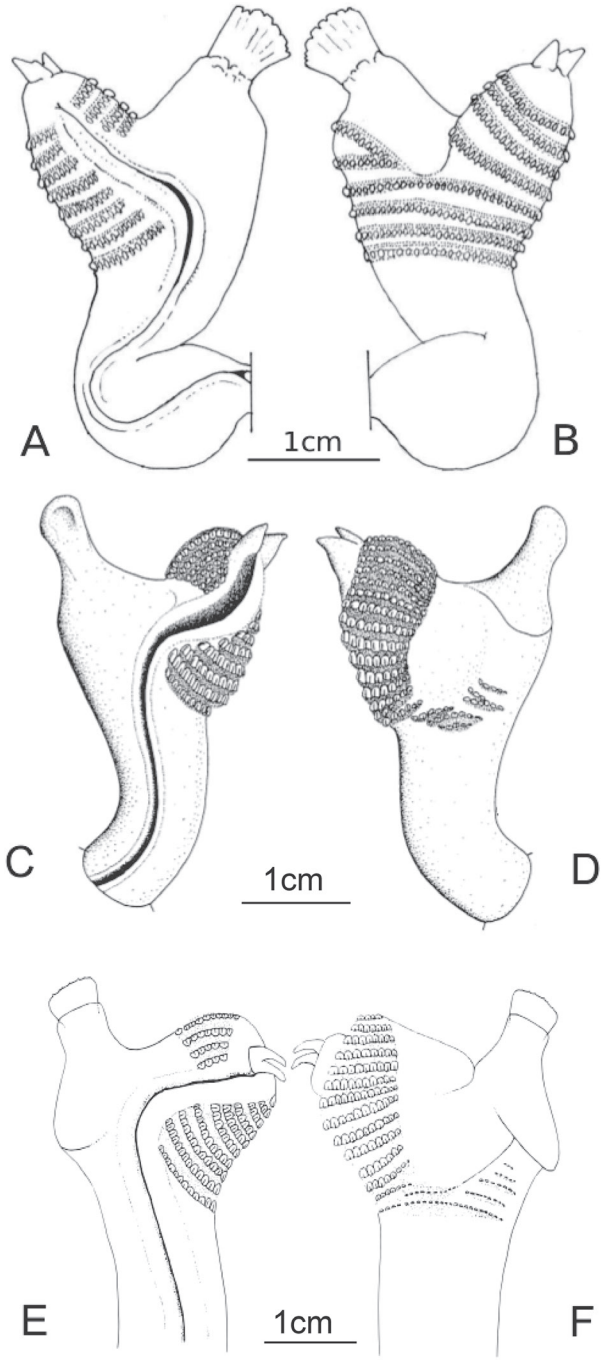

Figure 4. Drawings of the hemipenises of the male paratype ZMUT Sa178 of *V. semotus*, sulcal (**A**) and asulcal (**B**) (illustration by Sam Sweet), *V. doreanus* (ZFMK 26341) sulcal (**C**) and asulcal (**D**), and *V. finschi* (ZMB 14596) sulcal (**E**) and asulcal view (**F**) (**C–F** illustrated by Thomas Ziegler, reproduced from Ziegler et al. 1999).

Suprafemorals and suprabrachials oval, keeled and surrounded by 1–2 rows of granules. Supratibials irregularly round to oval, polished or keeled and surrounded by 2–3 rows of granules. Infrafemorals round to slightly oval and usually equipped with a row of granules along the distal edge. Infratarsals round to polygonal, highly domed and with a few granules around the corners. Most are light in color and only few have dark pigmented centers. There are rows of 9 enlarged postdigital scales along the outer margin of the fourth hind toe. Infracarpals similar in color to infratarsals, round to slightly polygonal, domed and with granules around the corners.

Dorsal head scales irregularly sized and polygonal, flattened, and equipped with numerous pits. There are seven enlarged supraocular scales on each side, bordered by 1-3 rows of smaller scales. Rostral scale, paired. There are 25+25 enlarged pentagonal supralabial scales equipped with as much as 30 pits. There are 26+26 irregularly shaped infralabials densely covered with pits. Temporal scales square or polygonal, polished and covered with up to ten pits. Two rows of scales separate the supralabials from the nostrils. The occipital scale is enlarged and roundish.The scales on the chest are enlarged, irregularly polygonal, flat and surrounded by only few granules. Ventral scales from the lower chest and down to the abdomen are rectangular, irregularly elongate, bordered by granules along the posterior margin, and with a single pit at the posterior end. The oviducts are translucent white and contains series of ovarian follicles about 10–15mm long.

Scale counts, measurements and proportion indices of the type series. Are presented in Table 3.

Hemipenal morphology. The hemipenis of the male paratype ZMUT Sa178 was everted prior to fixation (Fig. 4). The trunks are dark grey pigmented on the asulcal side excluding the lobes. The sulcus spermaticus runs medially on the trunk, turns to the lateral lobe and deflates at the base of the hemibaculum. There are four paryphasmata rows running across the asulcal side of the trunk proximally to the bifurcation of the lobes. About seven additional rows of paryphasmata continues up on the lateral lobe towards the apex. Two rows of paryphasmata runs on the lateral side of the medial lobe as a continuation of the truncal ornamentation. The medial hemibaculum is ossified, quadrangular and slightly decurved. The lateral hemibaculum is smaller, triangular, and with two sharp ends.

Variation and color in life. The type series is relatively uniform in coloration and pattern. The ground color of the dorsum, tail, legs and head is black. The dorsum and femurs are densely covered by yellow-orange scales, most aggregated in groups of 1–10 (mostly 2–4) scales forming lines, half circles or more rarely complete rings. The markings becomes denser on the neck and changes in color to brown-grey-yellow on the upper neck and head. On the dorsal side of the hands, feet, digits, supratibials and distal 2/3 of the tail most of the light markings are of a blue-green color. On the distal half of the tail these are arrayed in several indistinct transversal bands. The venter is white-pinkish, and with a blue hue on the infratibial surfaces. The upper chest and gular region has an orange-pink hue and is densely marmorated with black on the anterior half. The black markings are paler half adjacent to the gular fold. Photographs

Table 3. Measurements, proportion indices and scalecounts of the type series of *V. semotus*.

Measurements	ZMUT Sa176 (holotype)	ZMUT Sa177 (paratype)	ZMUT Sa178 (paratype)	ZMUC 4272 (paratype)	ZMUC 4273 (paratype)
SVL	390	400	400	45	48
F	620	610	640	69	69
TL	101	101	104	114	117
E	236	228	235	-	-
D	135	140	150	-	-
A	66	68.5	70	78	80
B	39	39	40.5	48	48
C	27	24	26.5	32	34
G	19	21	23	25	26
H	14	14	14	16	17
I	33	33.5	35	-	-
Proportion indices					
1	1.59	1.53	1.6	1.53	1.44
2	1.36	1.5	1.64	1.56	1.53
9	1.74	1.67	1.52	-	-
10	1.69	1.76	1.73	1.63	1.67
11	2.44	2.85	2.64	2.44	2.35
Scalation					
S	161	162	152	167	160
XY	153	147	149	150	152
T	89	87	87	89	89
X	40	39	38	39	43
N	93	89	85	92	91
m	116	114	108	119	118
P	47	47	47	49	51
Q	100	97	99	103	103
DOR	166	162	164	165	164
VEN	107	108	105	110	113

from the field allows for a description of coloration of a juvenile (Fig. 3C). This specimen is black with bright orange and yellow spots on the dorsum, white spots from the shoulder and anteriorly, more or less arrayed in 16 transverse rows between the venter and the head. On the distal 2/3 of the tail these spots turns into 16 complete, well defined whitish crossbands. On the dorsal sides of the legs and around the tailbase the spots are yellow-green. The head is decorated with white patches, and the lips have five white bars on both sides. The iris is dark brown.

Distribution. *Varanus semotus* is known so far only from Mussau, an island of 414 km^2 in the northern Bismarck Sea (Fig. 5). According to some of the locals on Mussau, monitors are absent from Emirau, the second largest island of the St. Matthias group, but this needs confirmation from fieldwork. It is also unknown whether this species occurs on the other two nearby islands Emananus and Eloaua.

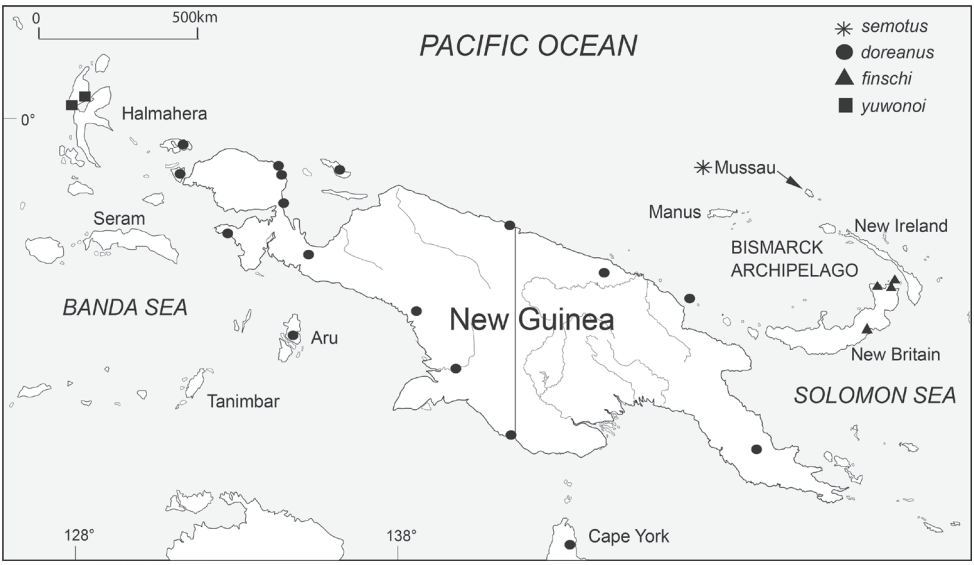

Figure 5. Map of New Guinea and surrounding islands showing the distribution of the members of the yellow-tongued monitors.

Natural history. A total of 16 observations were made during fieldwork on Mussau, all of them along the coast near the village of Nai at the SE corner of the island. Searches in the secondary growth forest of the interior of the island and in the mangrove forests near Palakau did not produce any observations. The relatively dry coastal vegetation near Nai comprises a mixture of coconut palms, pandanus and other trees and shrubs able to persist in the karst, limestone and salt spray affected area (Fig. 6). In this vegetation type monitors appeared to be relatively common. Just south of the village there is a freshwater spring with a small area of Sago palms which was also a popular site for monitors. The lizards were usually spotted either as they were foraging on the ground and quickly fled up in trees, or while they were basking on the trunks of palms or other trees. The specimens collected as vouchers were noosed from trees with a long pole. As is typical of the closely related *V. doreanus*, *V. finschi* and *V. yuwonoi* the specimens were exceedingly aggressive and inclined to bite when captured and handled. Stomach content analysis of the three ZMUT specimens revealed a total of five reptile eggs (3,2,0) and one small skink. All stomachs contained the remains of crabs. Philipp et al. (2007) recorded a bird as the stomach content of ZMUC 4272.

Morphology. The PCA resolved group structure and only partly overlapping morpho-areas for the four species included (Fig. 7ab). *Varanus semotus* shows no area overlap on component axes 1–2 and 1–3 while the other three species show full or partial overlap on axes 1–3 (Fig. 7b). Potential sexual dimorphism in scalation characters have not been reported and were not taken into account. PC1 and PC2 accounted for over 80% of the variation with highest loadings on characters S, XY and m (Table 4). *Varanus yuwonoi* and *V. finschi* associate closely as a result of the mutually high scale counts.

Figure 6. Typical vegetation of coastal karst areas of Mussau Island where several *Varanus semotus* were observed (photo by VW).

Table 4. Factor loadings, proportion of variance and eigenvalues for the three first components in the PCA. The two highest loading factors on each component are shown in bold.

Factor	Comp 1	Comp 2	Comp 3
P	0.019	-0.017	-0.312
Q	0.023	0.279	**0.548**
S	**0.291**	0.427	-0.304
T	0.141	0.292	0.292
XY	**0.861**	-0.170	0.083
m	0.198	**0.653**	0.362
N	0.143	0.222	0.222
R	-0.136	**0.457**	**0.457**
Proportion of variance	54%.2	29.1%	6.4%
Eigenvalue	435.4	233.9	51.6

The population from Mussau is at the opposite extreme with lower scale counts than the other members. *Varanus doreanus*, for which the largest sample size was available (all from West Papua), demonstrate a considerable amount of intraspecific variation.

Molecular genetic analysis. Using PartitionFinder, we selected three data partitions: *16S rRNA* + *ND4* 1st codon positions + $tRNA^{HIS}$, *ND4* 2nd codon positions and

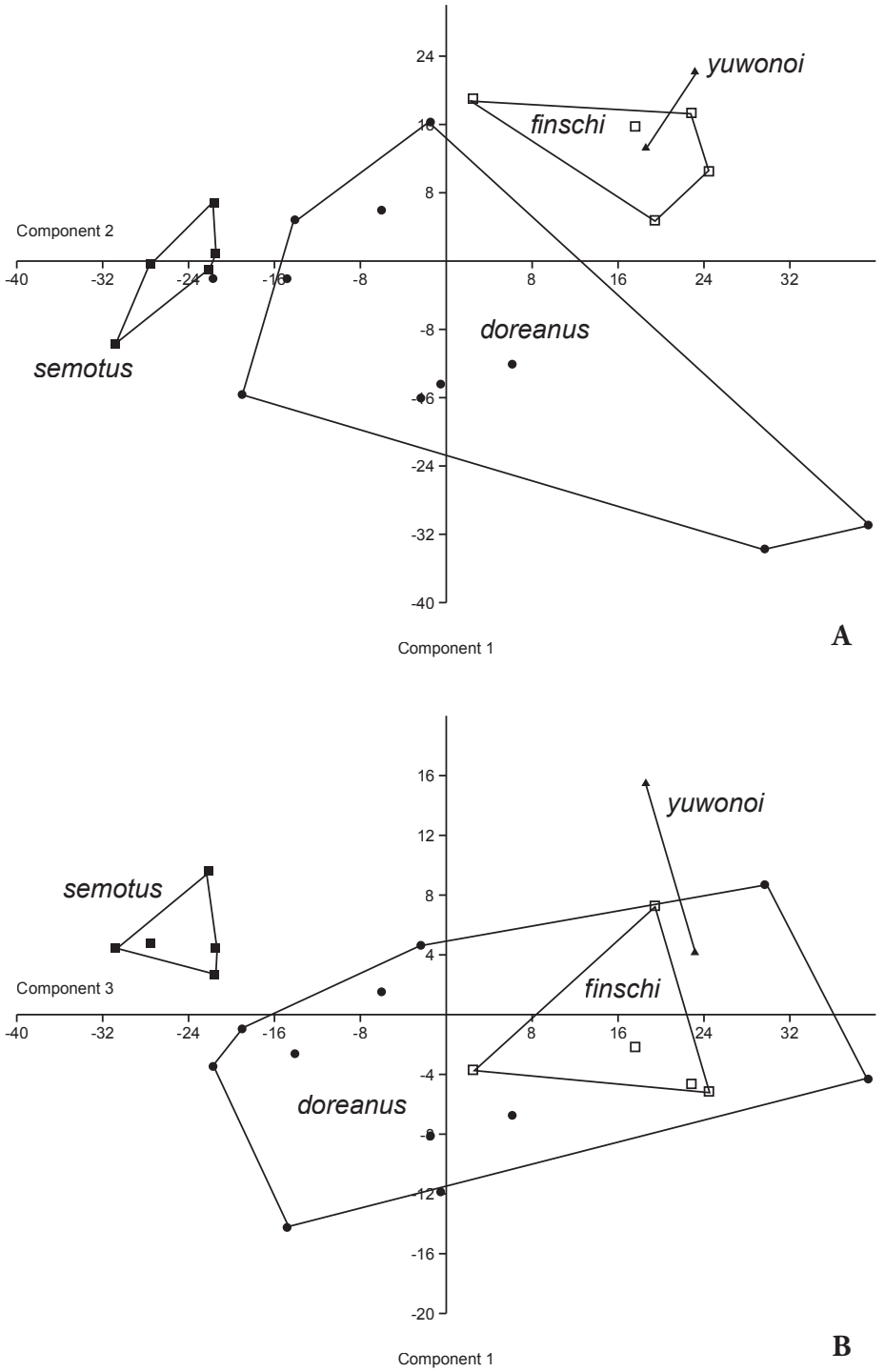

Figure 7. Principal Components Analysis of 9 scalation characters of the yellow-tongued monitors showing axis 1–2 (**A**) and 1–3 (**B**). Voucher information and scale counts are found in Appendix.

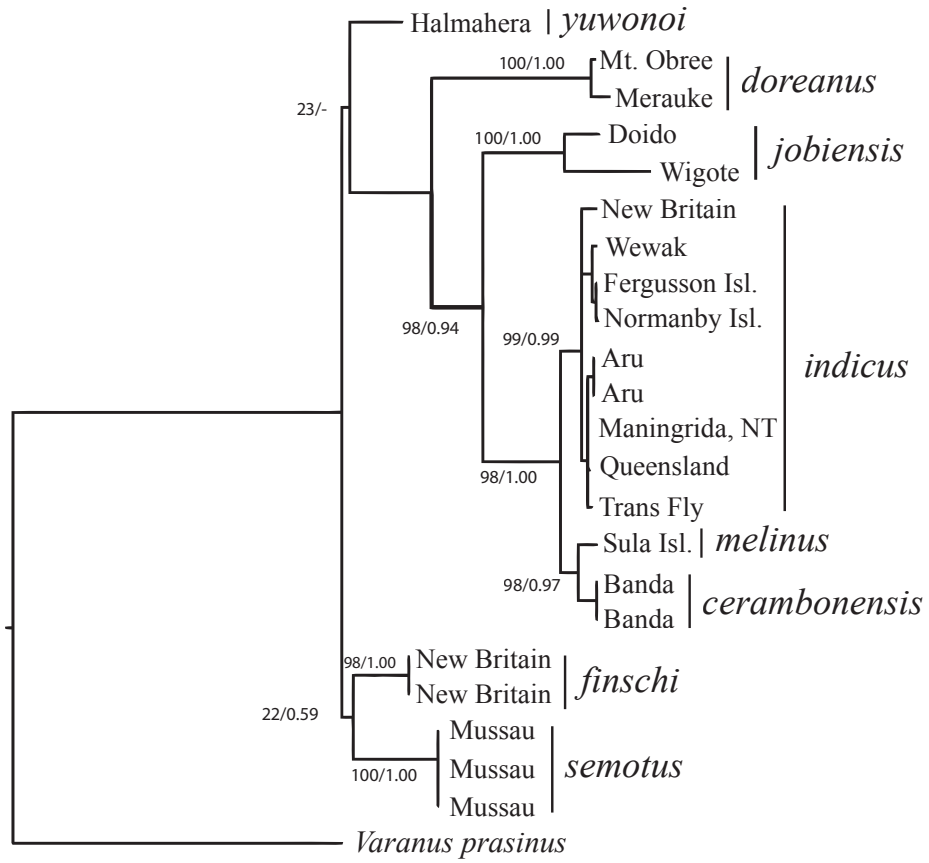

Figure 8. RaxML phylogeny of the Pacific monitors based on the combined mitochondrial *16S rRNA* and *ND4* regions; values show maximum likelihood bootstrap- and Bayesian posterior node support. Voucher information and GenBank accession numbers are presented in Table 2.

ND4 3rd codon positions with the following nucleotide substitution models respectively: TrN+G, HKY+I and TrN. Bootstrap proportions and Bayesian posterior probabilities strongly supported monophyly of conspecific sequences for each taxon where we had more than one sequence available (Fig. 8). Relationships between the taxa were also strongly supported for the most part except for the nodes placing *V. finschi*, *V. semotus* and *V. yuwonoi*, which effectively comprise a polytomy along with a clade comprising the remaining taxa.

A single haplotype was observed for the concatenated *16S rRNA* and *ND4* sequences among the three *V. semotus* sequenced. Net average uncorrected sequence divergence (*dA*) between *Varanus* sister species pairs for *ND4* ranged from 1.9% to 14.3% with a mean of 8.7% (Table 5). Net average uncorrected sequence divergence was 6.4% between *V. finschi* and *V. semotus* sp. n. and 2.3% between *V. cerambonensis* and *V. melinus*.

Table 5. Net average sequence divergence (*dA*) A) between sister species pairs of varanids and B) among members of the *V. indicus* species group.

A

Sister species pair	*dA (%)*
V. finschi-semotus sp. n.	6.4
V. cerambonensis-melinus	2.3
V. brevicauda-sparnos	13.4
V. eremius-sparnos	14.3
V. brevicauda-eremius	8.5
V. komodoensis-varius	12.5
V. mitchelli-semiremex	12.1
V. gouldii-rosenbergi	11.2
V. bushi-gilleni	6.6
V. pilbarensis-hamersleyensis	6.3
V. acanthurus insulanicus-baritji	1.9

B

Taxon	c	i	d	f	m	s	y	j
V. cerambonensis (c)	-							
V. indicus (i)	3.4	-						
V. doreanus (d)	11.5	11.4	-					
V. finschi (f)	11.7	11.1	11,4	-				
V. melinus (m)	2.3	3.6	12.3	12.9	-			
V. semotus (s)	10.2	10.2	11.5	6.4	11.1	-		
V. yuwoni (y)	11.1	11.7	11.7	7.0	12.6	6.7	-	
V. jobiensis (j)	6.8	6.2	9.4	8.6	7.4	8.3	8.9	-

Discussion

Biogeography. The members of the *V. indicus* species group have been extraordinarily successful at colonizing the islands of the SW Pacific. *Varanus indicus* and its closest relatives, which are adept at oversea dispersal, have reached most islands between the western Moluccas and eastern Solomon islands. The yellow-tongued monitors on the other hand have, been far less adept at oversea dispersal. *Varanus doreanus* populations are with few exceptions (such as Biak) restricted to the land bridge islands of New Guinea. *Varanus yuwonoi* to Halmahera, a geologically complex island which was much more closely associated with parts of western New Guinea during the Miocene and Pliocene (Hall 1998) when it may have been easier to colonize by monitors and other terrestrial animals. *Varanus finschi* likely reached the nearby New Britain through oversea dispersal as this island has no known historical landbridges to New Guinea. *Varanus semotus* is notable as it is separated from its closest relatives by hundreds of kilometers of open sea and must have colonized the oceanic Mussau Island through long distance oversea dispersal, most likely by rafting. Vidal et al. (2012) estimate the

age of *V. indicus* species group at around 6–11.5 mya. With this time reference the subsequent lineage diversification of species group should have occurred sometime in the late Miocene to early Pleistocene during which it is also likely that Mussau was colonized.

The St. Matthias group is situated on northern arc of the Bismarck Archipelago and has never had land connections to larger landmasses. It has three known endemic species of passerine birds; the Mussau monarch (*Symposiachrus menckei*), the Mussau triller (*Lalage conjuncta*) and the Mussau fantail (*Rhipidura matthiae*), but this number was most likely greater prior to human colonization (Steadman and Kirch 1998). There are no known native terrestrial mammals on Mussau but three still undescribed species of bats have recently been discovered (Flannery 1995, Aplin et al. 2015). Very little has been published on the herpetofauna of Mussau (e.g. Brown 1955, Mys 1988, Richards and Aplin 2015) and most of the recorded species are either widespread tramp species or endemics shared by Mussau and Manus. A recent (2014) faunal survey conducted by the Wildlife Conservation Society discovered a new endemic species of frog of the genus *Cornufer* (which constitute half of the known amphibian fauna). All nine species of skinks (single species of *Carlia*, *Eugongylus*, *Lamprolepis*, *Lipinia* and *Sphenomorphus* and 4 species of *Emoia*) recorded by the same expedition are widespread while one of the four species (2 *Gehyra*, 1 *Gekko* and 1 *Nactus*) of gekko (*Gehyra* sp.) is reported to be a new species endemic to Manus and Mussau Island (Richards and Aplin 2015). According to Richards and Aplin (2015) it is likely that additional species occur in the still unexplored fragments of primary forest of the interior. For now *V. semotus* is the only endemic lizard known from Mussau.

The absence of *Varanus indicus s.l.* which is otherwise almost universal on islands in the Southwest Pacific, including Manus and New Hanover, is more difficult to explain. The lack of widespread mangrove swamps around the coastlines seems an insufficient explanation as most island populations of Mangrove monitors are habitat generalists that occur in various coastal and inland habitat types (Weijola and Sweet 2015).

Varanus finschi. Virtually nothing has been published on the biology of *V. finschi* since its initial description over two decades ago. In 1988 SCD collected a specimen at Amelei on the south coast of New Britain (AMS 129614). In 2012 VW visually identified four and collected two specimens in the vicinity of Rabaul, Kokopo and Nodup at the northern end of East New Britain (ZMUT Sa186 & 190). These new samples allowed us to include the species in a larger molecular phylogeny of the *V. indicus group* for the first time. The samples of alleged *V. finschi* (BPBM 17250 & 19510) from Milne Bay Province used by Ziegler et al. (2007) were re-identified as *V. cf. jobiensis* (by VW). Examination of live specimens also showed that the tongue color of *V. finschi* is yellow rather than pink/light as reported earlier (Sprackland 1997, Harvey and Barker 1998). According to VW's field observations *V. finschi* is most numerous along the coast. Attempts to find monitors higher up in the Baining Mountains (500-700 m. elev.) were unsuccessful despite local testimonies of occasional observations. *Varanus indicus* is common along the coast and in the mangroves of New Britain and there appears to be at least partial habitat overlap between the two species.

Varanus finschi has been reported to have an extensive range outside of New Britain including New Ireland, New Guinea (Ziegler et al. 1999), northern Australia (Ziegler et al. 2001) and the Kei Islands (Philipp et al. 2004). However, as the only records from New Guinea (ZMB 18838 & 18839) and Queensland (NMW 12329-6 & 12429-8) are based on colonial-era museum vouchers without detailed collection information we consider them unreliable. The records for the Kei islands and New Ireland stem from misidentification of populations of *V.* cf. *indicus* with high scale-counts, pink tongue and similar dorsal pattern to *V. finschi* (Weijola pers. obs.). There is a single record from the Duke of York Islands (AMS R5618) but VW was not able to verify its occurrence there during a field survey in 2012. Thus, as far as we are aware, all verifiable records of *V. finschi* are from New Britain.

Conservation. The field observations indicate that *V. semotus* doesn't occur, or possibly only at low densities, in the highly degraded secondary forest/bush of large parts of the interior of the island. It is likely that the species occurred throughout Mussau prior to the large scale logging activities of the past three decades (Venter and Arihafa 2015). Thus the species is now mostly restricted to the coastal strip of a relatively small isolated island. Possible threats to the future survival of this species would be the introduction of cane toads which were widely established in the PNG islands during WW2 (Zug et al. 1975). According to unconfirmed accounts by locals they already occur on Emirau Island which also according to local inhabitants on Mussau lack monitor lizards. *Varanus semotus* is the only large-sized terrestrial generalist predator and scavenger on the island, and may well fill an important ecological function, making it of particular conservation concern. The new species is unusual inasmuch as it fills a role normally occupied by Mangrove monitors on isolated Pacific islands and it can well be considered a biogeographical oddity.

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Appendix

Scale counts of specimens included in the Principal Components Analysis.

Catalogue nr.	Locality	P	Q	S	T	X	XY	m	N	R
V. doreanus										
ZMA10193	Sabang	56	107	165	83	57	173	106	88	61
ZMA10194a	Noord R.	55	96	173	82	48	180	110	87	57
ZMA10199	Sermonai R.	43	103	161	92	39	161	95	88	67
ZMA12125	Hollandia	52	100	163	90	44	176	104	95	73
RMNH5164	Digoel R.	54	114	180	97	43	163	120	100	57
RMNH7035	Manokwari	52	102	171	94	40	164	118	97	60
RMNH21029	Gariau-lake	53	113	169	85	42	159	107	87	59
RMNH21051	Fak Fak	49	103	168	89	38	158	118	88	56
RMNH21055b	Manokwari	55	102	158	91	40	153	112	87	56
Mean		**52.1**	**104.4**	**167.6**	**89.2**	**43.4**	**165.2**	**110**	**90.8**	**60.7**
V. semotus										
ZMUT Sa176	Mussau	47	100	161	89	40	153	116	93	74
ZMUT Sa177	Mussau	47	97	162	87	39	147	114	89	66
ZMUT Sa178	Mussau	47	99	152	87	38	149	108	85	67
ZMUC 4272	Mussau	49	103	167	89	39	150	119	92	66
ZMUC 4273	Mussau	51	103	160	89	43	152	118	91	69
Mean		**48.2**	**100.4**	**160.4**	**88.2**	**39.8**	**150.2**	**115**	**90**	**68.4**
V. finschi										
ZMUT Sa186	New Britain	50	106	188	94	50	188	128	92	54
AMR5618	Duke of York	45	103	172	105	54	185	125	98	58
AMR129614	New Britain	49	121	181	99	46	187	131	100	57
MNHN 00 192	New Britain	48	108	174	97	46	165	129	100	45
MNHN 00 195	New Britain	48	108	184	99	51	179	129	98	54
Mean		**48**	**109.2**	**179.8**	**98.8**	**49.4**	**180.8**	**128.4**	**97.6**	**53.6**
V. yuwonoi										
Harvey & Barker (1998)	Halmahera	47	98	174	100	-	-	-	103	
Ziegler et al. (2007a)	Halmahera	53	108	188	101	45	184	137	-	
Mean		**50**	**103**	**181**	**100.5**	**45**	**184**	**137**	**103**	