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Occurrence of *Diopatra marocensis* (Annelida, Onuphidae) in the eastern Mediterranean

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Abstract

The present study deals with the presence of *Diopatra marocensis* in the eastern Mediterranean. This species is small-sized and inhabited muddy bottom near the opening of rivers or lagoons [salinity range: 33–39‰] in the Aegean and Levantine Seas, and reached a maximum density of 90 ind.m^{-2} in Mersin Bay. This species might be an alien species that was introduced from the East Atlantic (near Gibraltar) to the eastern Mediterranean via ballast water of ships, as it has never been reported from the western Mediterranean Sea.

Keywords

Polychaeta, Levantine Sea, Aegean Sea, Turkey

Introduction

The genus *Diopatra* Audouin & Milne-Edwards, 1833 is represented by 59 species world-wide (Paxton 2014) and only by two species (*Diopatra neapolitana* Delle Chiaje, 1841 and *Diopatra micrura* Pires, Paxton, Quintino & Rodrigues, 2014) in the Mediterranean Sea (Delle Chiaje 1841; Arias and Paxton 2014). *Diopatra*

neapolitana is a large species, maximally reaching a length of 39 cm in Izmir Bay (Dagli et al. 2005), 50 cm on the coast of France (Fauvel 1923) and 73 cm on the coast of Portugal (Pires et al. 2012). It occurs in vegetated and unvegetated muddy sand bottoms in the shallow-water benthic environments of the Mediterranean Sea and has ecological and economic importance in the region. It forms dense populations in organically enriched sediments (i.e. 198 ind.m^{-2} and 408 g.m^{-2} in the Aegean Sea) and has been overexploited by diggers for the fish bait trade (Dagli et al. 2005). The large and strong tube of this species, which partly protrudes from the sediment surface, acts as a sediment and substrate stabilizer that enhances local biodiversity (Thomsen and McGlathery 2005).

Diopatra neapolitana was previously regarded as a widely distributed species from the Atlanto-Mediterranean to the Indo-Pacific regions (see Dagli et al. 2005). After re-examinations of the specimens that were previously identified as *D. neapolitana* from Japan (Okuda 1938; Choe 1960) and Singapore (Tong and Chou 1996), it was revealed that they in fact belonged to *D. sugokai* Izuka, 1907 and *Diopatra claparedii* Grube, 1878, respectively (Paxton 1998), indicating its restricted distributional pattern. Since the study made by Paxton (1993), 14 new *Diopatra* species have been described in the world's oceans, some of which co-occurred with *D. neapolitana* such as *D. biscayensis* Fauchald, Berke & Woodin, 2012, *D. cryptornata* Fauchald, Berke & Woodin, 2012, *D. marocensis* Paxton, Fadlaoui & Lechapt, 1995 and *D. micrura* (Paxton et al. 1995; Fauchald et al. 2012; Pires et al. 2012)

During the biodiversity and pollution-monitoring projects performed along the coasts of Turkey between 2005 and 2012, we came across small individuals of a *Diopatra* species, especially in the eastern part of the Levantine Sea (in Iskenderun and Mersin Bays) that were first identified as *Diopatra* sp. (Çinar et al. 2012) and later as *D. marocensis*. The present study re-describes this species and gives some notes on its ecological and biological features.

Material and methods

Specimens of *Diopatra marocensis* were collected at 7 stations in the Levantine Sea (stations K41, 71, G11, 34, D13, D14 and 11) and at 4 stations in the Aegean Sea (stations 3, 15, G40, 37) between 2005 and 2012, using a Van Veen grab (sampling an area of 0.1 m^{-2}) and a dredge (stations D13 and D14) (Figure 1). Benthic material taken from stations was passed through 0.5 mm mesh and the retained material was fixed with 4% formaldehyde. In the laboratory, the material was washed with tap water, sorted under a stereomicroscope and preserved in 70% ethanol. The biometrical features of the *Diopatra* specimens such as the body length (excluding palps), width (excluding parapodia), H+10 (length comprising the head and the first 10 chaetigers) and the length of chaetae were measured by using an ocular micrometer. Drawings were made with the aid of a camera lucida. Specimens were deposited in the Museum of Faculty of Fisheries, Ege University (ESFM).

Figure 1. Map of the study area.

Description of species

***Diopatra marocensis* Paxton, Fadlaoui & Lechapt, 1995**
Figures 2–5

Diopatra marocensis: Paxton et al. 1995: 949–955, fig. 1; Fauchald et al. 2012: 51, fig. 2a–b.

Material examined. ESFM-POL/2009-196, 29.04.2009, Mersin Bay, near the opening of Seyhan River [salinity: 34‰], station 34, 36°43'33"N, 34°52'11"E, 9 m, mud; ESFM-POL/2005-1511, 1 September 2005, Kale, K41, near Beymelek Lagoon [salinity: 39‰], 36°14'29"N, 30°01'33"E, 5 m, gravelly sand, 1 specimen; ESFM-POL/2005-107, 10 September 2005, D13, near the opening of Ceyhan River [salinity: 39‰], 36°33'22"N, 35°34'17"E, 10 m, muddy sand, 8 specimens; ESFM-POL/05-295, 10 September 2005, İskenderun Bay, D14, near the opening of Ceyhan River [salinity: 38‰], 36°32'51"N, 35°34'37"E, 25 m, 3 specimens; ESFM-POL/2005-1396, 17 September 2005, Mersin Bay, G11, near the opening of Seyhan River [salinity: 38‰], 36°45'47"N, 34°51'54"E, 5 m, mud, 14 specimens; ESFM-POL/2005-2086, 8 October 2005, Kuşadası [salinity: 39‰], G40, 37°52'00"N, 27°15'27"E, 10 m, sandy mud, 3 specimens; ESFM-POL/2009-34, 4 February 2009, Mersin Bay, station 34, near the opening of Seyhan River [salinity: 38‰], 36°43'33"N, 34°52'11"E, 9 m, mud, 2 specimens; ESFM-POL/2009-300,

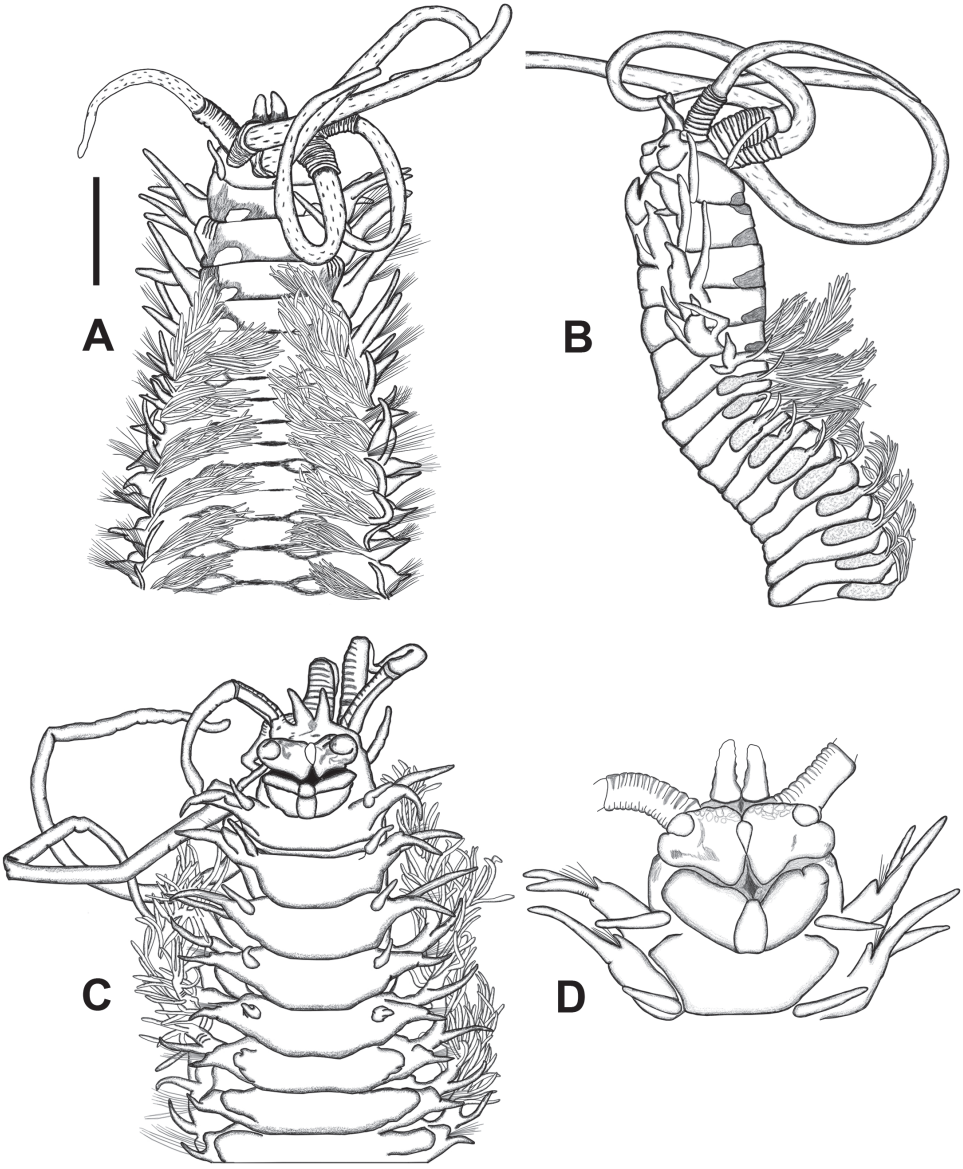

Figure 2. *Diopatra marocensis*. **A** Anterior part, dorsal view (ESFM–POL/2011–48) **B** Anterior part, lateral view **C** Anterior part, ventral view **D** Anterior end, ventral view. Scale bar: **A** = 2 mm, **B** = 1.80 mm, **C** = 1.88 mm, **D** = 0.73 mm.

29 April 2009, Mersin Bay, station 34, near the opening of Seyhan River [salinity: 34‰], 36°43'33"N, 34°52'11"E, 9 m, mud, 4 specimens; ESFM–POL/2009–281, 20 October 2009, Mersin Bay, station 34, near the opening of Seyhan River [salinity: 38‰], 36°43'33"N, 34°52'11"E, 10 m, mud, 2 specimens; ESFM–POL/2011–48, 23 August 2011, Enez, near the opening of Meriç River [salinity: 33‰], station 3,

40°43'41.5"N, 26°02'03.1"E, 4 m, sandy mud, 1 specimen; ESFM–POL/2011–256, 28 August 2011, near the opening of Bakırçay River [salinity: 39‰], station 15, 38°55'11"N, 26°58'50"E, 4 m, sandy mud, 1 specimen; ESFM–POL/2011–255, 1 September 2011, near the opening of Büyük Menderes River [salinity: 39‰], station 37, 37°32'39"N, 27°10'28"E, 5 m, sandy mud, 2 specimens (juvenile); ESFM–POL/2011–254, 10 September 2011, Mersin Bay, near the opening of Seyhan River, station 71 [salinity: 37‰], 36°47'00"N, 34°38'06"E, 6 m, sandy mud, 1 specimen; ESFM–POL/2012–1, 17 June 2012, Iskenderun Bay, Yumurtalık [salinity: 39‰], station 11, 36°50'27"N, 35°54'32"E, 12 m, mud, 6 specimens.

Description. All specimens incomplete, except for juvenile specimens from stations G40 and 37, and a mature specimen from station 71 (ESFM–POL/2011–254); having 27 mm body length, 1.3 mm body width (chaetiger 5) and 102 chaetigers. Large specimen incomplete, 35 mm long (H+10 = 5 mm), 2.1 mm wide, with 83 chaetigers. Body somewhat semicircular, anterio-ventral side flat in most specimens; a ventral groove in some highly contracted specimens. Anterior end including first 2 chaetigers larger than following chaetigers (Figures 2A, B, 5A). After chaetiger 15, cross section of segments becoming rectangular; ventrum of segments after chaetiger 15 with a vertical groove, extending back to posterior end. Dorsal side of prostomium, frontal lips, anterior faces of palpophores and antennophores with brown pigmentation (Figures 2A, 5A). In most specimens, a "w"-shaped brownish marking present on anterior side of prostomium (Figure 5C). Palpostyles and antennostyles with bar-shaped brownish pigments scattered on surface (Figure 2A, 5A). Body color pale brownish with a distinct dark brown color pattern on dorsal side of anterior segments (on first 20–25 chaetigers). Color pattern like eyeglasses, lying on posterior part of each segment upside down (Figure 2A, 5A). Only one specimen (ESFM–POL/2009–281) having irregular dark brown pigmentations on anterio-ventral side, other specimens without color markings on ventral side.

Antennae and palps covering dorsum of prostomium, emerging near posterior part of prostomium. One pair of pyriform, subulate or digitiform frontal lips on anterior part of prostomium; large specimen having an anomaly; with three frontal lips; on left side of prostomium, two lips attached with each other at base present. Upper lips, massive, medially distinctly separated; with a distinct, elongated papilla between lips (no ridge between halves); each lip cushion shaped, with large, bulbous distal papilla (Figure 2C, D, 5B). Mouth bordered by high ridges. Lower lips tripartite, with a triangular median section and paired high wings laterally (Figure 2D). Palps reaching posterior part of chaetiger 3. Tips of antennae missing in large specimen, reaching chaetiger 11 in other specimens. Palpophores with 10 rings; maximally 11. Antennophores with 12 rings. Palpophores and antennophores without lateral projections. One pair of small sphaerical eyes clearly discernable on juveniles, located near bases of lateral antennae (Figure 5C); due to dense pigmentation, eyes indiscernible in larger worms. Two large, crescentic nuchal organs present on posterio-dorsal sides of prostomium.

Peristomium shorter than first chaetiger, narrow on dorsal side, becoming larger laterally; ventral side as long as dorsal side. Peristomial cirri present, emerging ante-

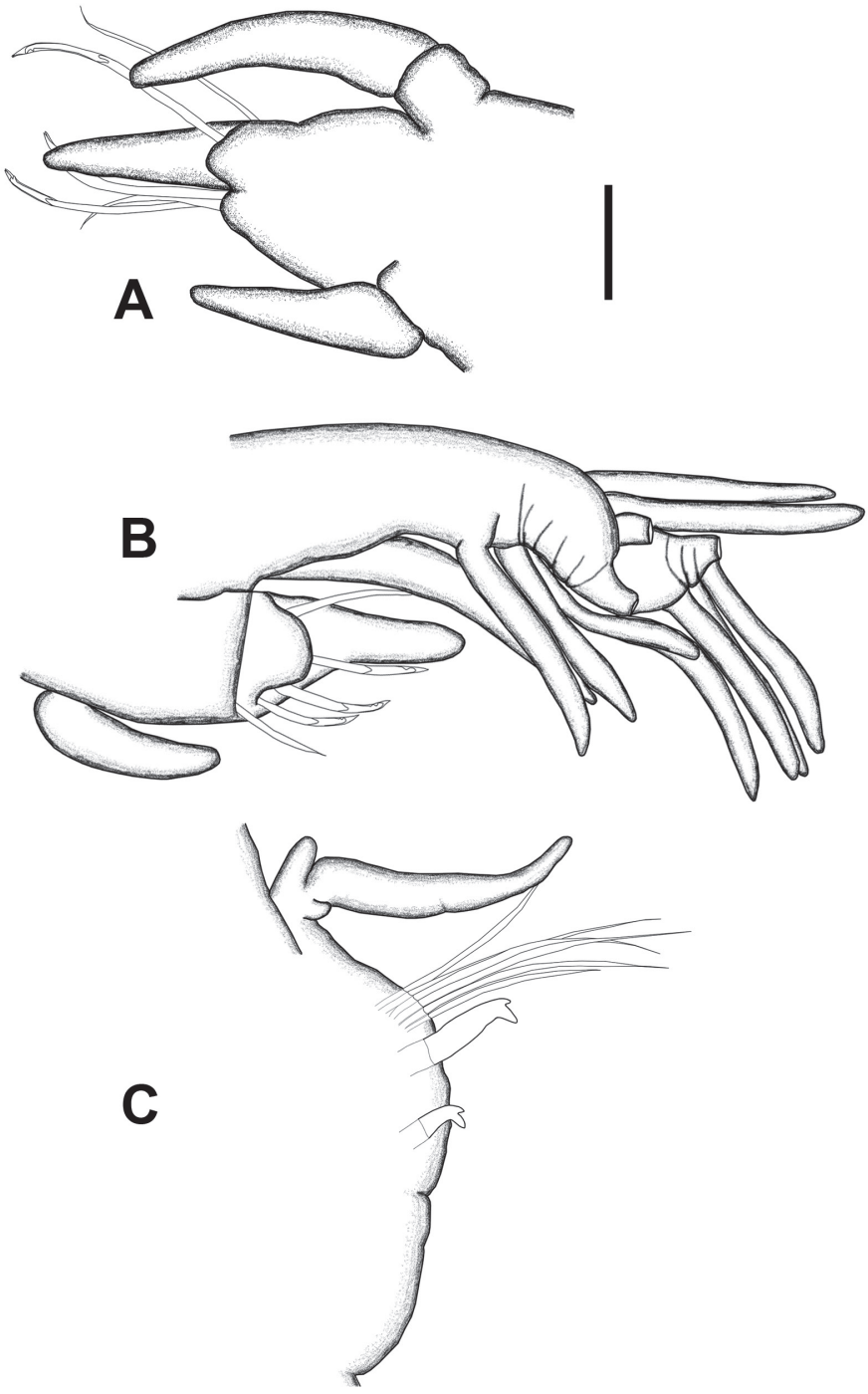

Figure 3. *Diopatra marocensis*. **A** Parapodium of chaetiger 1 (ESFM-POL/2009-300), anterior view **B** Parapodium of chaetiger 4, anterior view **C** Parapodium of chaetiger 43, anterior view. Scale bar: **A** = 200 μm, **B** = 96 μm, **C** = 173 μm.

rio-dorsal side of peristomium; digitiform, longer than peristomium, extending to posterior part of prostomium (Figure 2A). First four or five parapodia projecting laterally; from chaetiger 6 to end of body, parapodia mainly projecting dorsally. First four or five parapodia with distinct, thick proximal part, enlarging distally; posterior ones conical, decreasing in length, becoming short cones in last chaetigers. First parapodia located dorsally, projecting laterally, second to fifth parapodia placed and directed laterally. In first three chaetigers, postchaetal lobe digitiform, extending beyond tips of dorsal cirri (Figure 3A); following chaetigers with shorter postchaetal lobe (Figure 3B, C). Prechaetal lobe of chaetigers 1–3, bilobed; dorsal part larger than ventral one. Dorsal cirri digitiform, emerging on dorsal side of parapodia; cirrophores well developed.

Ventral cirri digitiform on chaetigers 1–4, emerging from posterio-ventral side of parapodia (Figure 3A, B); tips extending just beyond prechaetal lobe; those on chaetiger 5 shorter, more or less triangular with bulbous base; those on chaetiger 6 and remaining ones as thick glandular flattened pads. Pads becoming larger on chaetiger 18–20, then gradually decreasing in size and remaining as rounded swellings in posterior parapodia (Figure 3C).

Upper fascicle of chaetiger 1 with 2 slender limbate chaetae (Figure 4A), ca. 400 μm long; lower fascicle with 4 pseudocompound hooks; distinctly bidentate, distance between pseudocompound fracture and tip of chaeta maximally 90 μm long; having long (distance between tip of chaeta and tip of hood 15 μm long), pointed hoods (Figure 4B). One aciculum penetrating within dorsal cirri and three aciculae in parapodia; tips extending beyond postchaetal lobe, resembling short simple chaetae. Parapodia 2 with 2 limbate chaetae and 5 pseudocompound hooks; resembling those on chaetiger 1. Superior fascicle of chaetiger 4 with 2 limbate chaetae, ca. 325 μm long. Inferior fascicle of chaetiger 4 with 1 limbate chaeta (225 μm long) and 3 pseudocompound hooks; distance between pseudocompound fracture and tip of chaeta maximally 65 μm long. Unmodified parapodia with serrated limbate chaeta, pectinate chaeta (first appeared on chaetiger 6) and subacicular hook (first appearing on chaetiger 13). Chaetiger 13 with 8 serrated limbate chaetae, 2 pectinate chaetae and one subacicular hook; limbate chaetae maximally 175 μm long; pectinate chaetae with oblique tips, having 16–18 thin, equal teeth (Figure 4C); subacicular hook strongly bidentate, light amber-coloured, with truncated hood, becoming double from chaetiger 14 onwards (Figure 4D).

Branchiae starting from chaetiger 4 on majority of specimens; four specimens possessing first branchiae on chaetiger 5. Branchiae with more than 10 spiraled whorls of long filaments (15 spiraled whorls on large specimen); decreasing gradually in number of whorls and in size after chaetiger 12 (7 spiraled whorls on chaetiger 15, 3 spiraled whorls on chaetiger 30); having 2 filaments after chaetiger 35, one filament after chaetiger 37, becoming a short papilla between chaetiger 40 and 46, absent posteriorly.

Mandibles partly sclerotinized (proximal parts) with calcareous distal cutting plates, rounded tip with distal indentations. Maxillae distinctly sclerotinized along cutting edges; supporting structures barely sclerotinized. Maxillary formula: Mx I = 1 + 1; Mx II = 8 + 7; Mx III = 7 + 0; Mx IV = 7 + 9; Mx V = 1 + 1.

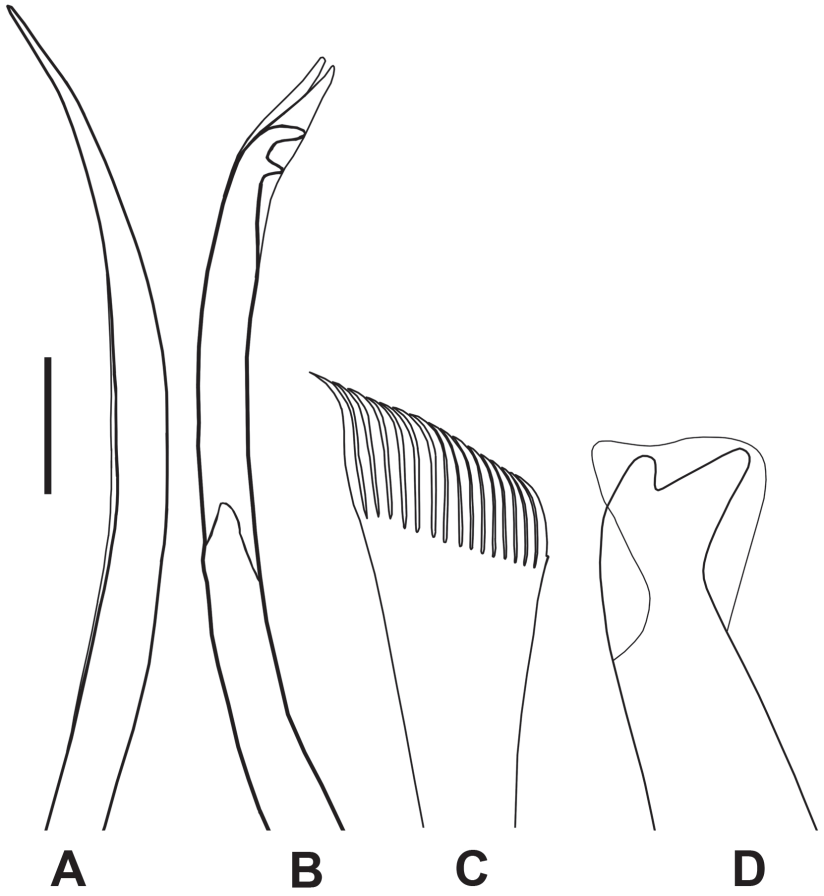

Figure 4. *Diopatra marocensis*. **A** Limbate chaeta, chaetiger 1 (ESFM–POL/2009–300) **B** Bidentate pseudocompound hook, chaetiger 1 **C** Pectinate chaeta, chaetiger 35 **D** Subacicular hook, chaetiger 35. Scale bar: **A** = 20 μm, **B** = 15 μm, **C** = 23 μm, **D** = 46 μm.

Pygidium with two pairs of pygidial cirri; ventral pair (0.5 mm) longer than dorsal pair (0.2 mm).

Tube parchment-like, cylindrical, surface covered by debris comprising mud with shell fragments of *Abra alba* (Wood W., 1802) and *Parvicardium exiguum* (Gmelin, 1791), and plant debris drifted from rivers.

Ecology. This species was found near the opening of rivers or lagoons [salinity range: 33–39‰] in the area between 4 and 25 m depth, and attained its highest density at station 34 (90 ind.m^{-2}).

Feeding. Gut content analysis of digestive tracks of some worms revealed that this species mainly feeds on algae.

Reproduction. One specimen has eggs in its coelomic cavity, measuring 200 μm in diameter on average. This species is known to be a simultaneous hermaphrodite, brooding large eggs (about 600 μm) in the parental tube (Pires et al. 2012; Arias et al. 2013).

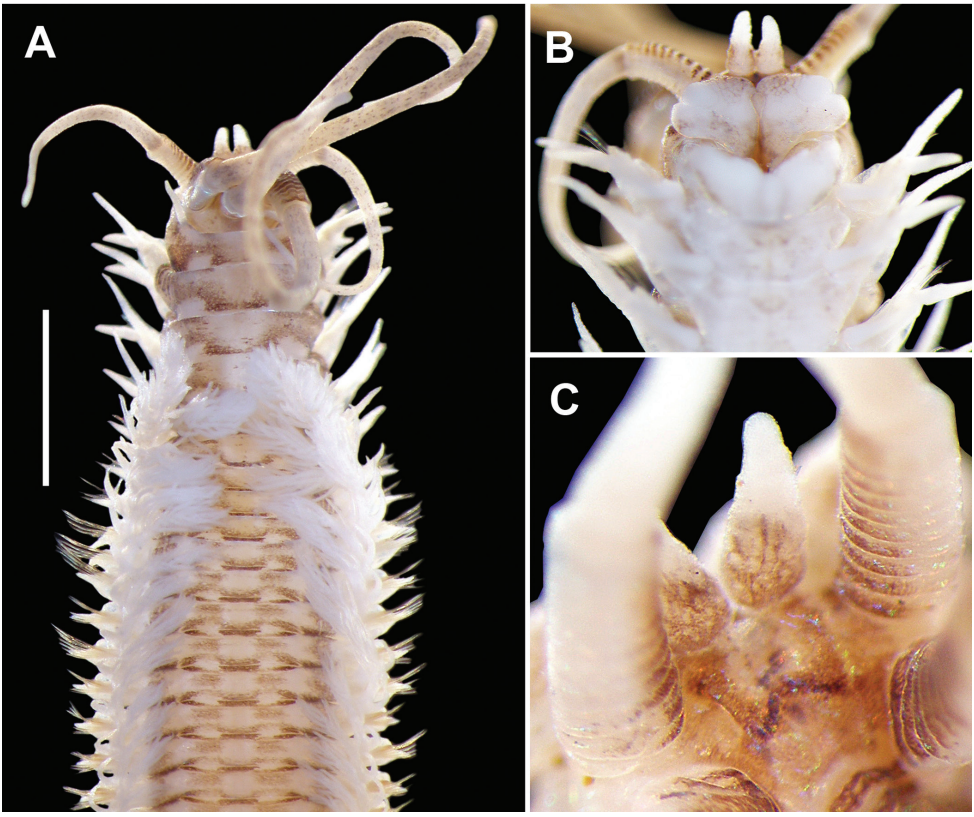

Figure 5. *Diopatra marocensis*. **A** Anterior part, dorsal view (ESFM–POL/2009–300) **B** Anterior end, ventral view **C** Anterior end, dorsal view. Scale bar: **A** = 2 mm, **B** = 1.18 mm, **C** = 0.18 mm.

Symbiotic relationship. Specimens of *Diopatra marocensis* collected from stations D14 and 34 were densely infected by a parasite (?Entoprocta). The parasite is attached to the dorsal side, parapodia and branchiae of the worms. One specimen (ESFM–POL/2009–34) with 15 chaetigerous segments (posterior part is missing) had almost 40 parasites. Arias et al. (2010) found a symbiotic relationship between specimens of *D. marocensis* and a peritricous protozoan (the genus *Epistylis*) from northern Spain.

Geographical distribution. This species is only known from the East Atlantic (near Gibraltar; Morocco, Spain and Portugal) and the eastern Mediterranean. The presence of this species in the eastern Mediterranean and absence in the western Mediterranean is interesting. There could be two reasonable explanations for its presence in the eastern Mediterranean; 1) this species might have been introduced to the area by ballast water of ships; 2) it might be an Atlanto-Mediterranean species that widely occurs near openings of river mouths in the area, but has been overlooked up to now, or misidentified as a juvenile specimen of the large Mediterranean species *D. neapolitana*. These explanations can be refuted or proved when more data regarding this species in different basins of the Mediterranean are accumulated. As there is a big gap between

the Atlantic and Mediterranean populations of this species, this species can be regarded as a new alien species for the Mediterranean Sea, at least for now.

Discussion. *Diopatra marocensis* can be distinguished from other *Diopatra* species (*D. neapolitana*, *D. marocensis*, *D. micrura*, *D. biscayensis* and *D. cryptornata*) reported from the north-eastern coast of the Atlantic Ocean and Mediterranean Sea, in having pectinate chaetae that have oblique combs with 16–18 thin teeth. The original description of *D. marocensis* differs from the re-description of the species based on the eastern Mediterranean specimens in having different pigmentation in the anterior end (generally pale, but small specimens having palps with irregular brownish specks, and peristomium and anterior chaetigers with mid-dorsal bars on the posterior part of segment) and branchiae with fewer numbers of whorls (max. 8–9 whorls in the original description vs. 15 in the eastern Mediterranean specimens). However, such characters of the eastern Mediterranean specimens were also noted on the Spanish specimens (H. Paxton, pers. comm.).

Acknowledgments

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A new species of *Harpacticella* Sars, 1908 (Copepoda, Harpacticoida), from a tidal pool on Jeju Island, Korea

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Abstract

A new species of the genus *Harpacticella* Sars, 1908 is described from a tidal pool on Jeju Island, Korea. *Harpacticella jejuensis* **sp. n.** is closely related to *H. itoi* Chang & Kim, 1991, with regard to the structure of P1 exp-1 and enp-1, the length of P1 exp-1 and exp-2, and the setal number of the P5 exopod in males. However, the new species is clearly distinguishable from *H. itoi* by the combined following characters: six setae on the P5 exopod in females, one naked seta on the inner margin of P1 exp-2, the short endopod of P1 compared to the exopod, and a naked long seta on the proximal inner margin of the P5 exopod of males. The mtCOI partial sequence is provided as a DNA barcode for the new species.

Keywords

Harpacticidae, taxonomy, zoogeography, DNA barcode, marine, new species

Introduction

Harpacticella Sars, 1908 is a genus of harpacticoid copepods, family Harpacticidae Dana, 1846. The genus has been reported from various habitats (fresh water, brackish water, and marine), mostly in Asian waters (Itô and Kikuchi 1977; Chang and Kim 1991), and has also been recorded from the Pacific Northwest, USA (Cordell et al. 2007).

The first *Harpacticella* species report was by G.O. Sars (1908) who examined sandy littoral sediment of Lake Baikal. He proposed a new genus for this species, based on a reduced number of antennule segments and a two-segmented antennary exopod. So far, six species in the genus *Harpacticella* have been reported (Wells 2007). Among these species, *H. inopinata* Sars, 1908, *H. paradoxa* (Brehm, 1924), and *H. amurensis* Borutzky, 1952 were described from freshwater, *H. lacustris* Sewell, 1924 and *H. itoi* Chang & Kim, 1991 from brackish water, and *H. oceanica* Itô, 1977 from the marine environment.

During a study of the harpacticoid community along the coast of Jeju Island of Korea, we collected a new species of *Harpacticella* from a tidal pool. Here, we describe the new species and provide a key to species in the genus *Harpacticella*. Partial mtCOI sequence was also obtained as a DNA barcode for the new species.

Materials and methods

Samples were collected by hand net (63 μm mesh-size) from a tidal pool on the coast of Jeju Island, Korea. Specimens were preserved in 99% ethanol. Specimens were dissected in lactic acid, and the dissected parts were mounted on slides with lactophenol mounting medium. Preparations were sealed with transparent nail varnish. All drawings were prepared using a drawing tube attached to an Olympus BX51 differential interference contrast microscope.

Descriptive terminology is adopted from Huys et al. (1996). Abbreviations used in the text are as follows: A1, antennule; A2, antenna; ae, aesthetasc; exp, exopod; enp, endopod; P1-P6, first to sixth thoracopod; exp (enp)-1(2,3), proximal (middle, distal) segment of a three-segment ramus; CR, caudal rami. Specimens were deposited in the National Institute of Biological Resources, Incheon, Korea (NIBR). Scale bars in figures are indicated in μm.

Molecular analysis. For DNA extraction, fixative materials (99% Et-OH) were removed from specimens by washing with distilled water, and DNA was extracted using a tissue DNA purification kit (COSMO GENETECH Co. Ltd., Korea). Amplifications were performed in 20 μl reactions volumes containing extracted tissue DNA, primers LCO-1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO-2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994), and PCR premix (BioNEER Co), using a TP600 thermal cycler (TAKARA). PCR conditions comprised initial denaturation at 94 °C for 300 s, followed by 40 cycles of denaturation at 94 °C for 60 s, annealing at 46 °C for 120 s, and extension at 72 °C for 180 s; a final extension step was then performed at 72 °C for 600 s. PCR products were evaluated by electrophoresing amplification products on a 1% agarose gel containing ethidium bromide. Purification of amplified products was performed using a PCR purification kit (COSMO GENETECH Co. Ltd., Korea), and both strands were sequenced using an ABI 3730XL sequencer (COSMO GENETECH Co. Ltd., Korea).

Systematics

Order Harpacticoida Sars, 1903
Family Harpacticidae Dana, 1846
Genus *Harpacticella* Sars, 1908

***Harpacticella jejuensis* sp. n.**
http://zoobank.org/77BE96F4-597C-44D0-A07E-03A4D993A5F2
Figs 1–6

Type locality. A tidal pool (33°13.949'N; 126°30.653'E) on Beophwan beach, Seoguipo-shi, Jeju Island, Korea.

Materials examined. Holotype: 1♀ (NIBRIV0000304111) in 70% ethanol from the type locality. Paratypes: 5♀♀ (NIBRIV0000304112) in 70% ethanol, 2♀♀ (NIBRIV0000304113 – NIBR0000304114) dissected on 11 and 10 slides, respectively, and 2♂♂ (NIBRIV0000304115 – NIBR0000304116) dissected on 11 and 2 slides, respectively. All from the type locality and collected by R. Jeong on 3 March 2013.

DNA-barcode (mtCOI) sequence and trace were submitted to GenBank (KM272559, 619 bp).

Description. Female. Total body length 720–810 μm (mean = 759 μm; n = 10, measured from anterior margin of cephalosome to posterior margin of caudal rami). Maximum width at posterior margin of cephalosome (mean = 380 μm; n = 10). Urosome gradually tapering posteriorly. Body surface armed with some sensilla (Figs 1A–B).

Prosome (Fig. 1A–B) 4-segmented, comprising cephalosome and 3 free pedigerous somites. P1-bearing somite fused to cephalosome. Cephalosome (Fig. 1A) with few sensilla and smooth posterior margin. Other prosomites with few sensilla on dorsal and lateral surfaces. Dorsal tegumental windows of elongated oval shape, one on cephalosome and one on three succeeding prosomites; 2 windows on genital double somite (Fig. 1A). Pleural areas well developed and rounded, without lobate posterolateral angles.

Rostrum (Fig. 1A–C) well developed, trapezoid with smooth anterior apex, clearly defined at base. Dorsal surface smooth with pair of sensilla at apical margin.

Urosome (Figs 1A–B, 2C) 5-segmented, comprising the P5 somite, genital double somite, 2 free abdominal somites, and anal somite. All urosomites with row of spinules ventrally. P5-bearing somite with smooth dorsal surface and row of spinules along lateral margins.

Genital double somite (Figs 1A–B, 2C, 7A) subdivided by distinct chitinous structure laterally indicating original segmentation. Genital field located ventrally near anterior margin with copulatory pore positioned medially. P6 as small protuberance bearing 1 plumose seta.

Anal somite (Fig. 2C, D) without anal operculum, but with well-developed pseudoperculum arising from penultimate somite.

Caudal ramus (Figs 1A–B, 2D) wider than long; seta I inserted at half length of caudal ramus, ventrolaterally; lateral seta II longer than seta I, inserted close to distal

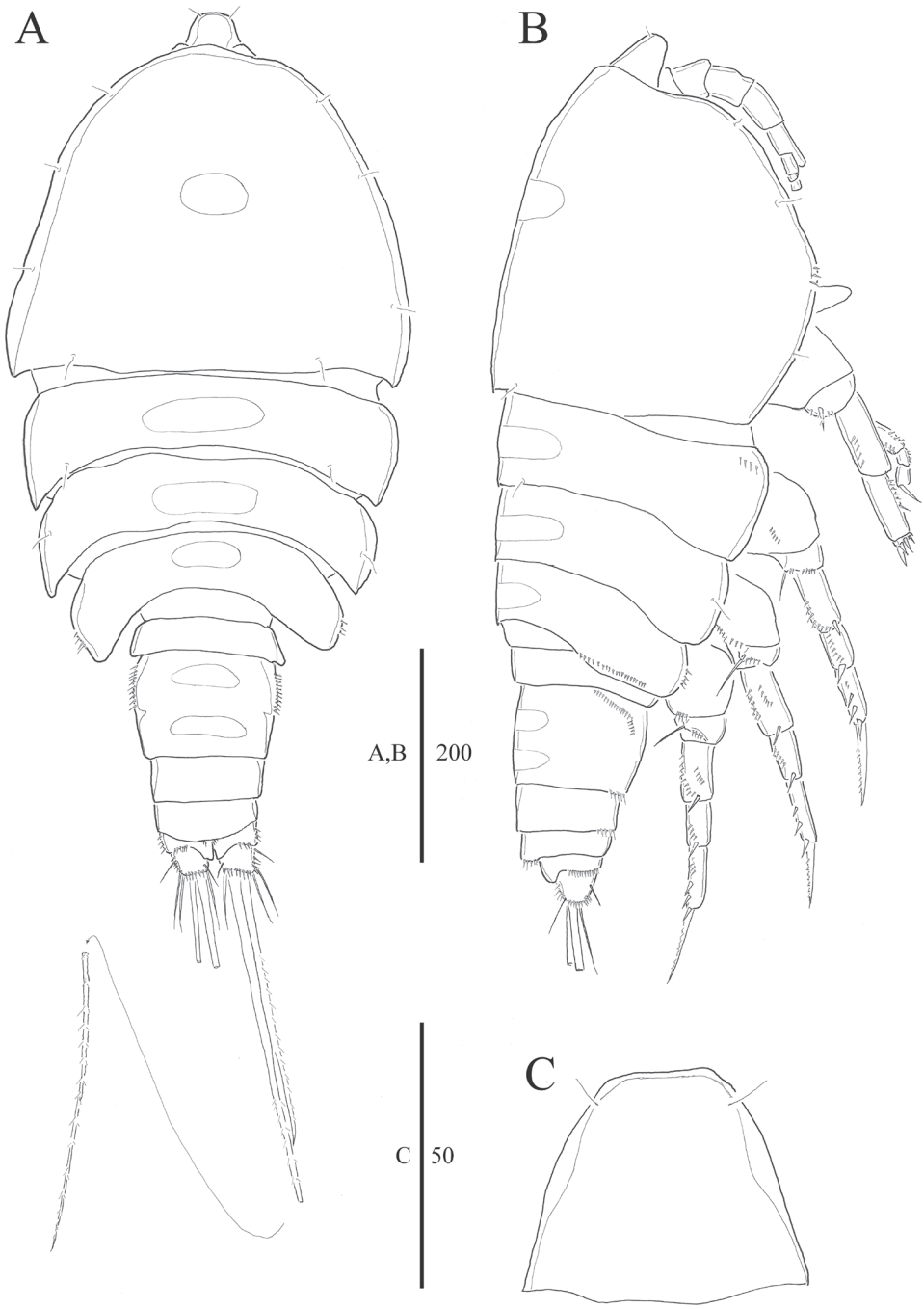

Figure 1. *Harpacticella jejuensis* sp. n. Female. **A** habitus, dorsal **B** habitus, lateral **C** rostrum. Scale bars in μm.

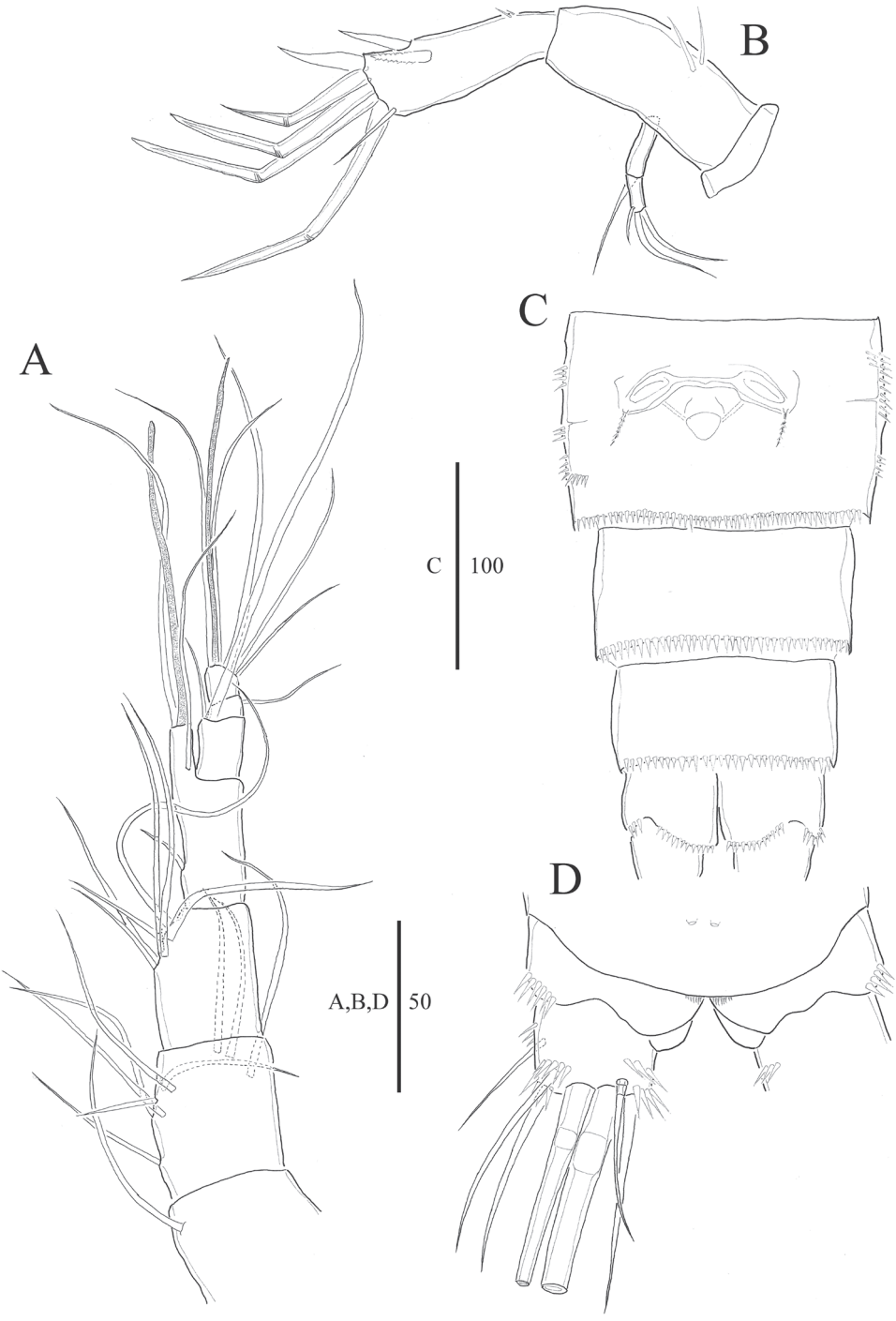

Figure 2. *Harpacticella jejuensis* sp. n. Female. **A** antennule **B** antenna **C** urosome (excluding P5-bearing somite), ventral **D** caudal rami, dorsal.

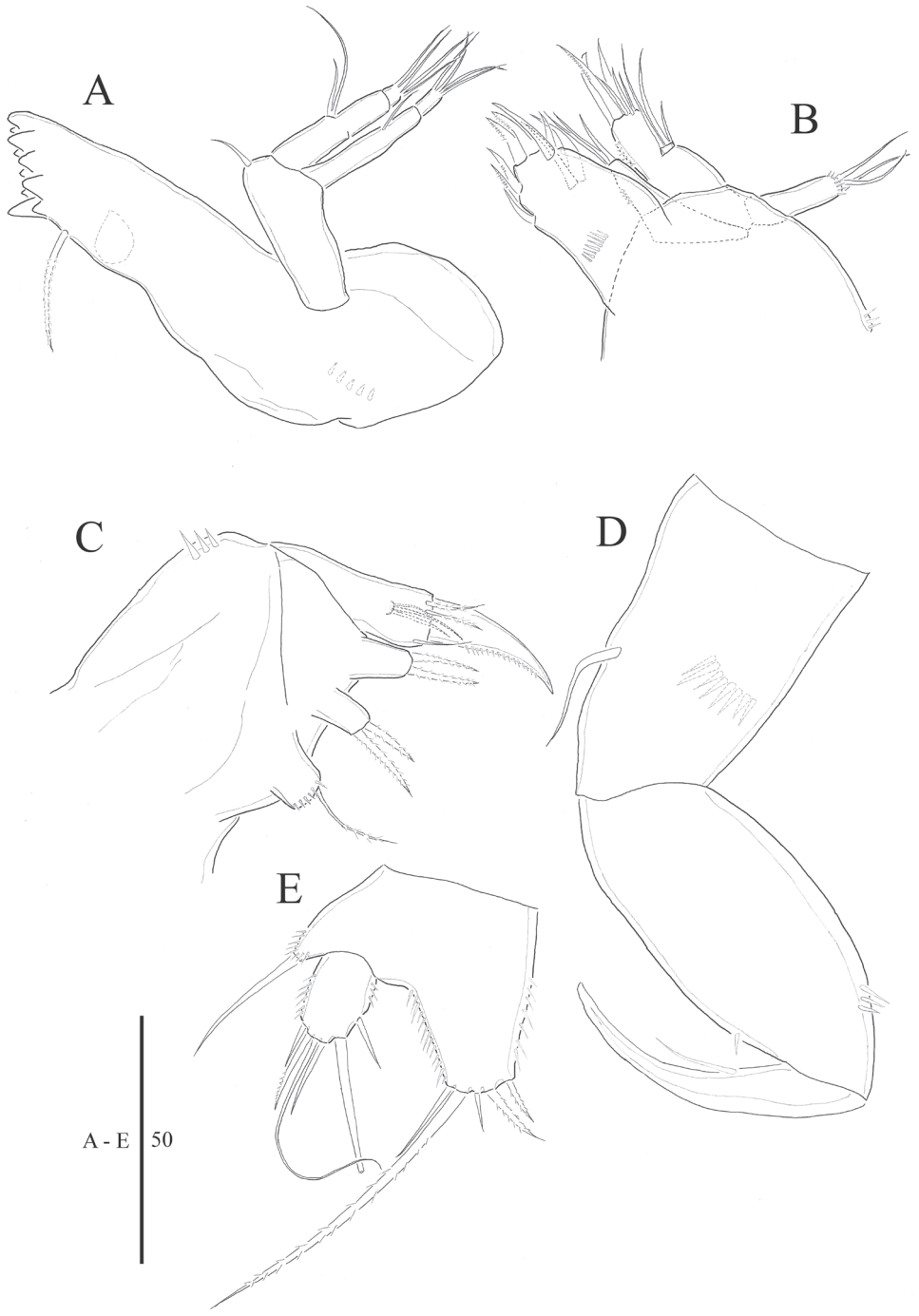

Figure 3. *Harpacticella jejuensis* sp. n. Female. **A** mandible **B** maxillule **C** maxilla **D** maxilliped **E** P5.

outer corner; seta III as long as lateral seta II; apical seta IV unipinnate, slightly longer than urosome; apical seta V bipinnate, as long as whole body; apical seta VI similar in length to seta III; dorsal seta VII bare and bi-articulate at its base.

Antennule 7-segmented (Fig. 2A). Aesthetascs on segments 4 and 7. All setae slender and bare. Armature formula: 1-[1], 2-[9], 3-[7], 4-[2 + (1+1 ae)], 5-[2], 6-[1], 7-[3+1 acrothek]. Apical acrothek consisting of well-developed aesthetasc fused basally to two slender naked setae.

Antenna (Fig. 2B) 3-segmented, comprising coxa, allobasis, 1-segmented endopod, and 2-segmented exopod. Coxa small, bare. Allobasis elongated with two spinules on surface. First exp segment with bare seta on distal end; second segment 3 bare setae. Endopod with 2 spinules near proximal area, pinnate spine on dorsal surface, naked spine laterally; apically bare spine; 4 geniculate spiniform setae and bare spiniform seta, apically.

Mandible (Fig. 3A) with large coxa and well-developed gnathobase; cutting edge with 10 blunt teeth overlapping each other; accessory plumose seta at dorsal corner. Mandibular palp well developed. Basis with naked seta on lateral distal margin. Endopod 2-segmented; enp-1 with 2 juxtaposed setae at the middle of inner margin; enp-2 with 4 juxtaposed setae on distal end. Exopod 2-segmented; exp-1 with naked seta at the middle of inner margin; exp-2 with 4 juxtaposed setae on distal end.

Maxillule (Fig. 3B) with praecoxa arthrite with spinular row near proximal area; 2 spines on anterior and 1 on posterior surface subterminally; 2 unipinnate spines and 1 bare spine apically, 2 unipinnate spines and bare spine on lateral margin. Coxa with endite bearing 3 naked setae apically. Basis endite with row of spinules laterally; 3 spines and 3 bare setae apically. Endopod small and 1-segmented with 3 bare setae. Exopod elongated, with spinules and 3 setae apically.

Maxilla (Fig. 3C): syncoxa with 3 endites: proximal endite with 1 plumose seta apically; medial and distal endite each with 2 pinnate spines; basis with unipinnated claw: accessory armature consisting of 2 pinnated setae and bare seta. Endopod represented by small protuberance with 3 bare setae.

Maxilliped (Fig. 3D): syncoxa with bare seta and oblique row of spinules. Basis with spinule on inner margin and 3 spinules on outer margin. Endopod 1-segmented, forming strong spine with seta.

Swimming legs 1–4 (Figs 4A–B, 5A–B) biramous, P1–P4 with 3-segmented exopod and 3-segmented endopod; spinules along inner and outer margins as illustrated. Intercoxal sclerites well developed.

P1 (Fig. 4A): coxa with row of spinules along outer lateral margin. Basis shorter than wide, with strong outer seta; inner spine inserted near inner distal corner, with several spinules. Endopod shorter than exopod reaching about half of exp-2; end-1 with plumose seta at the middle of the inner margin; enp-2 small without ornamentation but a row of spinules along outer lateral margin; enp-3 longer than wide with pinnate claw distally and inner naked seta on distal inner margin. Exp-1 as long as exp-2, with spinules along outer margin and spine on distal outer corner; exp-2 with short naked seta on distal part of inner margin and pinnate spine at middle part of the outer margin; exp-3 short with 4 curved pinnate claws and bare seta.

P2 (Fig. 4B): coxa with 2 rows of spinules along outer lateral margin. Basis shorter than wide, with slender outer seta and spinules on outer lateral margin. Endopod as long as exopod; row of spinules along outer margin of each segment. Exopodal segments with row of spinules along outer margin; exp-1 with 2 rows of spinules and pore on anterior surface; exp-2 with pore on anterior surface.

P3 (Fig. 5A): coxa with 2 rows of spinules along outer lateral margin. Basis shorter than wide, with slender outer seta and spinules along outer lateral margin. Endopod reaching to middle of exp-3; row of spinules along outer margin of each segment. Exopodal segments with row of spinules along outer margin; exp-1 with 2 rows of spinules and pore on anterior surface; exp-2 with pore on anterior surface.

P4 (Fig. 5B): coxa with 2 rows of spinules along outer lateral margin. Basis shorter than wide, with slender outer seta and spinules on outer lateral margin. Endopod shorter than exopod, reaching to proximal half of exp-3; row of spinules along outer margin of each segment. Exopodal segments with row of spinules along outer margin; exp-1 with 2 rows of spinules and pore on anterior surface; exp-2 with pore on anterior surface.

Armature formulae as follows:

Thoracopod	Exopod	Endopod
P1	0.1.050	1.0.110
P2	1.1.223	1.1.221
P3	1.1.323	1.1.321
P4	1.1.323	1.1.221

P5 (Fig. 3E): exopod and baseoendopod well separated. Baseoendopod with slender and bare outer lateral seta. Endopodal lobe larger than exopod and extended beyond distal margin of exopod; with 3 pinnate and 2 bare setae. Exopod oval, with 6 setae; rows of spinules along inner and outer margins.

Male. Total body length of examined samples 631–650 μm (mean = 643 μm; n = 5, measured from anterior margin of cephalosome to posterior margin of caudal rami). Greatest width at posterior margin of cephalosome. Cephalosome with sensilla along lateral margin. Other prosomites also with sensilla along lateral margin (Fig. 6A).

Prosome (Fig. 6A), 4-segmented, comprising cephalosome bearing first pedigerous somite and 3 free pedigerous somites. Cephalosome slightly narrower than in female, with smooth posterior margin. Prosomites 3 and 4 with some spinules along lateral proximal margin. Dorsal tegumental window elongated oval shape on cephalosome, three succeeding prosomites, and two genital somites (Fig. 6A). Rostrum well developed with pair of sensilla.

Urosome (Figs 6A, C, 7E) 6-segmented, with P5-bearing somite, genital somite, and 4 free abdominal somites. Free abdominal somites with rows of spinules ventrally. Caudal setae as in female. Sexual dimorphism in A1, P5, P6, and genital field.

Antennule (Fig. 6B) 6-segmented, chirocerate; geniculation between segment 5 and 6, segment 5 swollen and largest. Aesthetasc on segments 5 and 6. All setae slender and bare. Armature formula: 1-[1], 2-[1], 3-[8], 4-[9], 5-[7+(1+ae)], 6-[2+acrothek]. Apical acrothek with aesthetasc and 2 bare setae.

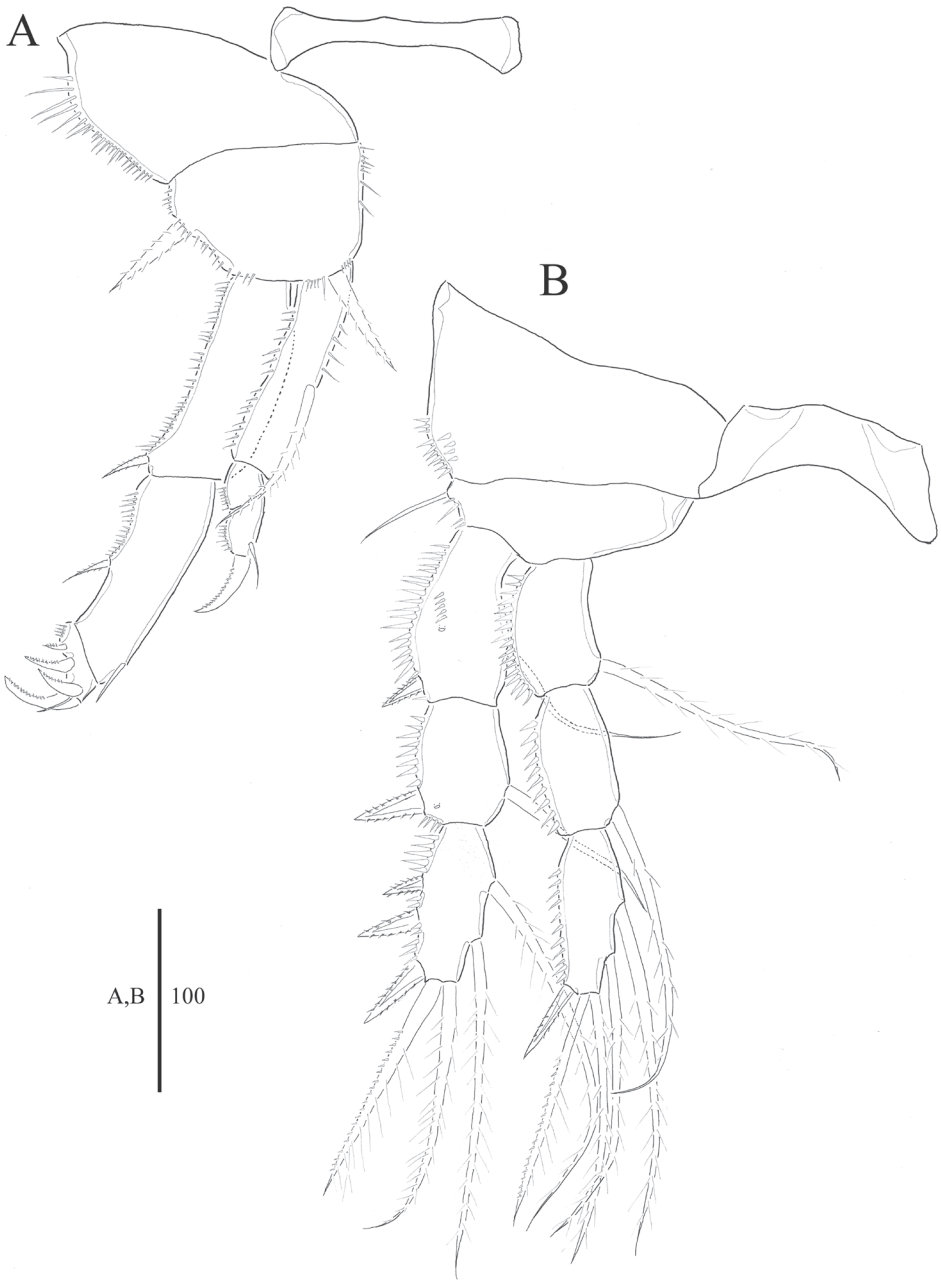

Figure 4. *Harpacticella jejuensis* sp. n. Female. **A** P1 **B** P2.

P5 (Figs 6C, D, 7D), baseoendopod fused medially, forming large transverse plate; each of them with slender and bare outer lateral seta. Exopod quadrangular, with 4 bare setae: inner, 2 terminal, outer bare setae.

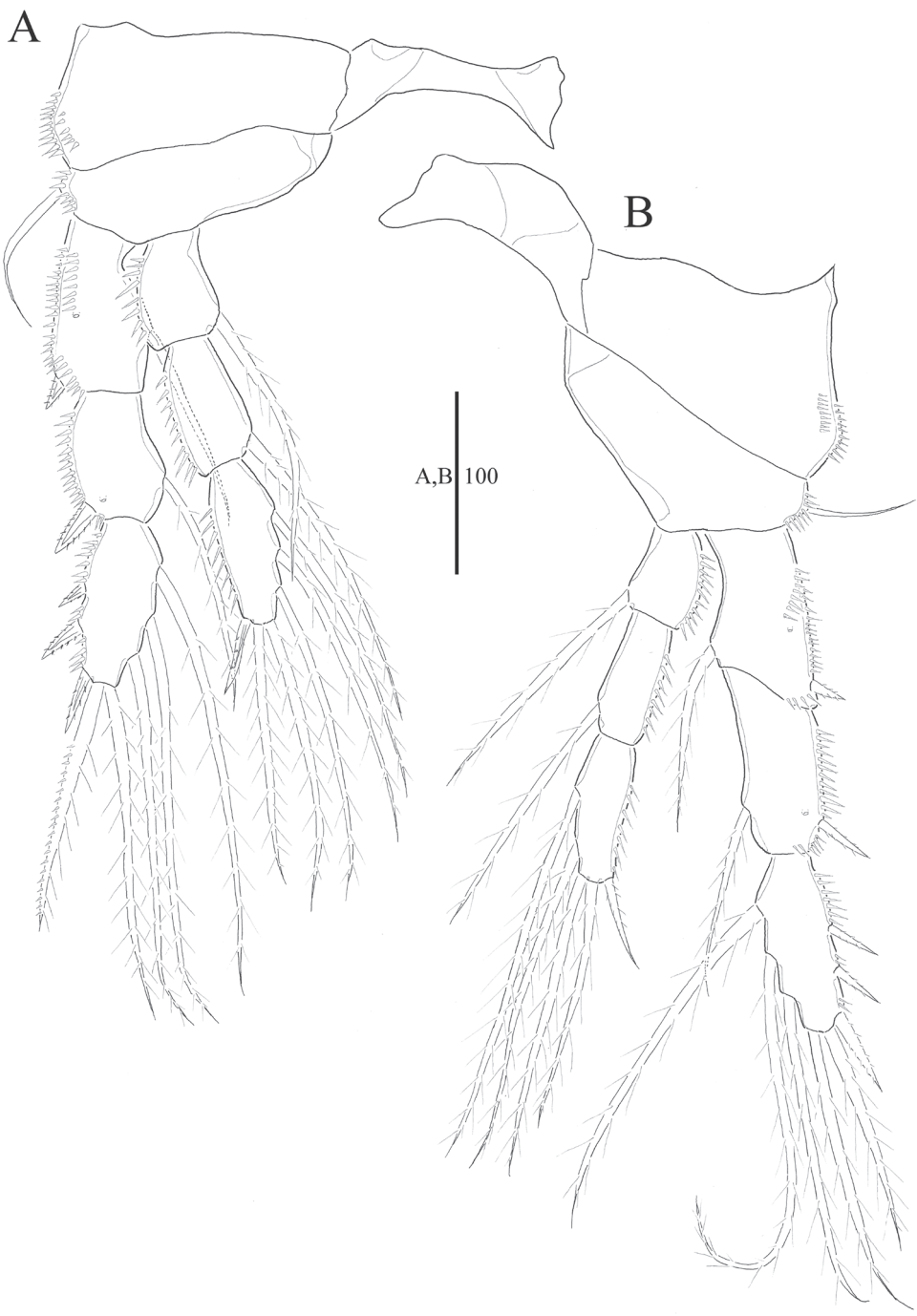

Figure 5. *Harpacticella jejuensis* sp. n. Female. **A** P3 **B** P4.

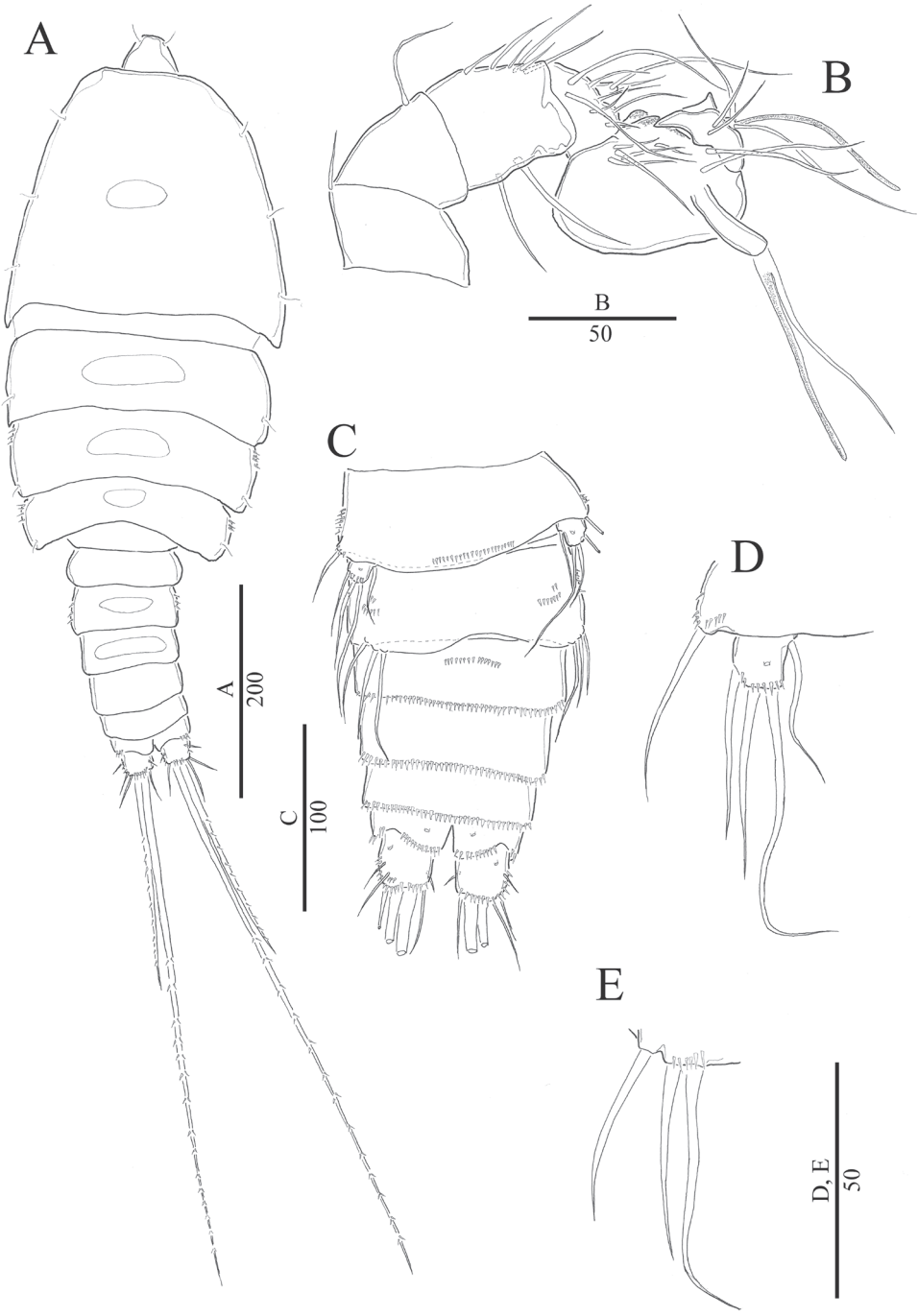

Figure 6. *Harpacticella jejuensis* sp. n. Male. **A** habitus, dorsal **B** antennule **C** urosome, ventral **D** P5 **E** P6.

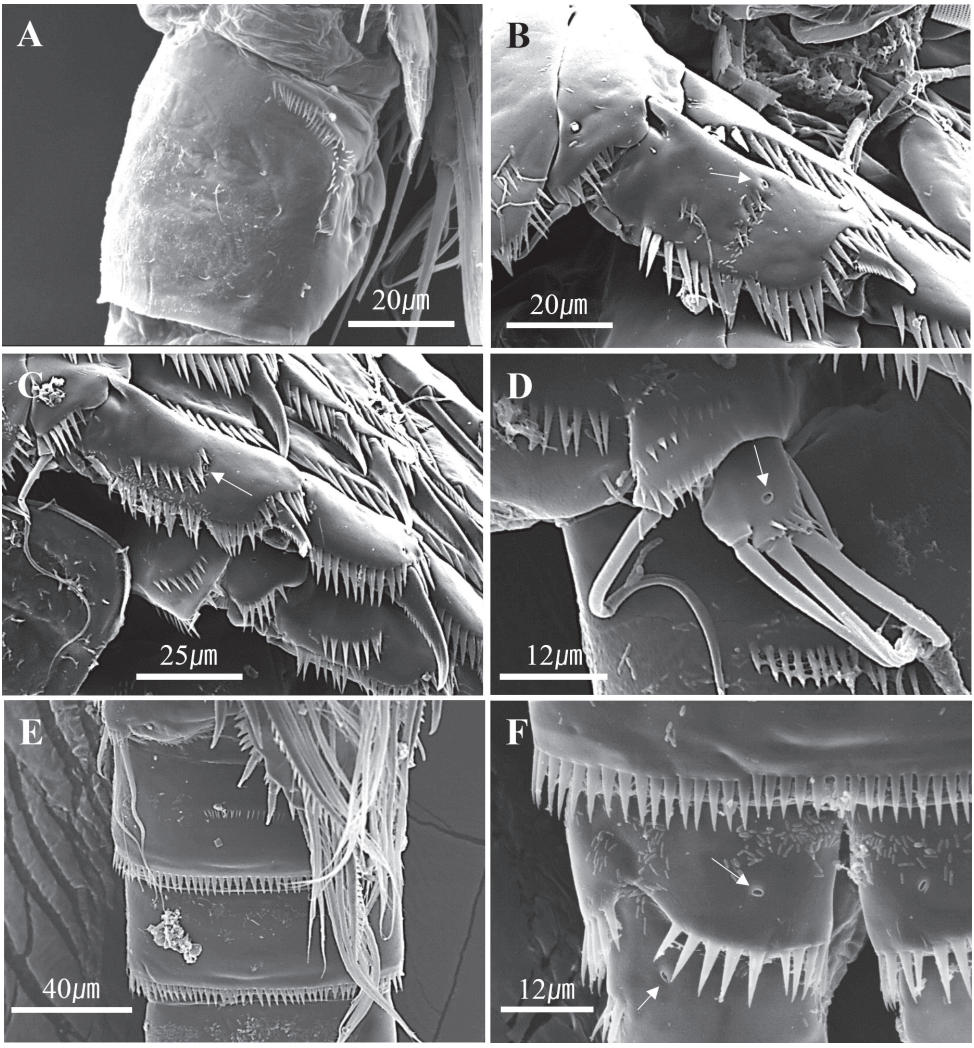

Figure 7. *Harpacticella jejuensis* sp. n. Scanning electron micrographs. Female: **A** genital double somite, lateral; Male. **B** P2 exp-1 **C** P3 exp-1 and 2 **D** P5 exp **E** 2 and 3 free abdominal somites, ventral **F** anal somite, ventral. Arrow indicates a pore on the surface.

P6 (Fig. 6C, E): small plate with 3 bare setae; inner seta longest, outer seta shortest; row of spinules near base of setae.

DNA barcode. mitochondrial oxidase subunit I; partial cds; 619 bp

ACTTTATATCTTTTAAGGGGGATATGAGCGGGAGT-
TATGGGGGCGGCAATAAGAGTTATTATTCGGCTTGAATTAGGACA-
GCCTGGGACTTTAATTAAGGATGAGCAAATTTATAATGTTTTAGT-
GACTTCGCATGCTTTTATTATAATTTTCTTTATGGTTATACCAATTT-
TAATTGGGGGGTTTGGAAACTGGTTAGTTCCTTTAATATTAG-
GAGCTCCTGATATGGCCTTTCCTCGATTAAATAATTTGAGATTCT-

GATTTTTGATGCCCTCTCTTATATTAATAATTATTAGAAGAGTTGTT-
GAAGGCGGGGCAGGGACAGGGTGAACTGTTTACCCCCCTTTAAGAA-
GAAATTTAGCACATGCAGGAGGCTCGGTGGATTTAGTAATTTTTTCTT-
TACATTTAGCAGGAGTTTCTTCCTTATTAGGGGCTGTAAATTTTATT-
AGGACTTTAAGAAATCTTCGAGTATTCGGGATGTATTTTGACCAAGT-
GCCGTTATTTTGTTGATCTGTCTTGGTTACAGCTGTTCTATTACTTT-
TATCACTGCCTGTATTAGCGGGGGCAATTACTATATTGTTGACCGATC-
GAAACATTAATTCAAGCTTCTATGATGTTA

Etymology. The specific name refers to the type locality of Jeju Island, Korea.

Discussion

The new species clearly fits in the genus *Harpacticella* based on the combination of following character sets: a) 7-segmented antennule in the female, b) 2-segmented antennary exopod, c) 3-segmented P1 endopod and exopod, d) only one seta on the inner edge of P2 enp-2 and e) spiniform outer spine of exp-3 P3 and P4 (Table 1). *Harpacticella jejuensis* sp. n. is closely related to *H. itoi* Chang & Kim, 1991 based on the length of P1 exp-1 and enp-1, the length of P1 exp-1 and exp-2, and the four setae on the P5 exopod of males. However, *H. jejuensis* can be distinguished from *H. itoi* by the following distinctive characters: (1) six setae on the P5 exopod of females (Fig. 2E) compared to five setae in *H. itoi* (see Fig. 3A; Chang and Kim 1991; this character is a unique character within the genus); (2) one bare seta on inner margin of P1 exp-2 (Fig. 4A), which is absent in *H. itoi* (see Fig. 1C; Chang and Kim 1991); (3) P1 endopod much shorter than exopod (ratio = 0.64:1), but in *H. itoi* it is as long as exopod (see Fig. 1C; Chang and Kim 1991); (4) naked seta on proximal inner margin of male P5 (Fig. 6D), but plumose-type in *H. itoi* (see Fig. 3E; Chang and Kim 1991); (5) naked seta on proximal inner margin of male's P5 is three times longer than length of male's P5 exopod (Fig. 6D), but it is almost two times longer in *H. itoi* (see Fig. 3E; Chang and Kim 1991).

Harpacticella species have a wide distribution ranging from freshwater to true marine environments, and have been found in Asian waters, American waters, and the Aldabra Atoll in the Indian Ocean (Fig. 8). All species in this genus except *H. amurensis* Borutzky, 1952 and *H. inopinata* Sars, 1908 have been recorded from at least two localities; the former two species have been recorded only in the type locality (Itô and Kikuchi 1977; Evstigneeva 1993). *H. itoi* Chang & Kim, 1991 has been found in several locations in the southeastern part of Korean peninsula and *H. oceanica* Itô, 1977 was documented in Korean and Japanese marine waters (Itô 1977; Chang and Kim 1991; Song and Chang 1993). *H. lacustris* Sewell, 1924 has a discontinuous distribution and has been recorded in India, China, and Japan (Sewell 1924; Wells and McKenzie 1973; Ishida 1989b). *H. paradoxa* (Brehm, 1924) is the most ubiquitous species; it has been recorded in China, Japan, and the northwest coast of the USA (Brehm 1924; Pesta 1930; Itô and Kikuchi 1977; Ishida 1987; Ishida 1989a; Ishida

Table 1. Morphological comparison of species in the genus *Harpacticella* Sars, 1908.

Characters	*H. amurensis*	*H. inopinata*	*H. itoi*	*H. lacustris*	*H. oceanica*	*H. paradoxa*	*H. jejuensis*
A2							
No. of exp segments	1	2	2	2	2	2	2
No. of exopodal setae	3	3	4	3	4	4	4
Md							
No. of basal setae	unknown	2	1	2	2	1	1
P1							
Length of exp / enp	1.3	1.4	1.3	1.3	1.2	1.5	1.5
Length of exp-1 / enp-1	1.1	1.4	0.9	0.9	0.9	1.1	1.1
P2							
Length of exp / enp	1.2	1	1	1.1	1.2	1	1
Exp	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323
Enp	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221
P3							
Exp	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323
Enp	1.1.321	1.1.321	1.1.321	1.1.321	1.1.321	1.1.321	1.1.321
P4							
Exp	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323	1.1.323
Enp	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221	1.1.221
P5 female							
No. of exopodal seta	4	5	7	5	5	5	6
Length of exp / enp	0.6	0.6	0.7	0.7	0.4	0.4	0.7
P5 male							
No. of exopodal seta	unknown	3	4	3	4	3	4
Body size (μm)							
Female	700	800	650	650	620	850	780
Male	unknown	700	530	550	560	unknown	650
Type locality	Amur River (Borutzky 1952)	Lake Baikal (Sars 1908)	Tamjin River, Korea (Chang and Kim 1991)	Chilka Lake near Calcutta, India (Sewell 1924)	Bonin Island, Japan (Itô 1977)	Talifu Lake in Yunnan Province, China (Brehm 1924)	Seogwipo in Jeju Island, Korea

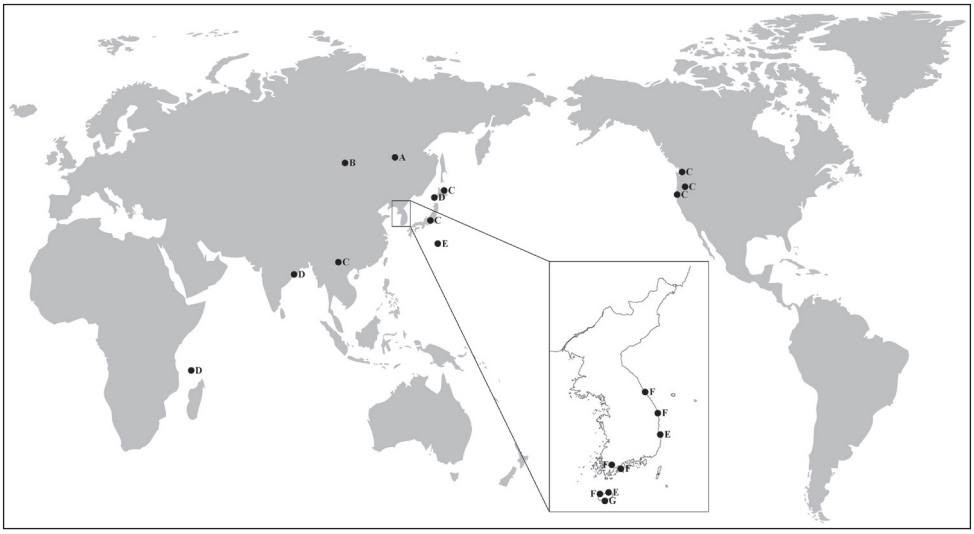

Figure 8. Distribution of *Harpacticella* species. **A** *H. amurensis* **B** *H. inopinata* **C** *H. paradoxa* **D** *H. lacustris* **E** *H. oceanica* **F** *H. itoi* **G** *H. jejuensis* sp. n.

2003; Ishida et al. 2004; Cordell et al. 2007). Cordell et al. (2007) suggested that the introduction of *H. paradoxa* may have been due to anthropogenic factors such as ballast waters. Small marine invertebrates have been shown to be introduced into new marine ecosystems via ballast water (Orsi and Ohtsuka 1999). Recently, molecular approaches have been used to determine the origin of these invasive organisms (Le Roux and Wieczorek 2009; Simon et al. 2011)

DNA barcoding is an efficient tool to identify species, especially morphologically similar species (Floyd et al. 2002; Hebert et al. 2003; Guidetti et al. 2005; Bhadury et al. 2006). This barcode can also be used for biogeographical analysis of invasive or widely distributed species (Garrick et al. 2004). We obtained a 619-bp partial sequence of mtCOI (KM272559) for use in future studies; no sequences have been obtained from congeners to date, even though it would be interesting to determine the phylogenetic relationships among congeners based on analysis of mtCOI sequences.

Key to Species in the Genus *Harpacticella*, 1908

1	A2 exp with 3 setae	**2**
–	A2 exp with 4 setae	**4**
2	A2 exp 1-segmented	***H. amurensis***
–	A2 exp 2-segmented	**3**
3	P1 exp-1 much longer than P1 enp-1	***H. inopinata***
–	P1 exp-1 as long as P1 enp-1	***H. lacustris***

4	Md basis with 1 seta; P2 exp as long as P2 enp **5**
–	Md basis with 2 setae; P2 exp longer than P2 enp..... ***H. oceanica***
5	P5 exp of female with 5 setae; P5 exp of male with 3 setae..... ***H. paradoxa***
–	P5 exp of female with 6 setae; P5 exp of male with 4 setae.. ***H. jejuensis* sp. n.**
–	P5 exp of female with 7 setae; P5 exp of male with 4 setae..... ***H. itoi***

Acknowledgements

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Taxonomic revision of *Aegista subchinensis* (Möllendorff, 1884) (Stylommatophora, Bradybaenidae) and a description of a new species of *Aegista* from eastern Taiwan based on multilocus phylogeny and comparative morphology

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Abstract

Aegista subchinensis (Möllendorff, 1884) is a widely distributed land snail species with morphological variation and endemic to Taiwan. Three genetic markers (partial sequence of the mitochondrial cytochrome c oxidase subunit I [COI], the 16S rDNA and the nuclear internal transcribed spacer 2 [ITS2]) were analysed to infer phylogenetic relationships and genetic divergence of closely related species of the genus *Aegista*, *A. vermis* (Reeve, 1852) and *A. oculus* (Pfeiffer, 1850). A new species from *A. subchinensis* has been recognized on the basis of phylogenetic and morphological evidences. The nominal new species, *A. diversifamilia* **sp. n.** is distinguished from *A. subchinensis* (Möllendorff, 1884) by its larger shell size, aperture and apex angle; wider umbilicus and flatter shell shape. The northernmost distribution of *A. diversifamilia* **sp. n.** is limited by the Lanyang River, which is presumed to mark the geographic barrier between *A. diversifamilia* **sp. n.** and *A. subchinensis*.

Keywords

Stylommatophora, Helicoidea, Southern Ryukyu Islands, Yaeyama Islands, new species

Introduction

Traditional morphology-based taxonomy provides a window to explore biodiversity and evolutionary history. The application of molecular genetic markers opens new avenues to discover biodiversity. In recent years, it was found that the species richness of land snails bearing comparably few morphological characteristics and exhibiting limited abilities of dispersal had been underestimated once molecular tools were applied (Hirano et al. 2014, Nantarat et al. 2014, Nishi and Sota 2007, Prevot et al. 2013, Wu et al. 2008). In contrast, taxonomic revision based merely on a single molecular locus may lead to an overestimation of number of taxa. Integrative taxonomy was therefore proposed to integrate multiple independent lines of evidence for objective taxonomic treatment (Dayrat 2005, Padial et al. 2010, Schlick-Steiner et al. 2010).

Taiwan is a continental island that was formed through the collision of the Philippine Sea plate and the Eurasian plate. This collision uplifted the Central Mountain Range (CMR), forming a major physical barrier for animals inhabit lowland areas. The CMR has contributed to evolutionary divergences between organisms on either side of the CMR both on interspecific and intraspecific levels (Huang and Lin 2010, Jang-Liaw et al. 2008, Kuo et al. 2014, Lin et al. 2012, Wang et al. 2007, Yu et al. 2014). *Aegista subchinensis* (Möllendorff, 1884) is one of the most widely distributed species of the genus *Aegista* in Taiwan and is commonly found in lowland hardwood forests near the CMR (Hsieh 2003, Hsieh et al. 2006, Hsieh et al. 2013, Lee and Chen 2003, Lee and Wu 2004). The morphological differences observed between western and eastern populations indicate that the evolutionary diversification of this species complex may be underestimated and requiring further investigation (Lee and Chen 2003, Lee and Wu 2004). Based on multilocus sequence analyses and comparative morphology, we demonstrate that *Aegista* snails from eastern Taiwan, originally identified as *A. subchinensis*, represent a new species which is herein described as *A. diversifamilia* sp. n.

Materials and methods

Sample collection and molecular techniques

Live snails identified as *A. subchinensis* were collected from ten localities in Taiwan. Similar species, *A. vermis* (Reeve, 1852) of Ishigaki Island and *A. oculus* (Pfeiffer, 1850) of Miyako Island, were collected from two and four localities, respectively, on the southern Ryukyu Islands. Four congenic species, *A. mackensii* (Adams & Reeve, 1850), *A. granti* (Pfeiffer, 1865), *A. inrinensis* (Pilsbry & Hirase, 1905), and *A. shermani* (Pfeiffer, 1865), distributed in Taiwan were used as outgroups to root the phylogenetic tree. Global positioning system (GPS) coordinates of sampling sites (including latitude, longitude and altitude) were recorded using Garmin GPSmap 60CSx with an uncertainty of less than 10 metres (Figure 2 and Table 1). Vouchers and type specimens of *A. subchinensis* and *A. diversifamilia* sp. n. were deposited in the National Museum

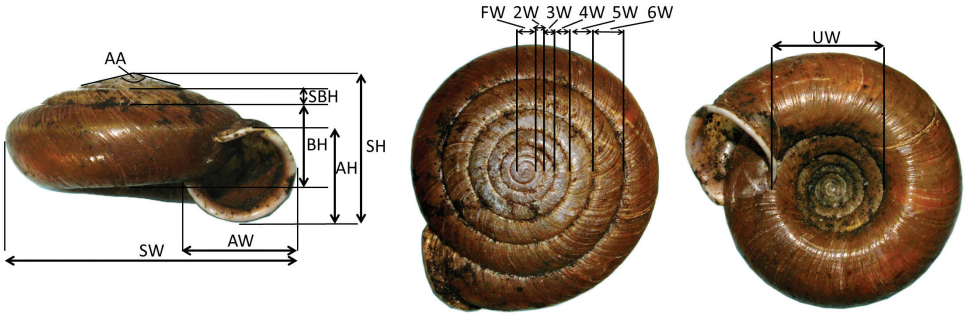

Figure 1. Morphometric measurement of shell size variation of *Aegista diversifamilia* sp. n. (shown in this figure) in top view, apertural view and umbilical view. **AA**: angle of apex; **AH**: aperture height; **AW**: aperture width; **BH**: body whorl height; **FW**: first whorl width; **SBH**: secondary body whorl height; **SH**: shell height; **SW**: shell width; **UW**: umbilicus width; **2W–6W**: 2nd–6th whorl width.

of Natural Science, Taiwan (NMNS, NMNS-7276) and the Natural History Museum, United Kingdom (NHMUK 20140070–20140074). Snails were relaxed in water for at least 6 hours, quickly fixed by submersion in boiling water and then preserved in 95% ethanol. DNA was extracted from 10 mg of foot tissue using AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Bioscience, USA) following the manufacturer's protocol. A partial sequence of mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified using the LCO1490 and HCO2198 universal primers (Folmer et al. 1994). Partial 16S ribosomal DNA was amplified using the 16Sar and 16Sbr universal primers (Palumbi et al. 1991). Complete nuclear internal transcribed spacer 2 (ITS2) was amplified using the LSU1 and LSU3 primers (Wade et al. 2006). The PCR mixture was composed of 10 ng DNA template, 1 μM primers, 1X Taq DNA polymerase 2.0 Master mix kit (Ampliqon, Denmark) and water. The total volume of the PCR mixture was 20 μl. PCR was performed under the following conditions: initial denaturation at 94 °C for 1 min followed by 36 cycles of denaturing at 94 °C for 30 s, annealing at 48 °C or 52 °C for 30 s and a final extension at 72 °C for 30 s. Primer annealing temperature was 48 °C for COI and 52 °C for 16S and ITS2. The size of the PCR products was checked under ultraviolet light after gel electrophoresis. The PCR mixture was purified using Genomics Universal DNA Purification kit (Genomics BioSci and Tech, Taiwan). Sanger sequencing was performed on an ABI PRISM 3730 DNA Analyzer at Institute of Cellular and Organismic Biology, Academia Sinica.

Phylogenetic reconstruction

Sequences were visually checked using BIOEDIT version 7.2.5 (Hall 1999) and deposited in GenBank (KJ574281–KJ574400, Table 1). The sequence of ingroups made available by Hirano et al. (2014) were incorporated into the phylogenetic reconstruction (Table 1). Sequences were aligned by MAFFT online version 7 (Katoh and Standley 2013) using default settings. PARTITIONFINDER version 1.1.1 (Lanfear

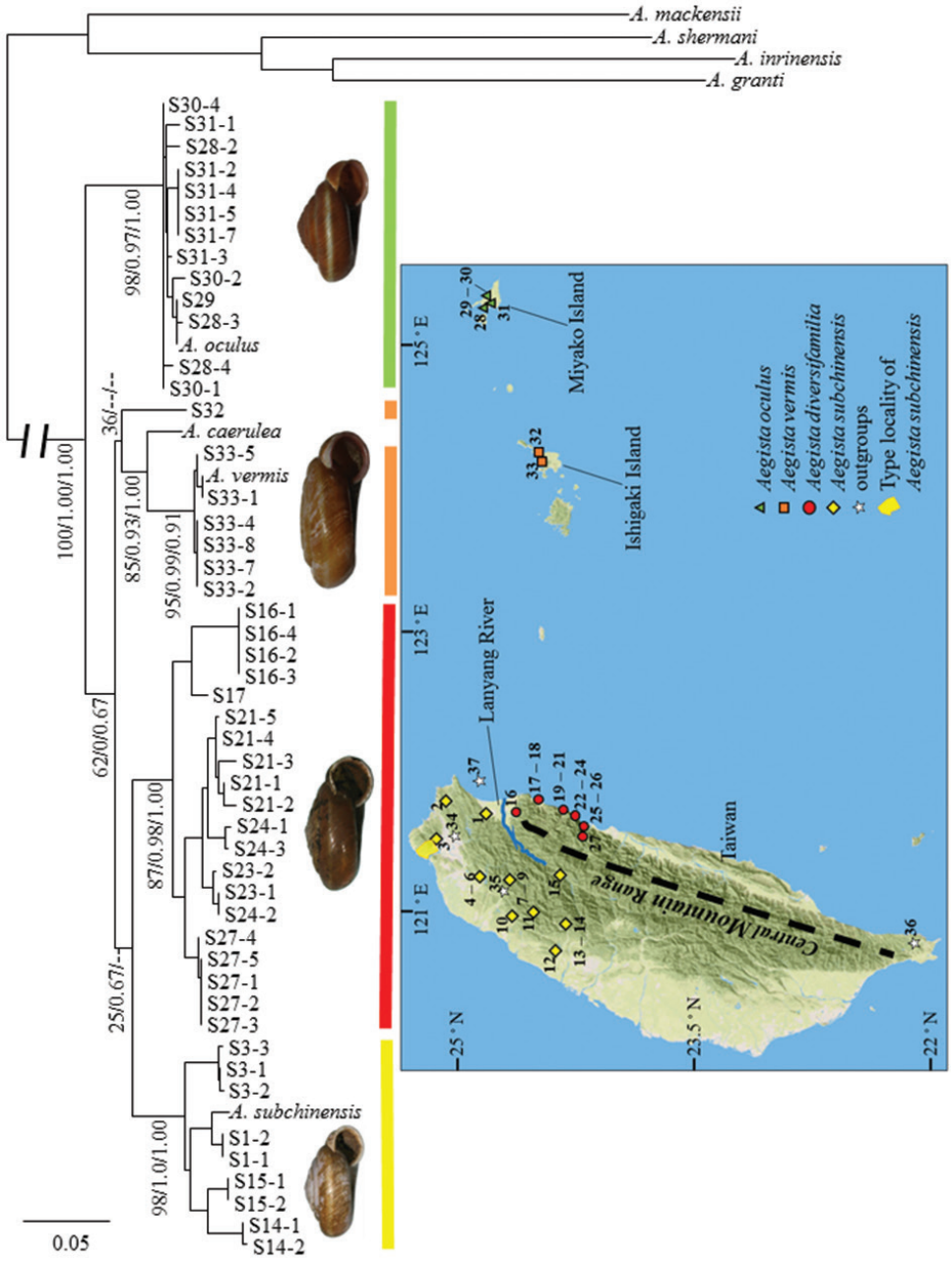

Figure 2. Maximum likelihood phylogeny and sampling sites of *Aegista* spp. Reconstructed phylogeny was based on concatenated sequences of mitochondrial COI, 16S and nuclear ITS2 genes. Branch support confidences of clades are shown in bootstrap, approximate likelihood-ratio test and Bayesian posterior probability, respectively. The log likelihood of maximum likelihood tree = -6584.1713. The numbered sampling sites are detailed in Table 1.

Table 1. Sampling information and GenBank accession number of DNA sequences

	GPS coordinates			Sample size		GenBank accession number		
Sampling locality	**Latitude**	**Longitude**	**Altitude**	**N_G**	**N_M**	**COI**	**16S**	**ITS2**
Aegista subchinensis								
# Wulai, Taipei	NA.	NA.	NA.	1	0	AB852655	NA.	AB852922
1. Linmei Shipan Trail, Jiaoxi Twp., I-Lan Co., Taiwan	24°49'41.88"N	121°43'56.34"E	286	2	2	KJ574361	KJ574321–KJ574322	KJ574382–KJ574383
2. Houtong, Ruifang District, New Taipei City, Taiwan	25°05'10.8"N	121°49'44.5"E	105	0	3	NA.	NA.	NA.
3. Zhishanyan, Taipei City, Taiwan	25°06'10.8"N	121°31'47.0"E	53	3	5	KJ574358–KJ574360	KJ574318–KJ574320	KJ574380–KJ574381
4. Datieliao Trail, Daxi Twp., Taoyuan City, Taiwan	24°50'59.22"N	121°18'46.20"E	433	0	1	NA.	NA.	NA.
5. Shimen Reservoir 1, Daxi Twp., Taoyuan City, Taiwan	24°49'08.58"N	121°16'27.72"E	323	0	5	NA.	NA.	NA.
6. Shimen Reservoir 2, Daxi Twp., Taoyuan City, Taiwan	24°48'57.24"N	121°15'09.90"E	198	0	1	NA.	NA.	NA.
7. Frog Rock, Jianshi Twp., Hsinchu Co., Taiwan	24°41'12.3"N	121°13'43.2"E	468	0	5	NA.	NA.	NA.
8. Fuxing Coal Mine, Jianshi Twp., Hsinchu Co., Taiwan	24°40'58.3"N	121°14'01.3"E	512	0	1	NA.	NA.	NA.
9. Jinping, Jianshi Twp., Hsinchu Co., Taiwan	24°40'41.5"N	121°15'15.5"E	638	0	1	NA.	NA.	NA.
10. Shishan Trail, Nanzhuang Twp., Miaoli Co., Taiwan	24°38'33.0"N	121°00'30.6"E	344	0	5	NA.	NA.	NA.
11. Fengmei, Nanzhuang Twp., Miaoli Co., Taiwan	24°32'44.8"N	121°01'41.7"E	695	0	2	NA.	NA.	NA.
12. Dacaopai, Sanyi Twp., Miaoli Co., Taiwan	24°22'29.10"N	120°47'52.40"E	523	0	3	NA.	NA.	NA.
13. Wu Shi Branch School, Heping District, Taichung City, Taiwan	24°17'34.8"N	120°56'07.8"E	650	0	1	NA.	NA.	NA.
14. Wushikeng, Heping District, Taichung City, Taiwan	24°12'46.49"N	120°56''44.16"E	894	2	0	KJ574364–KJ574365	NA.	NA.
15. Huanshan, Heping District, Taichung City, Taiwan	24°19'11.27"N	121°17'18.33"E	1560	2	1	KJ574362–KJ574363	KJ574323	KJ574384
Aegista diversifamilia sp. n.								
16. Anpingkeng, Dongshan Twp., I-Lan Co., Taiwan	24°36'52.5"N	121°46'38.1"E	70	4	5	KJ574339–KJ574342	KJ574299–KJ574302	KJ574385
17. Wushibi, Su'ao Twp., I-Lan Co., Taiwan	24°29'13.5"N	121°50'02.9"E	382	1	0	KJ574343	KJ574303	KJ574386
18. Chaoyang Trail, Nan'ao Twp., I-Lan Co., Taiwan	24°27'35.9"N	121°48'53.9"E	42	0	2	NA.	NA.	NA.
19. Heren 1, Xiulin Twp., Hualien Co., Taiwan	24°14'49.1"N	121°43'06.4"E	36	0	1	NA.	NA.	NA.
20. Heren 2, Xiulin Twp., Hualien Co., Taiwan	24°14'54.8"N	121°42'51.4"E	55	0	7	NA.	NA.	NA.

	GPS coordinates			Sample size		GenBank accession number		
Sampling locality	**Latitude**	**Longitude**	**Altitude**	**N_G**	**N_M**	**COI**	**16S**	**ITS2**
21. Heren Trail, Xiulin Twp., Hualien Co., Taiwan	24°13'58.5"N	121°42'27.73"E	50	5	1	KJ574344–KJ574348	KJ574304–KJ574308	KJ574387–KJ574390
22. Jinwen Tunnel, Xiulin Twp., Hualien Co., Taiwan	24°12'28.7"N	121°40'23.5"E	128	0	8	NA.	NA.	NA.
23. Northern Chongde Tunnel, Xiulin Twp., Hualien Co., Taiwan	24°11'31.08"N	121°39'41.01"E	62	2	2	KJ574349–KJ574350	KJ574309–KJ574310	NA.
24. Southern Chongde Tunnel, Xiulin Twp., Hualien Co., Taiwan	24°11'22.0"N	121°39'36.8"E	56	3	5	KJ574351–KJ574352	KJ574311–KJ574313	KJ574394–KJ574396
25. Sanjianwu, Xiulin Twp., Hualien Co., Taiwan	24°10'55.3"N	121°37'34.3"E	165	0	6	NA.	NA.	NA.
26. Taroko Service Center, Xiulin Twp., Hualien Co., Taiwan	24°09'31.9"N	121°37'20.7"E	100	0	6	NA.	NA.	NA.
27. Badagang, Xiulin Twp., Hualien Co., Taiwan	24°10'36.8"N	121°33'43.6"E	421	5	0	KJ574353–KJ574357	KJ574314–KJ574317	KJ574391–KJ574393
Aegista oculus								
# Miyako Island, Japan	NA.	NA.	NA.	1	0	AB852642	NA.	AB852909
28. Shimozaki, Miyako Island, Japan	24°50'03.78"N	125°16'50.58"E	32	3	0	KJ574328	KJ574281–KJ574282	KJ574370
29. Hirara 1, Miyako Island, Japan	24°48'03.12"N	125°18'58.86"E	42	1	0	NA.	KJ574283	KJ574372
30. Hirara 2, Miyako Island, Japan	24°47'58.50"N	125°19'02.94"E	44	3	0	KJ574329	KJ574284–KJ574286	KJ574371, KJ574373
31. Shimozato, Miyako Island, Japan	24°47'15.24"N	125°17'11.10"E	44	6	0	KJ574330–KJ574335	KJ574287–KJ574291	KJ574374–KJ574375
Aegista vermis								
# IriomoteIsland, Japan	NA.	NA.	NA.	1	0	AB852660	NA.	AB852927
32. Tozato, Ishigaki Island, Japan	24°27'18.6"N	124°14'17.5"E	94	1	0	NA.	KJ574292	KJ574376
33. Fukai, Ishigaki Island, Japan	24°26'59.28"N	124°12'04.98"E	62	6	0	KJ574336–KJ574338	KJ574293–KJ574298	KJ574377–KJ574379
Aegista caerulea								
# Ishigaki Island, Japan	NA.	NA.	NA.	1	0	AB852626	NA.	AB852893

	GPS coordinates			Sample size		GenBank accession number		
Sampling locality	**Latitude**	**Longitude**	**Altitude**	**N_G**	**N_M**	**COI**	**16S**	**ITS2**
Outgroups								
Aegista granti								
34. Fuyang Park, Taipei City, Taiwan	25°0'56.66"N	121°33'26.82"E	32	1	0	KJ574368	KJ574326	KJ574398
Aegista inrinensis								
35. Neiwan, Hengshan Twp., Hsinchu Co., Taiwan	24°42'18.2"N	121°10'58.7"E	268	1	0	KJ574367	KJ574325	KJ574399
Aegista shermani								
36. Lanren Rd., Manzhou Twp., Pingtung Co., Taiwan	22°02'25.8"N	120°50'58.8"E	48	1	0	KJ574366	KJ574324	KJ574397
Aegista mackensii								
37. Gueishan Island, Taiwan	24°50'35.9"N	121°56'52.6"E	157	1	0	KJ574369	KJ574327	KJ574400

N_G: Number of specimen for Genetic analyses; N_M: Number of specimen for Morphological analyses; #: sequence obtained from Hirano et al. (2014); NA.: not available.

et al. 2012) was used to identify the best partition model for the protein-coding gene COI. The best-fit substitution model for each gene was evaluated via JMODELTEST version 2.1.4 (Darriba et al. 2012, Guindon and Gascuel 2003). The model filtering threshold of heuristic was set to 1.0 to increase the efficiency for evaluating 56 substitution models. The best-fit model was evaluated using BIC and DT criteria because their accuracy has been shown to outperform hLRT and AIC (Luo et al. 2010). Phylogenetic relationship was inferred from each gene and concatenated sequences using maximum likelihood and the Bayesian inference method. Phylogeny of maximum likelihood was inferred using PHYML 3.0 (Guindon et al. 2010) implemented in SEAVIEW version 4.4.2 (Gouy et al. 2010). Parameters were set to empirical nucleotide frequencies with optimized invariable sites; the number of rate categories equalled four; tree searching operation as the best of nearest neighbour interchange (NNI) and subtree pruning and regrafting (SPR); BioNJ with optimized tree topology as the starting tree. Branch support confidences were estimated via traditional bootstrap with 100 replicates (Felsenstein 1985) and an fast and accurate alternative method, approximate likelihood-ratio test (aLRT) (Anisimova and Gascuel 2006). Phylogeny of Bayesian inference was performed using MRBAYES version 3.2 (Ronquist et al. 2012). Two parallel runs of three heated chains and one cold chain were performed for 2–5 million generations and sampled every 1000 generations. Sampling was stopped when all chains reached stationarity and the runs converged (split frequencies < 0.01). The first 25% of sampling trees were discarded as burn-in and a 50% majority-rule consensus tree was computed. Branch support confidences were inferred from Bayesian posterior probability. Network is an alternative way to infer the phylogenetic relationship among haplotypes (Bandelt et al. 1999, Bapteste et al. 2013, Huson and Bryant 2006). Haplotype network was reconstructed by median-joining method (Bandelt et al. 1999) via NETWORK version 4.612 (available at http://www.fluxus-engineering.com/sharenet.htm). Interspecific and intraspecific genetic distances were calculated by using PAUP* version 4.0 (Swofford 2003) employing the best-fit substitution model but excluding sequences from Hirano et al. (2014).

Morphological analyses

For genital morphological comparison, we dissected two samples of one adult snail each from the western (Zhishanyan, Taipei City) and the eastern (Heren 1, Xiulin Township, Hualien County) population, respectively (Table 1). Snails were relaxed in water for more than 6 hours, fixed by submersion in boiling water for 20 seconds, and preserved in 70% ethanol. Shell was crushed before dissection. All soft tissues were preserved in 70% ethanol. Terminology of genital morphology follows Gómez (2001). Images of genitalia were obtained via Canon PowerShot A650IS digital camera. QP-card 201 was included in each image for colour and scale correction with PhotoImpact X3 (Corel Corp., Taipei, Taiwan). The following organs were measured in cm and scaled by their shell width: length of albumen gland (AG), hermaphroditic duct (HD),

spermoviduct (SOD), free oviduct (FO), vagina (V), dart-sac of auxiliary copulatory organ (DS), penis (P), epiphallus (E), and epiphallus flagellum (EF). The following ratios were calculated: HD/AG, AG/SOD, HD/SOD, FO/V, EF/E, E/P, and P/V.

Shell morphological comparisons were based on examination of 36 specimens of *A. subchinensis* from the west of the CMR and 43 specimens from the east (Table 1). Morphological differences between the western and the eastern *A. subchinensis* were analysed on the basis of measurements and shape outline coordinates. Shell images were obtained as mentioned above. The image's background was removed using CLIPPING MAGIC (available at https://clippingmagic.com/). Images were converted into thin-plate splines (TPS) format using TPSUTIL version 1.56 (Rohlf 2013). The Janssen's method of counting the number of whorls was used (Janssen 2007). Fifteen characteristics were measured: the number of whorls; width of the shell (SW), aperture (AW), umbilicus (UW), first whorl (FW), and 2nd–6th whorl (2W–6W); height of the shell (SH), aperture (AH), body whorl (BH), and secondary body whorl (SBH); and angle of apex (AA) (Figure 1). Measurements of length (in cm) and angle were conducted using TPSDIG version 1.4 (Rohlf 2004) and IMAGEJ version 1.47 (Abramoff et al. 2004), respectively. The shell flatness and aperture shape were expressed by calculating ratios of shell height divided by shell width (SH/SW), aperture height divided by aperture width (AH/AW) and umbilicus width divided by shell width (UW/SW), and additional ratios were calculated: AW/SW, BH/SW, SBH/SW, FW/SW, AH/SH, SBH/SH, SBH/SH, SBH/BH, SH/UW, AW/UW, AH/UW, BH/UW, SBH/UW, 2W/3W, 3W/4W, 4W/5W, 5W/6W. Summary statistics and 95% confidence intervals of these characteristics were calculated for the western and the eastern population of *A. subchinensis*. Statistical differences of each characteristic were analysed using Welch's *t* test or the Mann–Whitney *U* test when the distribution of characteristics did or did not follow a normal distribution, respectively. Multivariate analysis of variance (MANOVA) was conducted with the species as independent variables and the characteristics mentioned above as dependent variables. A Bonferroni correction was applied for *p*-values. All characteristics were log-transformed for principle component analysis (PCA) and discriminant analysis with Jackknifed classification via PAST version 3.01 (Hammer et al. 2001). Outline coordinates of each shell were digitalized using TPSDIG for geometric morphometric analysis of shell shape. Outline coordinates were approximated with 16 control points via MORPHOMATICA version 1.6 (Linhart et al. 2006). The outline coordinates of the western and the eastern *A. subchinensis* were used to calculate mean shell shape in MORPHOMATICA. A relative warps PCA was conducted using PAST.

Results and discussion

Molecular phylogeny

A total of 50 individuals were sequenced from the *Aegista* ingroup. The lengths of the COI, 16S and ITS2 after alignment were 655 bp, 280 bp and 750 bp, respectively. The

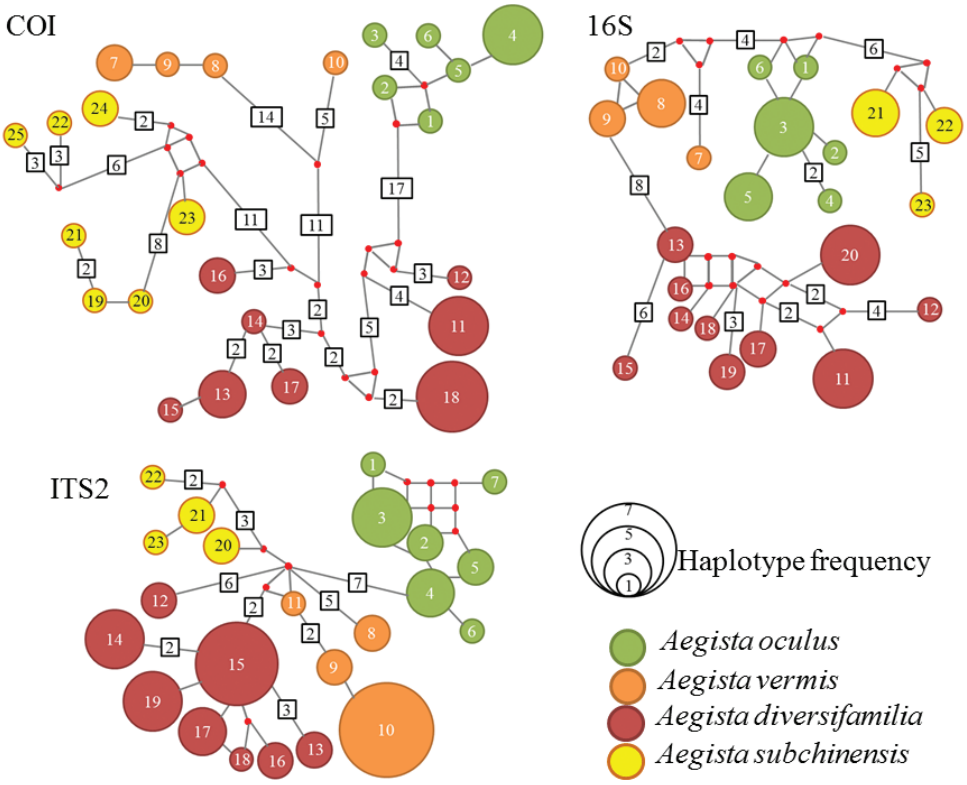

Figure 3. Haplotype networks of mitochondrial COI, 16S and nuclear ITS2 genes. Species are presented by colors. Haplotype frequency is shown by the size of the circular. Name of haplotype is numbered and presented inside the circle. Haplotypes are connected by simple line represent one mutation between haplotypes. Number of mutations between haplotypes are shown in square. Red dots are missing or hypothetical haplotypes.

best maximum likelihood tree and Bayesian consensus phylogram had similar topologies: *Aegista oculus* from Miyako Island formed a monophyly outside the other three species (Figure 2). *Aegista vermis* and *A. caerulea* from Ishigaki Island formed a sister pair with high branch support except for one individual of *A. vermis* from Tozato, Ishigaki Island (sampling site 32), which was found in an unresolved position in Bayesian phylogeny. The cluster of *A. subchinensis* revealed a basal bifurcation between eastern and western populations. The western and eastern *A. subchinensis* formed reciprocal monophyletic clades with highly supported by the bootstrap, aLRT and Bayesian posterior probability. However, the monophyly of the *A. subchinensis* clade was only weakly supported. Low statistical branch support for the monophyly of the *A. subchinensis* clade probably resulted from conflicting phylogenetic relationships between the COI and the 16S gene tree and unresolved phylogenetic relationships among species of the ITS2 gene tree (Suppl. material 1, Figures S1–6). Maximum likelihood and Bayesian COI gene trees suggested that the eastern *A. subchinensis* was the sister clade of *A. vermis* and the western *A. subchinensis* (Suppl. mate-

rial 1, Figures S1 and S4). Maximum likelihood 16S gene tree showed sister relationship between the western and the eastern *A. subchinensis* with low branch support (Suppl. material 1, Figure S2), but the eastern *A. subchinensis* was sister species to *A. vermis* with moderate branch support in Bayesian tree (Suppl. material 1, Figure S5). COI gene tree estimated by maximum likelihood and Bayesian inference and the 16S Bayesian gene tree suggested that the western and the eastern *A. subchinensis* were not sister clades.

No haplotypes were shared among species for COI, 16S or ITS2 genes (Figure 3). For COI haplotype network, the eastern *A. subchinensis* was nested with the other three species for at least 16 mutations, no clear sister species relationship could be inferred. Haplotype network of 16S gene suggested the western and the eastern *A. subchinensis* were distant related. The western *A. subchinensis* was nested with *A. oculus* at least 9 mutations. The eastern *A. subchinensis* was nested with *A. vermis* at least 8 mutations. For ITS2 haplotype network, the eastern *A. subchinensis* was more closely related to *A. vermis* that separated by at least 3 mutations. The eastern and western *A. subchinensis* were diverged at least 5 mutations.

Gene trees and haplotype networks suggested the western and the eastern *A. subchinensis* were not sister clades. The eastern *A. subchinensis* was more closely related to *A. vermis* that distributed in Ishigaki Island, Japan. The absence of shared haplotype between the eastern and the western *A. subchinensis* might suggest that they were diverged and currently no gene flow between them.

The mean genetic distance between the western and the eastern *A. subchinensis* clades was 5.9% for COI, 4.2% for 16S, and 0.8% for ITS2. This divergence corresponded to the divergence between other closely related congeneric species (Table 2). Based on phylogenetic trees, haplotype networks and genetic distance analyses, the western and the eastern *A. subchinensis* were diverged and might not be sister clades. More genetic markers are needed to resolve the low statistical support of phylogenetic relationships among species.

Morphological analyses

The major differences of genital morphology of the western and the eastern *A. subchinensis* were the length of AG, DS, EF, the shape of DS, and the number of lobes of mucus gland in the auxiliary copulatory organ (M) (Figure 4, Table 3). The length of AG of the eastern *A. subchinensis* (0.88, scaled by shell width) was three times longer than the western (0.24). The length of DS and EF were nearly two times longer in the eastern *A. subchinensis* (DS=0.45, EF=0.57) than the western (DS=0.23, EF=0.29). The shape of DS was more rounded and larger in the eastern *A. subchinensis* than the western. The eastern *A. subchinensis* had three lobes of M and the western *A. subchinensis* had two (Figure 4). The HD/AG ratio was the most different characteristics between the eastern and the western *A. subchinensis* that showed HD was three times longer than AG in the western *A. subchinensis* but HD was shorter than AG in the eastern (Table 3). The eastern *A. subchinensis* showed larger values than the western in AG/SOD, EF/E and E/P ratio.

Table 2. Interspecific divergence and intraspecific polymorphism of *Aegista* spp. from COI, 16S and ITS2 genes.

COI/16S/ITS2	***A. oculus***	***A. vermis***	***A. diversifamilia***	***A. subchinensis***
A. oculus	0.007/0.005/0.002			
A. vermis	0.075/0.036/0.015	0.002/0.008/0.004		
A. diversifamilia	0.067/0.051/0.014	0.064/0.034/0.007	0.023/0.014/0.004	
A. subchinensis	0.085/0.037/0.016	0.065/0.038/0.009	0.059/0.042/0.008	0.024/0.012/0.004

Table 3. Measurements for genital morphology of *Aegista subchinensis* and *A. diversifamilia* **sp. n.** The measurements are scaled by shell width, *A. subchinensis* (1.9 cm, collected from Zhishanyan, Taipei City) and *A. diversifamilia* **sp. n.** (2.3 cm, collected from Heren 1, Xiulin Township, Hualien County)

Measurement	**HD**	**AG**	**SOD**	**FO**	**V**	**DS**	**P**	**E**	**EF**
A. subchinensis	0.77	0.24	1.05	0.20	0.20	0.23	0.34	0.32	0.29
A. diversifamilia	0.73	0.88	1.38	0.22	0.33	0.45	0.33	0.45	0.57
Ratio	HD/AG	AG/SOD	HD/SOD	FO/V	EF/E	E/P	P/V		
A. subchinensis	3.19	0.23	0.73	1.00	0.89	0.94	1.75		
A. diversifamilia	0.84	0.63	0.53	0.66	1.25	1.37	1.01		

AG: albumen gland; DS: dart-sac of auxiliary copulatory organ; E: epiphallus; EF: epiphallial flagellum; FO: free oviduct; HD: hermaphroditic duct; P: penis; SOD: spermoviduct; V: vagina.

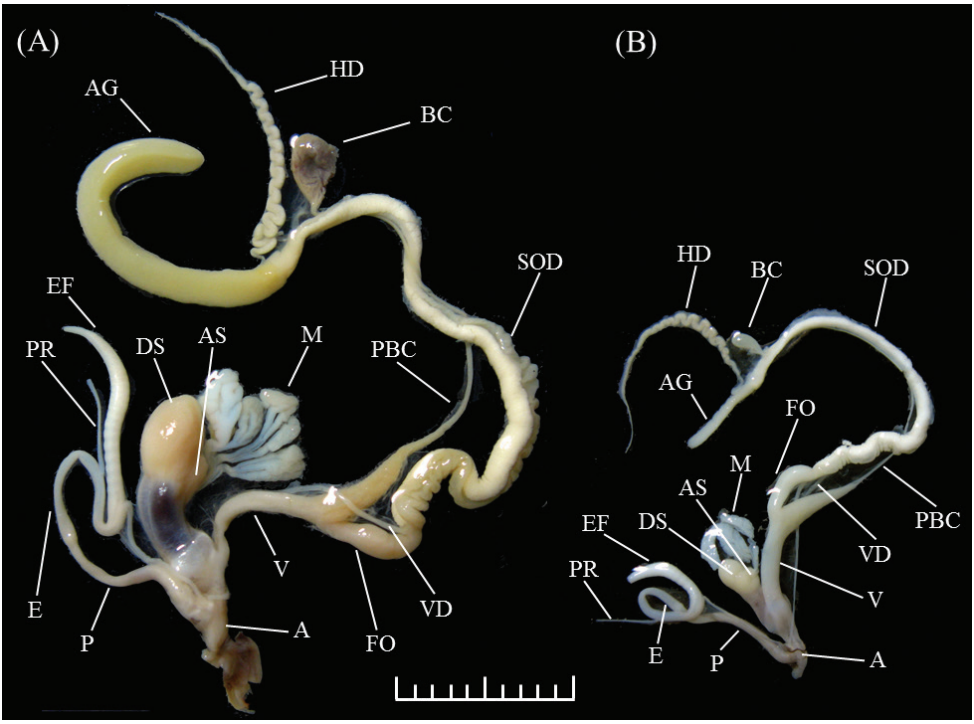

Figure 4. Genital morphology of **A** *Aegista diversifamilia* sp. n. and **B** *A. subchinensis*. Scale bar = 1 cm. **A**: atrium; **AG**: albumen gland; **AS**: accessory-sac of auxiliary copulatory organ; **BC**: bursa copulatory; **DS**: dart-sac of auxiliary copulatory organ; **E**: epiphallus; **EF**: epiphallial flagellum; **FO**: free oviduct; **HD**: hermaphroditic duct; **M**: gland of the auxiliary copulatory organ; **P**: penis; **PBC**: pedunculus of bursa copulatory; **PR**: penis retractor; **SOD**: spermoviduct; **V**: vagina; **VD**: vas deferens.

The eastern and western populations of *A. subchinensis* differed significantly from each other in all studied shell parameters ($p < 0.001$) except the number of whorls and the height of the secondary body whorl (Table 4, Suppl. material 1, Table S1). The eastern *A. subchinensis* had a similar number of whorls ($p > 0.05$) but shells were significantly larger than those of the western *A. subchinensis* and had a wider apex angle. The eastern and western populations also differed significantly in all morphometric ratios except UW/SW, AW/UW, 2W/3W, 3W/4W and 5W/6W. MANOVA suggested that the morphological difference between the western and the eastern *A. subchinensis* was statistical significant (Bonferroni-corrected *p*-value=1.12E-10). Results of the PCA of morphological characteristics suggested that principle component axis 1 (PC1) explained 46.32% of the total variation and had the highest loading scores for aperture height (0.284). PC2 explained 20.03% of the total variation and had the highest loading scores for height of the secondary body whorl (0.476). The PCA scores plot of PC1 and PC2 showed that morphology between the eastern and the western *A. subchinensis* were well distinguished with very limited overlap (Figure 5A). Discriminant analysis showed that specimens could be correctly classified (100%) into eastern

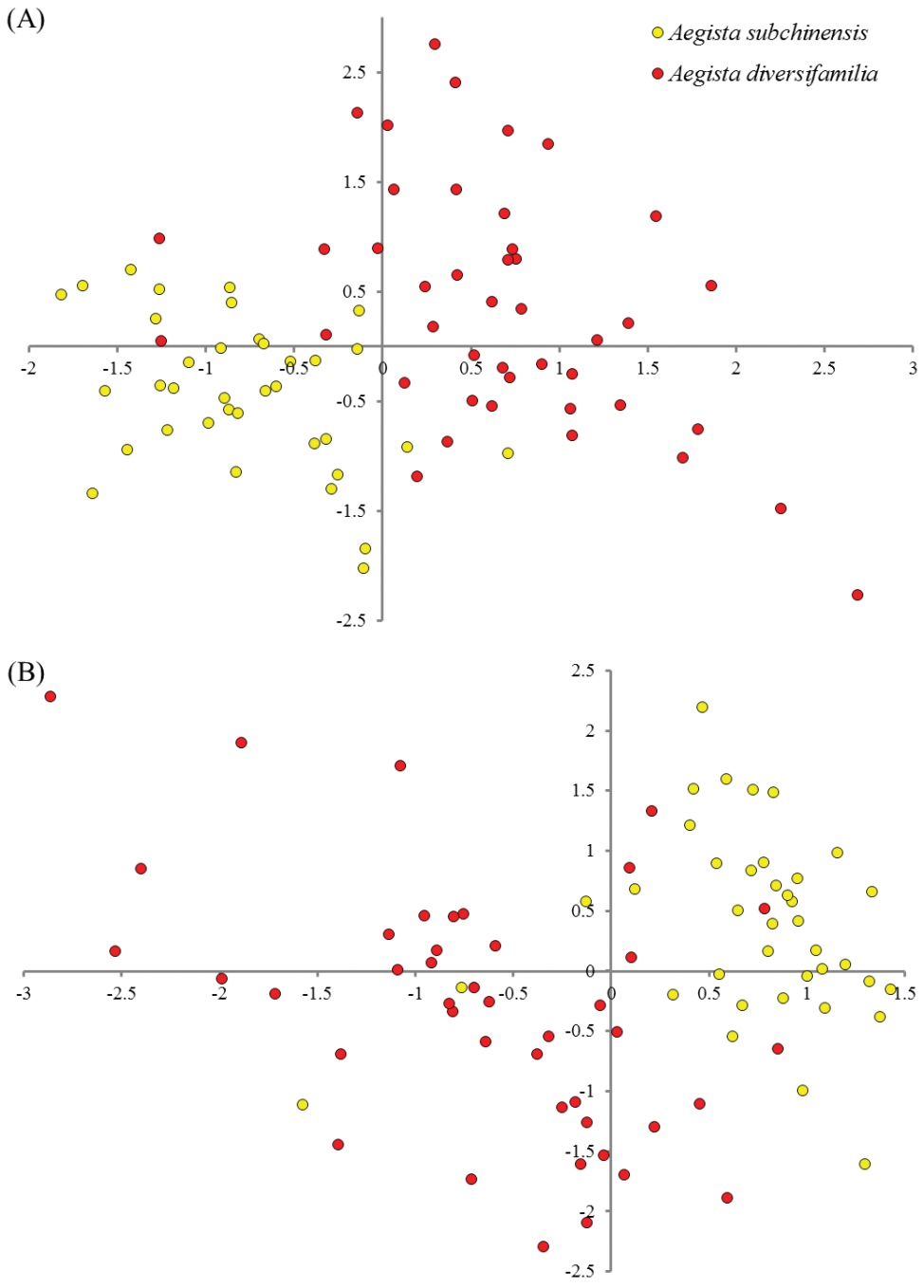

Figure 5. Principle component analysis (PCA) of *Aegista subchinensis* and *A. diversifamilia* sp. n. **A** PCA of measurements and ratios **B** relative warps PCA of shell shape coordinates.

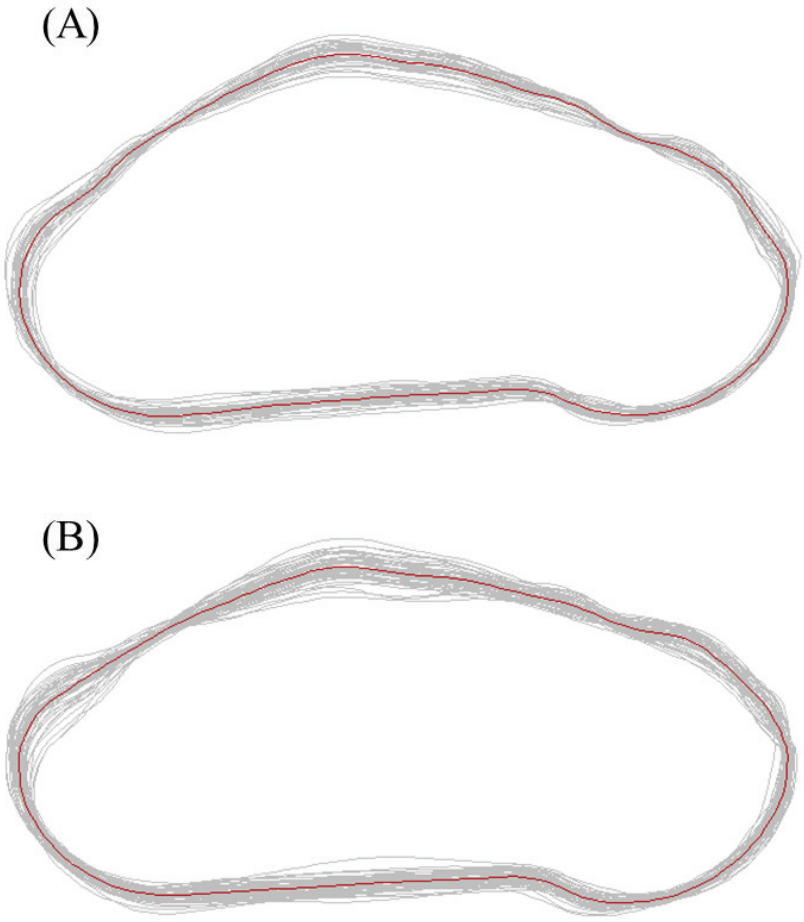

Figure 6. Mean shell shape of **A** *Aegista subchinensis* and **B** *A. diversifamilia* sp. n.

and western clades (93.67% using Jackknifed analysis). Relative warps PCA of shell outline coordinates suggested that PC1 and PC2 represented 94.43% and 3.07% of the total variation, respectively. The scores plot of relative warps PC1 and PC2 also showed prominent morphological differences between the eastern and the western clade (Figure 5B). The mean shape of the western and the eastern *A. subchinensis* were presented in Figure 6. The eastern *A. subchinensis* (Figure 6B) was more flat than the western *A. subchinensis* (Figure 6A). The type locality of *A. subchinensis* was Tamsui in northern Taiwan (Figure 2). The illustration of *A. subchinensis* presented in plate 7 figure 8 of the original description (Möllendorff 1884) showed higher conic shape that was similar to our analysed western populations of *A. subchinensis*. Despite we did not have sample from Tamsui, the type locality of *A. subchinensis*, we sampled much wider geographical region around Tamsui that included more variation of molecular and morphological characteristics.

Based on the observed amounts of morphological and genetic differentiation, we conclude that the eastern and western populations assigned to *A. subchinensis* have diverged into separate species. Phylogeny reconstructed from concatenated sequences supports monophyly of both clades corresponding to their allopatric distributional pattern that separated by the Lanyang River. The Lanyang River was a biogeographic barrier for a high elevation mammal, Formosan wood mouse *Apodemos semotus* (Hsu et al. 2001). Some researchers identified the Xueshan Mountain Range, located in the northern area of Lanyang River, as a biogeographic barrier for lowland animals (Lin et al. 2012, Shih et al. 2011, Yu et al. 2014). To our knowledge, this is the first study revealed that the Lanyang River as a barrier for lowland terrestrial animals. We suggested that the eastern *A. subchinensis* might be diverged from the western *A. subchinensis* by vicariance event.

Systematics

Superfamily Helicoidea Rafinesque, 1815
Family Bradybaenidae Pilsbry, 1939

Genus *Aegista* Albers, 1850

Type species. *Helix chinensis* Philippi, 1845, original designation.

***Aegista diversifamilia* sp. n.**
http://zoobank.org/B36A2814-2702-40B0-844B-AC4B12A8BD25
Figures 7, 8, Table 4, Suppl. material 1, Table S1

Aegista subchinensis Hsieh, 2003: 200, figs; Lee and Chen 2003: 234, figs above text, figs 1–2; Lee and Wu 2004: 13–14, figures 2A, 3D; Hsieh et al. 2006: 250, figs; Wu and Jian 2006: fig 33; Hsieh et al. 2013: 335, figs.

Aegista (*Aegista*) *subchinensis* Hemmen and Niederhöfer, 2007: figs 67, figs 80; Wen and Hwang 2014: fig 1.

Type material. Holotype NMNS-7276-001 (adult dry shell, Figure 7A). **Paratypes** NMNH-7276-002 (1 juvenile in EtOH) and NHMUK 20140070 (4 adult dry shells, Figure 7B–E) from the same locality of holotype. NMNH-7276-003 (1 adult dry shell) and NMNH-7276-004 (1 adult dry shell) from the northern entrance of Chongde Tunnel, Xiulin Township, Hualian County, 24°11'31.08"N, 121°39'41.01"E, elevation 62 m. NMNS-7276-005 (6 adult dry shells) and NHMUK 20140071 (2 adult dry shells) from Jinwen Tunnel, Xiulin Township, 24°12'28.7"N, 121°40'23.5"E, elevation 128m.

Type locality. Taiwan, Hualian County, Xiulin Township, Forest around the Chongde Tunnel, 24°11'22.0"N, 121°39'36.8"E, elevation 56 m.

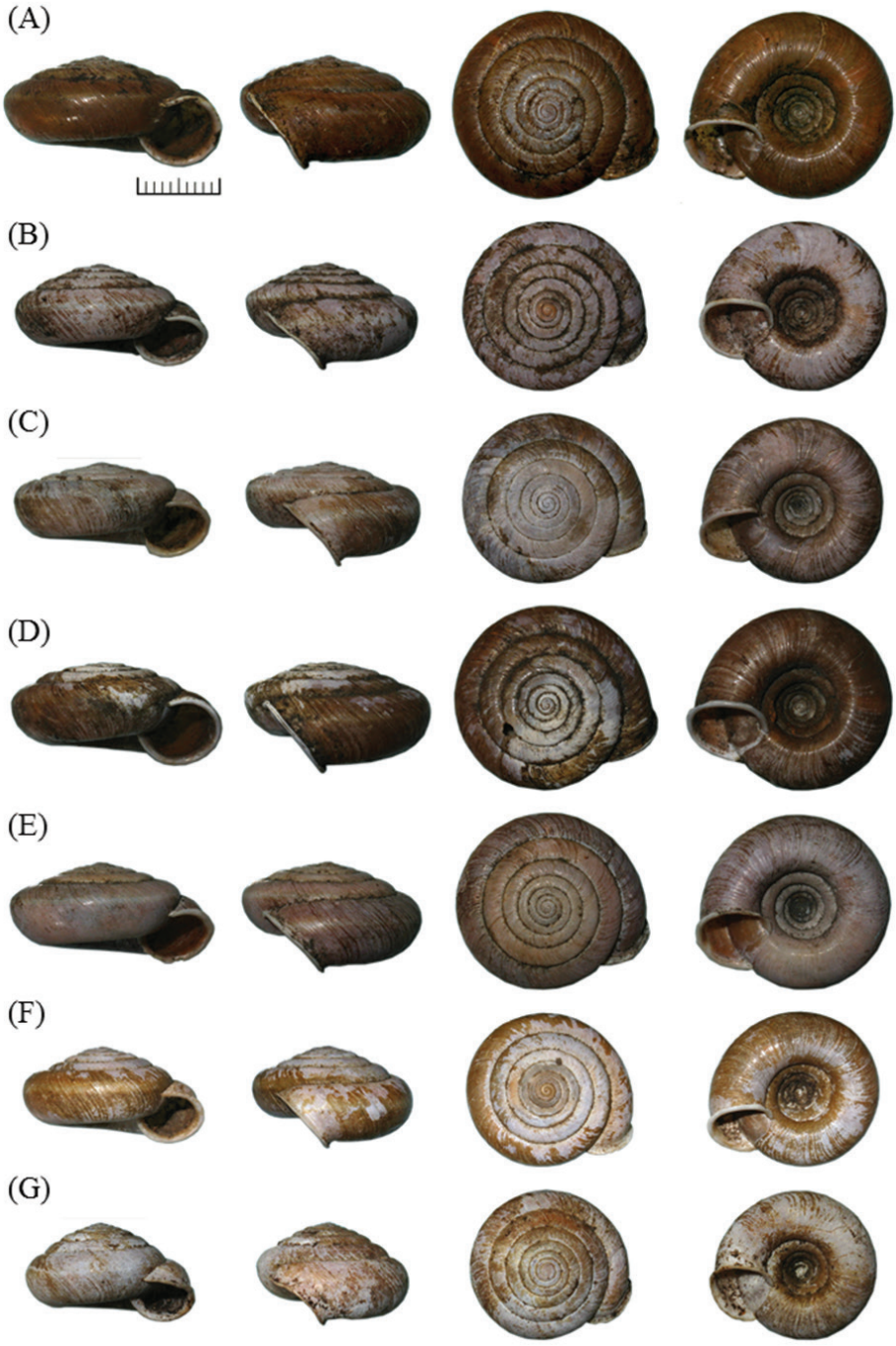

Figure 7. Shell images of *Aegista diversifamilia* sp. n. and *A. subchinensis*. *Aegista diversifamilia* sp. n.: **A** holotype, NMNS-7276-001 **B–E** paratype, NHMUK20140070, the same locality of holotype. *Aegista subchinensis*: **F** collected from Zhishanyan, Taipei City **G** collected from Linmei Shipan Trail, Jiaoxi Township, I-Lan County. Scale bar = 1 cm.

Other material examined. Anpingkeng, Dongshan Township, I-Lan County, 24°36'52.5"N, 121°46'38.1"E (3 adult dry shells); Wushibi, Su'ao Township, 24°29'13.5"N, 121°50'02.9"E (1 juvenile in EtOH); Chaoyang Trail, Nan'ao Township, 24°27'35.9"N, 121°48'53.9"E (2 adult dry shells); Heren 1, Xiulin Township, Hualien County, 24°14'49.1"N, 121°43'06.4"E (1 adult dry shells); Heren 2, 24°14'54.8"N, 121°42'51.4"E (7 adult dry shells); Heren Trail, 24°13'58.5"N, 121°42'27.73"E (1 adult and 4 juvenile in EtOH); Southern Chongde Tunnel, 24°11'22.0"N, 121°39'36.8"E (2 juvenile in EtOH); Sanjianwu, 24°10'55.3"N, 121°37'34.3"E (6 adult dry shells); Taroko Service Center, 24°09'31.9"N, 121°37'20.7"E (6 adult dry shells); Badagang, 24°10'36.8"N, 121°33'43.6"E (1 adult and 4 juvenile in EtOH, 6 adult dry shells) (materials mentioned above were deposited in NMNS, NMNH-7276); Hoping Forest Road, (1 adult in EtOH, NMNS-004875-00015 and 1 adult dry shell, NMNS-004962-00038); Sanzhan Northern Stream, (1 adult dry shell, NMNS-003348-00023).

Description. *Shell Morphology.* Shell depressed globosed, dextral, medium sized, shell width range 1.98–3.24 cm, shell height range 0.97–1.68 cm, shell height/shell width ratio range 0.43–0.55. Shell thin but solid, glossy with chestnut brown or yellowish-brown, usually with narrow and light brown spiral band on periphery. Shell surface with distinct oblique and curved growth lines. Apex obtuse, angle range 148.56°–165.02°. Spire depressed conic, slightly convex, suture depressed. Whorl range 6.6–8.2 in number, earlier whorl narrow then slowly increases regularly, and last whorl shouldered. Body whorl height range 0.53–0.88 cm. Aperture little descending, ovate or nearly circular, width range 0.78–1.32 cm, height range 0.48–1.05 cm. Peristome white, expanded and reflected. Umbilicus widely open, width range 0.77–1.59 cm. Mean and standard errors of each characteristics were provided in Table 4. Morphological measurements of all specimens were presented in Suppl. material 1, Table S1.

Genital morphology. Atrium thick and short. Penis slender and long. Epiphallus slender, longer than penis. Penis retractor muscle thin and long, attached to one-third part of epiphallus. Epiphallial flagellum thick and long, logner than epiphallus, wider than penis and epiphallus. Dart-sac of auxiliary copulatory organ thick and large, inserted into the base of vagina, with one small accessory-sac of auxiliary copulatory organ. Three mucus glands of the auxiliary copulatory organ. Vagina slender at the base of dart-sac, gradual wider and thick toward free oviduct, inflated at the connected region of free oviduct, about equal length of penis. Free oviduct thick, short,inflated. Pedunculus of bursa copulatory thin and long. Sac of bursa copulatory large and oval. Vas deferens thin and long, wider than penis retractor muscle. Spermoviduct long, about four times longer than penis and oviduct. Hermaphroditic duct slender and long, about half length of spermoviduct. Albumen gland thick and long, longer than hermaphroditic duct.

Etymology. Named after the recent efforts supporting equal marriage rights in Taiwan and around the world. Derived from "diversus" (Latin for different) and "familia" (Latin for family), adjective of feminine gender.

Distribution. Endemic to Taiwan and is currently known from I-Lan and Hualian Counties. *Aegista diversifamilia* sp. n. is absent from Gueishan Island based on our

Table 4. Measurements (in cm) of *Aegista subchinensis* and *A. diversifamilia* sp. n. Mean, standard error, statistical method and the *p*-value were provided.

	Aegista subchinensis (N=36)	***Aegista diversifamilia*** (N=43)	
	Mean±SE	**Mean±SE**	**Statistical difference**
whorls	7.21±0.04	7.23±0.05	M
shell width (SW)	1.97±0.02	2.46±0.04	W, *p*=9.80E-16
shell height (SH)	1.04±0.01	1.21±0.02	M, *p*=2.09E-6
aperture width (AW)	0.75±0.01	0.97±0.02	W, *p*=1.23E-15
aperture height (AH)	0.53±0.01	0.74±0.02	W, *p*=3.47E-15
umbilicus width (UW)	0.72±0.01	0.95±0.02	M, *p*=3.33E-13
body whorl height (BH)	0.57±0.01	0.68±0.01	W, *p*=3.83E-11
secondary body whorl height (SBH)	0.10±0.00	0.10±0.00	M
Angle of apex (AA)	150.8±0.65	154.7±0.67	M, *p*=2.23E-4
First whorl width (FW)	0.14±0.00	0.16±0.00	M, *p*=5.77E-5
2nd whorl width (2W)	0.06±0.00	0.07±0.00	M, *p*=1.21E-5
3rd whorl width (3W)	0.08±0.00	0.10±0.00	M, *p*=9.74E-11
4th whorl width (4W)	0.12±0.00	0.14±0.00	M, *p*=8.23E-8
5th whorl width (5W)	0.16±0.00	0.19±0.00	M, *p*=6.37E-12
6th whorl width (6W)	0.20±0.00	0.25±0.00	M, *p*=4.03E-11
SH/SW	0.53±0.01	0.49±0.00	W, *p*=7.71E-9
AW/SW	0.38±0.00	0.39±0.00	M, *p*=2.37E-3
UW/SW	0.37±0.00	0.39±0.01	M
AH/SH	0.51±0.01	0.62±0.01	W, *p*=9.84E-12
AH/AW	0.70±0.01	0.76±0.01	W, *p*=9.18E-6
SH/UW	1.45±0.02	1.28±0.03	M, *p*=5.42E-6
AW/UW	1.05±0.01	1.03±0.02	M
AH/UW	0.73±0.01	0.79±0.02	M, *p*=7.21E-4
BH/SW	0.29±0.00	0.28±0.00	M, *p*=4.70E-6
BH/SH	0.55±0.00	0.57±0.01	W, *p*=4.40E-3
BH/UW	0.79±0.01	0.73±0.02	M, *p*=2.43E-3
SBH/BH	0.68±0.01	0.78±0.01	W, *p*=7.14E-9
SBH/SW	0.05±0.00	0.04±0.00	W, *p*=6.84E-7
SBH/SH	0.10±0.00	0.08±0.00	W, *p*=2.35E-5
SBH/UW	0.15±0.01	0.11±0.00	W, *p*=2.76E-8
FW/SW	0.07±0.00	0.06±0.00	M, *p*=4.80E-6
2W/3W	0.75±0.03	0.68±0.01	M
3W/4W	0.68±0.02	0.72±0.01	M
4W/5W	0.76±0.01	0.70±0.01	M, *p*=6.09E-4
5W/6W	0.79±0.01	0.77±0.01	W

M: Mann-Whitney *U* test; W: Welch's *t* test.

field investigation (Huang et al. 2013). The northernmost distribution is limted by the Lanyang River. We suggest that the Lanyang River is the putative biogeographic boundary between *A. diversifamilia* sp. n. and *A. subchinensis*.

Figure 8. Living snail *Aegista diversifamilia* sp. n. from Heren, Xiulin Township, Hualien County, Taiwan (sampling site 19 in Table 1).

Ecology. Live snails are generally found on the ground or under leaf litter in shady, moist environments in lowland hardwood forests (Figure 8). Eggs white and round, approximately 3 mm in diameter with 20–30 eggs in each spawn (personal observation of reared snail in laboratory).

Remarks. *Aegista diversifamilia* sp. n. can be distinguished from *A. subchinensis* by its overall larger shell width (1.98–3.24 cm), whorl width and aperture, more depressed shell, and wider umbilicus (0.77–1.59 cm) and larger apex angle (148.56°–165.02°) (see Suppl. material 1, Table S1). For the genital morphology, *A. diversifamilia* sp. n. was distinguished from *A. subchinensis* by thicker and about three times logner albumen gland, larger and about two times longer dart-sac of auxiliary copulatory organ and epiphallial flagellum. The length of hermaphroditic duct/ albumen gland ratio was three times larger in *A. diversifamilia* sp. n. than in *A. subchinensis*.

The morphological divergence between the eastern and the western *A. subchinensis* was firstly noticed by Lee and Chen (2003), who found that the shells from the western population were roughly one third smaller than those from the eastern population. When newly describing *A. caperata*, Lee and Wu (2004) suggested the presence of cryptic species within *A. subchinensis* from different sides of the CMR. Wen and Hwang (2014) compared reproductive system between subgenus *Aegista* and

Plectotropis. *Aegista* (*Aegista*) *subchinensis* and *A.* (*Plectotropis*) *mackensii* were dissected as representative species. Wen and Hwang (2014) mentioned there were two lobes of mucus glands of *A. subchinensis*. According to the shell masurements, sampling locality (Xiulin Township, Hualien County) and the illustration of genital morphology figure 1 of *A. subchinensis* was actually the nominal new species presented here, *A. diversifamilia* sp. n. It might suggested that the number of lobes of mucus glands is a variable characteristic in *A. diversifamilia* sp. n. .

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Supplementary material 1

Gene trees of maximum likelihood and Bayesian inference and the morphological measurements of *Aegista diversifamilia* sp. n. and *A. subchinensis*.

Authors: Chih-Wei Huang, Yen-Chen Lee, Si-Min Lin, Wen-Lung Wu

Data type: phylogenetic tree/measurement

Explanation note: Figure S1. Maximum likelihood phylogeny of mitochondrial COI gene. Branch support confidences are shown in bootstrap and approximate likelihood-ratio test. Figure S2. Maximum likelihood phylogeny of mitochondrial 16S gene. Branch support confidences are shown in bootstrap and approximate likelihood-ratio test. Figure S3. Maximum likelihood phylogeny of nuclear ITS2 gene. Branch support confidences are shown in bootstrap and approximate likelihood-ratio test. Figure S4. Bayesian phylogeny of mitochondrial COI gene. Figure S5. Bayesian phylogeny of mitochondrial 16S gene. Figure S6. Bayesian phylogeny of nuclear ITS2 gene. Table S1. Morphological measurements of *Aegista diversifamilia* sp. n. and *A. subchinensis*.

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A new quadrannulate species of *Orobdella* (Hirudinida, Arhynchobdellida, Orobdellidae) from central Honshu, Japan

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Abstract

A new quadrannulate species of *Orobdella*, *Orobdella masaakikuroiwai* **sp. n.**, from the mountainous region of central Honshu, Japan is described. This is only the second small species known within this genus, with a body length of less than 4 cm for mature individuals. Phylogenetic analyses using nuclear 18S rDNA and histone H3 as well as mitochondrial COI, $tRNA^{Cys}$, $tRNA^{Met}$, 12S, $tRNA^{Val}$, 16S, and ND1 markers showed that *O. masaakikuroiwai* **sp. n.** is the sister species of the quadrannulate *O. whitmani* Oka, 1895. Phylogenetic relationships within *O. masaakikuroiwai* **sp. n.** conducted using mitochondrial markers reveled a distinction between eastern and western phylogroups.

Keywords

Hirudinea, Hirudinida, *Orobdella*, new species, gastroporous, molecular phylogeny, Japan

Introduction

The genus *Orobdella* Oka, 1895 is an East Asian terrestrial macrophagous leech taxon assigned to the family Gastrostomobdellidae Richardson, 1971, along with the Southeast Asian terrestrial macrophagous genus *Gastrostomobdella* Moore, 1929 (Richardson 1971). Gastrostomobdellidae was once classified within the suborder Hirudiniformes, which includes jawed blood-feeding taxa (Sawyer 1986). Recent molecular phyloge-

netic studies revealed that *Orobdella* is part of the suborder Erpobdelliformes, which contains only predaceous leech taxa (Nakano et al. 2012, Oceguera-Figueroa et al. 2011). The monotypic family Orobdellidae Nakano, Ramlah & Hikida, 2012 was established for *Orobdella* based on both morphological differences between *Orobdella* and *Gastrostomobdella* (gastroporal duct of *Orobdella* is tubular and positioned on top of the female organ while in *Gastrostomobdella*, this duct is columnar and vertical in position to the gastropore) and the results of an analysis by Nakano et al. (2012) which rejected the monophyly of these two taxa. Despite their failure to reconstruct the precise phylogenetic relationships of *Orobdella* and *Gastrostomobdella* within Erpobdelliformes, the classification by Nakano et al. (2012) is followed here.

Orobdella now consists of 11 nominal leech species from East Asia: nine species known from the Japanese Archipelago (Nakano 2010, 2011b, 2012a, b, c); one present in the Korean Peninsula and adjacent islands (Nakano 2011a, Nakano and Seo 2012, 2014); and the remainder in Taiwan (Nakano and Lai 2012). Species of *Orobdella* are usually large in size, with the body length of mature individuals reaching to around 10 cm (e.g. Nakano (2011b)). The largest species in this genus is the octannulate *O. octonaria* Oka, 1895 recorded from Honshu, Japan, with a body length often greater than 20 cm (Nakano 2012c, Oka 1895). In contrast, the smallest species is the quadrannulate *O. koikei* Nakano, 2012b found in Hokkaido, Japan with a body length of less than 4 cm, but which were considered to be mature due to the presence of developed testisacs. It is also noteworthy that the distribution of *O. koikei* in Hokkaido overlaps with that of the quadrannulate *O. kawakatsuorum* Richardson, 1975, which is present in Hokkaido as well as its adjacent islands and attains a body length of ca. 10 cm (Nakano 2012b, Nakano and Gongalsky 2014).

Several small *Orobdella* leeches were recently collected from east-central Honshu, Japan. Although the bodies of the specimens are up to 3.5 cm in length, some of them already possess an obvious clitellum and they are thus considered to be mature individuals. These leeches are described herein as a new species. The phylogenetic position of this new species was reconstructed using nuclear 18S and histone H3 (H3), and mitochondrial COI, $tRNA^{Cys}$, $tRNA^{Met}$, 12S, $tRNA^{Val}$ and 16S rDNA, and ND1 sequence data.

Materials and methods

Sampling and morphological examination

Leeches were collected from seven localities in east-central Honshu, Japan (Fig. 1). These seven collection localities are numbered referring to the locality name listed in Table 1. When possible, altitudes above sea level and geographical coordinates for localities were obtained using a Garmin eTrex® GPS unit.

The specimens were relaxed by the gradual addition of absolute ethanol to fresh water. For DNA extraction, botryoidal tissue was taken from the posterior part of the body around the caudal sucker of every specimen, and then preserved in abso-

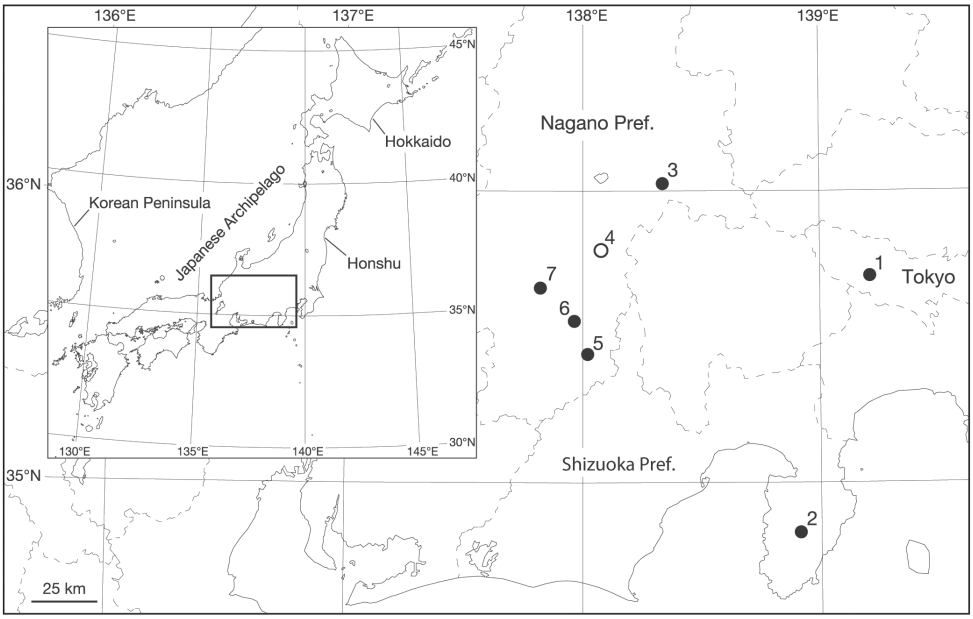

Figure 1. Map showing the collection localities of the specimens examined in this study. Open circle (4) indicates the *Orobdella masaakikuroiwai* sp. n. type locality, and closed circles (1–3, 5–7) indicate additional localities.

Table 1. Collection localities in this study with the information on locality names.

Locality number	Locality name
1	Akiruno, Tokyo Metropolis, Japan
2	Namesawakeikoku Valley, Izu Shizuoka Prefecture, Japan
3	Shibunoyu, Kitayama, Chino, Nagano Prefecture, Japan
4	Mt. Mitsugaisan, Ina, Nagano Prefecture, Japan
5	Shirabisotoge Pass, Ida, Nagano Prefecture, Japan
6	Ikuta, Matsukawa, Nagano Prefecture, Japan
7	Shiojidaira Nature Park, Iizuna, Nagano Prefecture, Japan

lute ethanol. The rest of the body was fixed in 10% formalin and then preserved in 70% ethanol. Four measurements were taken: body length (BL) from the anterior margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width (BW), caudal sucker length (CL) from the anterior to the posterior margin of the sucker, and caudal sucker width (CW) from the right margin to the left margin of the sucker. Examination, dissection, and drawing of the specimens were accomplished using a stereoscopic microscope with a drawing tube (Leica M125). Specimens used in this study have been deposited in the Zoological Collection of Kyoto University (KUZ).

The numbering convention is based on Moore (1927): body somites are denoted by Roman numerals, and the annuli in each somite are given alphanumeric designations.

PCR and DNA sequencing

The extraction of genomic DNA from botryoidal tissues preserved in absolute ethanol followed Nakano (2012b). Primer sets for the PCR and cycle sequencing (CS) reactions used in this study were as follows: for 18S, A and L (PCR and CS), C and Y (PCR and CS), and O and B (PCR and CS) (Apakupakul et al. 1999); for histone H3 (H3), H3aF and H3bR (PCR and CS) (Colgan et al. 1998); for COI, LCO 1490 (PCR and CS) and HCO 2198 (CS) (Folmer et al. 1994), and LCO-in (CS) and HCO-out (PCR and CS) (Nakano 2012b); for $tRNA^{Cys}$, $tRNA^{Met}$, 12S, $tRNA^{Val}$, and 16S ($tRNA^{Cys}$–16S), 12SA-out (PCR and CS) and 12SB-in (CS), and 12SA-in (CS) and 12SB-out (PCR and CS) (Nakano 2012b); for $tRNA^{Leu}$ and ND1 ($tRNA^{Leu}$–ND1), LND3000 and HND1932 (PCR and CS) (Light and Siddall 1999). The PCR reaction and DNA sequencing were performed using the modified methods outlined by Nakano (2012a). The 18S, H3 and ND1, and COI and $tRNA^{Cys}$–16S reactions were respectively performed using a GeneAmp PCR System 2700 and a GeneAmp PCR System 9700 (Applied Biosystems). The PCR reaction mixtures were heated to 94 °C for 5 min, followed by 40 cycles at 94 °C (10 s each), 48 °C for 18S, H3, and $tRNA^{Leu}$–ND1 or 45 °C for COI and $tRNA^{Cys}$–16S (20 s), and 72 °C (48 s for 18S, H3 and $tRNA^{Leu}$–ND1 or 1 min 12 s for COI and $tRNA^{Cys}$–16S), and a final extension at 72 °C for 6 min. The sequencing mixtures were heated to 96 °C for 2 min, followed by 40 cycles at 96 °C (10 s each), 50 °C (5 s each), and 60 °C (48 s each). The obtained sequences were edited using DNA BASER (Heracle Biosoft S.R.L.). The DNA sequences listed in Table 2 were newly obtained in this study, and were deposited with the International Nucleotide Sequence Database Collaboration (INSDC).

Molecular phylogenetic and genetic distance analyses

Sixty-six previously published sequences (Nakano 2012a, b, Nakano and Gongalsky 2014, Nakano and Lai 2012, Nakano et al. 2012, Nakano and Seo 2014) were obtained from the INSDC and used for the molecular phylogenetic analyses (Table 2). Four erpobdelliform species, *Erpobdella japonica* Pawłowski, 1962, *G. monticola* Moore, 1929, *Mimobdella japonica* Blanchard, 1897, and *Odontobdella blanchardi* (Oka, 1910), were used as outgroup taxa.

The phylogenetic position of the new species within the genus *Orobdella* was estimated based on sequences of nuclear 18S and H3 and mitochondrial COI, $tRNA^{Cys}$–16S, and ND1. Sequences of nuclear H3 and mitochondrial COI were aligned by eye because there were no indels. Nuclear 18S and mitochondrial $tRNA^{Cys}$–16S and $tRNA^{Leu}$–ND1 were aligned using MATTF L-INS-I (Katoh et al. 2005). Then, the $tRNA^{Leu}$ region was removed from each sequence of $tRNA^{Leu}$–ND1. The length of the aligned 18S sequences was 1845 bp, that of H3 was 327 bp, that of COI was 1266 bp, that of $tRNA^{Cys}$–16S was 1107 bp, and that of ND1 was 633 bp. The concatenated sequences thus yielded 5,124 bp positions.

Table 2. Samples used for the phylogenetic analyses. The information on the vouchers is accompanied by the collection locality numbers for *Orobdella masaakikuroiwai* sp. n. (see Fig. 1 and Table 1) and the INSDC accession numbers. Acronym: KUZ, the Zoological Collection of Kyoto University; UNIMAS, the Universiti Malaysia Sarawak.

Species	Voucher	18S	Histone H3	COI	tRNACys–16S	tRNALeu–ND1
Orobdella masaakikuroiwai sp. n.	KUZ Z694 Holotype (4)	AB938003	AB938013	AB938006	AB937997	AB938016
Orobdella masaakikuroiwai sp. n.	KUZ Z684 (1)			AB938010	AB938001	AB938020
Orobdella masaakikuroiwai sp. n.	KUZ Z687 (2)			AB938011	AB938002	AB938021
Orobdella masaakikuroiwai sp. n.	KUZ Z689 (5)			AB938005	AB937996	AB938015
Orobdella masaakikuroiwai sp. n.	KUZ Z696 (7)			AB938007	AB937998	AB938017
Orobdella masaakikuroiwai sp. n.	KUZ Z697 (6)			AB938008	AB937999	AB938018
Orobdella masaakikuroiwai sp. n.	KUZ Z699 (3)			AB938009	AB938000	AB938019
Orobdella dolichopharynx Nakano, 2011b	KUZ Z120 Holotype	AB663665e	AB698876a	AB679680b	AB679681b	AB828558f
Orobdella esulcata Nakano, 2010	KUZ Z29 Holotype	AB663655e	AB698873a	AB679664b	AB679665b	AB828555f
Orobdella ijimai Oka, 1895	KUZ Z110 Topotype	AB663659e	AB698877a	AB679672b	AB679673b	AB828559f
Orobdella kawakatsuorum Richardson, 1975	KUZ Z167 Topotype	AB663661e	AB698878a	AB679704b	AB679705b	AB828561c
Orobdella ketagalan Nakano and Lai, 2012	KUZ Z208 Holotype	AB704785d	AB704786d	AB704787d	AB828582f	AB828563f
Orobdella koikei Nakano, 2012b	KUZ Z156 Holotype	AB698883e	AB698882a	AB679688b	AB679689b	AB828560c
Orobdella mononoke Nakano, 2012a	KUZ Z224 Holotype	AB698868e	AB698869a	AB698866a	AB698867a	AB828564f
Orobdella octonaria Oka, 1895	KUZ Z181 Topotype	AB698870e	AB698871a	AB679708b	AB679709b	AB828562f
Orobdella shimadae Nakano, 2011b	KUZ Z128 Holotype	AB663663e	AB698875a	AB679676b	AB679677b	AB828557f
Orobdella tsushimensis Nakano, 2011a	KUZ Z134 Holotype	AB663653e	AB698872a	AB679662b	AB679663b	AB828554f
Orobdella whitmani Oka, 1895	KUZ Z45 Topotype	AB663657e	AB698874a	AB679668b	AB679669b	AB828556c
Erpobdella japonica Pawłowski, 1962	KUZ Z178	AB663648e	AB698879a	AB679654b	AB679655b	AB828542f
Gastrostomobdella monticola Moore, 1929	UNIMAS/A3/BH01/10	AB663649e	AB698880a	AB679656b	AB679657b	AB828543f
Mimobdella japonica Blanchard, 1897	KUZ Z179	AB663650e	AB698881a	AB679658b	AB679659b	AB828544f
Odontobdella blanchardi (Oka, 1910)	KUZ Z180	AB663651e	AB938012	AB938004	AB937995	AB938014

Sources: a Nakano (2012a); b Nakano (2012b); c Nakano and Gongalsky (2014); d Nakano and Lai (2012); e Nakano et al. (2012); f Nakano and Seo (2014).

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) models. ML phylogenies were calculated using TREEFINDER v. October 2008 (Jobb et al. 2004) with the PHYLOGEARS v. 2.0 tool package (Tanabe 2008), followed by nonparametric bootstrapping (BS) (Felsenstein 1985) conducted with 1,000 replicates. The best-fit models for each partition were selected based on the Akaike Information Criterion (Akaike 1974) using KAKUSAN4 (Tanabe 2011): for 18S, TN93 with gamma distribution (+G) and proportion of invariant sites (+I); for the first, second, and third positions of H3, respectively, a homogenous (+H) TN93 model, JC69+H, and J2+G; for the first, second, and third positions of COI, respectively, TN93+G+I, TVM+I, and TIM+G; for $tRNA^{Cys}$–16S, GTR+G; and for the first, second, and third positions of ND1, respectively, GTR+G+I, HYK85+G, and J2+G. BI and Bayesian posterior probabilities (BPPs) were estimated using MRBAYES v. 3.2 (Ronquist et al. 2012). The best-fit models for each partition were identified with the Bayesian information criterion (Schwarz 1978) using KAKUSAN4: for 18S, K80+G; for the first, second and third positions of H3, respectively, JC69+H, JC69+H, and HKY+G; for the first, second, and third positions of COI, respectively, GTR+G+I, F81+I, and HKY+G; for $tRNA^{Cys}$–16S, GTR+G; and for the first, second, and third positions of ND1, respectively, GTR+G, HKY85+G, and HKY85+G. Two independent runs of four Markov chains were conducted for 10 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using TRACER v. 1.5 (Rambaut and Drummond 2009), and the first 25,001 trees were discarded based on these results.

The phylogenetic relationships of the specimens of the new species were reconstructed based on sequences of mitochondrial regions. The alignment of the sequences as well as the reconstruction of the ML and BI phylogenies was accomplished followed the methods described above. The length of the aligned COI was 1266 bp, that of $tRNA^{Cys}$–16S was 1056 bp, and that of ND1 was 579 bp. Thus, the concatenated sequences yielded 2,901 bp positions. The best-fit models for each partition selected for the ML phylogenies were as follows: for the first, second, and third positions of COI, respectively, TN93+G, TVM+H, and TN93+G; for $tRNA^{Cys}$–16S, GTR+G; and for the first, second, and third positions of ND1, respectively, TN93+G, HKY85+H, and HYK85+G. The best-fit models identified for each partition for the BI analyses were as follows: the first, second, and third positions of COI, respectively, GTR+G, F81+H, and HKY85+G; for $tRNA^{Cys}$–16S, GTR+G; and for the first, second, and third positions of ND1, respectively, GTR+G, F81+H, and HKY85+G. For BI and BPPs, two independent runs of four Markov chains were conducted for 6 million generations, and the tree was sampled every 100 generations. The first 15,001 trees were eliminated based on the results of the parameter estimates and convergence.

Nodes with BS values higher than 70% were considered sufficiently resolved (Hillis and Bull 1993). Nodes with BPPs higher than 95% were considered statistically significant (Leaché and Reeder 2002).

Pairwise comparisons of the Kimura-2-parameter (K2P) distance (Kimura 1980) for the COI sequences (1266 bp) obtained from the specimens of the new species were calculated using MEGA5 (Tamura et al. 2011).

Taxonomy

Family Orobdellidae Nakano, Ramlah & Hikida, 2012
http://zoobank.org/5F5BABE8-BD26-4FC7-9593-F73E62E26122
Genus *Orobdella* Oka, 1895
http://zoobank.org/FA8333ED-8C17-41FD-AFC1-62A4F98D4AC1

***Orobdella masaakikuroiwai* sp. n.**
http://zoobank.org/72F9627C-763A-49D9-9C97-E15B2FD856AA
Figs 2–5

Diagnosis. Body length of mature individual less than 4 cm. Somite IV uniannulate, somites VIII–XXV quadrannulate. Clitellum in XI b5 to XIII a2. Pharynx reaching to XIV. Gastropore conspicuous in middle of XIII a1. Gastroporal duct bulbous, winding at junction with gastropore. Male gonopore in middle of XI b6, female gonopore inconspicuous in middle of XIII a1, behind gastropore, gonopores separated by 1/2 + 4 + 1/2 annuli. Paired epididymides in XV/XVI–XVI b5/b6 to XVII b5/b6–XVIII/XIX, occupying 7–10 annuli (i.e. one and a half to two and a half somites). Atrial cornua developed, ovate.

Type materials (see Fig. 1 for the locality number). Holotype. KUZ Z694, holotype, dissected, collected from under a rock along a forest road at Mt. Mitsugai-san, Ina, Nagano Pref., Japan (35°47.72'N, 138°04.70'E; Alt. 875 m; locality number 4), by TN on 20 July 2012.

Paratypes. Four paratypes from the type locality by TN on 20 July 2012: KUZ Z690, Z691 (35°47.72'N, 138°04.69'E; Alt: 872 m), and KUZ Z692, Z693 (35°47.74'N, 138°04.69'E; Alt: 872 m). KUZ Z693, dissected.

Additional materials (see Fig. 1 and Table 1 for the locality numbers). In total, 11 specimens examined. KUZ Z684–Z686 (three specimens), collected from under rocks in Akiruno (locality number 1), by TN: KUZ Z684, from along a mountain trail at Mt. Kariyoseyama (35°42.37'N, 139°12.03'E; Alt. 341 m) on 29 March 2010; KUZ Z685, from along Ohikagedori Road (35°43.33'N, 139°11.98'E; Alt. 230 m) on 30 March 2010; KUZ Z686, from along Bonborisen Forest Road (35°47.73'N, 139°11.01'E; Alt. 284 m) on 30 March 2010. KUZ Z687, Z688 (two specimens), from under rocks along a forest road in Namesawakeikoku Valley (locality number 2), by TN on 9 July 2011: KUZ Z687 (34°50.59'N, 138°54.69'E; Alt. 551 m); KUZ Z688 (34°50.50'N, 138°54.59'E; Alt. 576 m). KUZ Z689, from under fallen leaves along a forest road at Shirabisotoge Pass (35°26'N, 138°01'E; Alt. 1840 m; locality number 5), by Yoshiko Yamane on 14 October 2011. KUZ Z695, Z696 (two specimens), from under rocks in Shiojidaira Nature Park (locality no 7), by TN on 10 August 2012: KUZ Z695 (35°40.62'N, 137°50.48'E; Alt. 1304 m); KUZ Z696 (35°40.66'N, 137°50.48'E; Alt. 1315 m). KUZ Z697, Z698 (two specimens), from under rocks along a mountain stream in Ikuta (locality no 6), by TN on 10 August 2012: KUZ Z697 (35°33.67'N, 138°00.04'E; Alt. 1098 m); KUZ Z698 (35°33.68'N, 138°00.04'E; Alt. 1099 m). KUZ Z699, from under fallen leaves near Shibunoyu

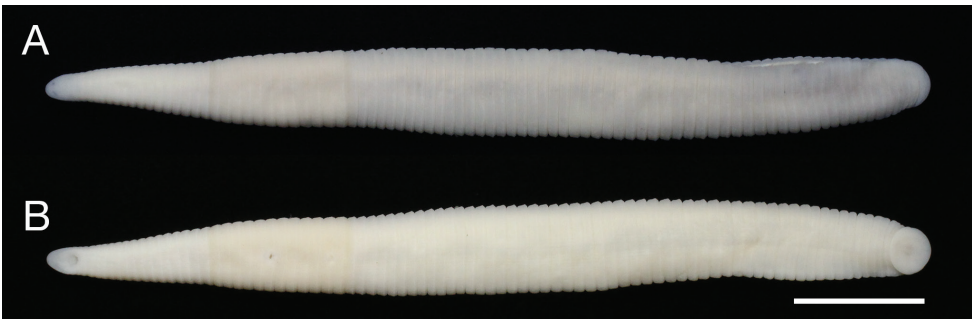

Figure 2. *Orobdella masaakikuroiwai* sp. n., holotype, KUZ Z694. **A** Dorsal and **B** ventral views. Scale bar, 5 mm.

(36°02.1'N, 138°19.5'E; Alt. 1860 m; locality number 3), by Yume Imada on 6 October 2012. KUZ Z684, Z687, Z689, Z696, Z697 and Z699 (six specimens), dissected.

Etymology. The specific name is a noun in the genitive case formed directly from the name of Mr Masaaki Kuroiwa, who generously accompanied the field survey in Nagano Prefecture.

Description of holotype. Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 34.0 mm, BW 3.42 mm (Fig. 2). Caudal sucker ventral, elliptic, CL 1.7 mm (minor axis), CW 1.9 mm (major axis) (Figs 2B, 3D).

Somite I completely merged with prostomium (Fig. 3A). Somites II–IV uniannulate, II not separated from I (Fig. 3A). Somite V biannulate, (a1 + a2) = a3; a3 forming posterior margin of oral sucker (Fig. 3A, B). Somites VI, VII triannulate, a1 = a2 = a3 (Fig. 3A, B). Somites VIII–XXV quadrannulate, a1 = a2 = b5 = b6 (Fig. 3A–E); b5 of X and a2 of XIII respectively being first and last annuli of clitellum (Fig. 3E). Somite XXVI triannulate, with slight furrow in a3, a1 > a2 < a3 (b5 = b6); a3 being ventrally last complete annuls (Fig. 3C, D). Somite XXVII biannulate, with slight dorsal furrow in last annulus; anus behind it with no post-anal annulus (Fig. 3C).

Anterior ganglionic mass in VI a2 and a3. Ganglia VII–X, of each somite, in a2 (Fig. 4A). Ganglion XI in a2 and b5 (Fig. 4A). Ganglia XII–XVIII, of each somite, in a2 (Fig. 4A). Ganglia XIX, XX, of each somite, in a1 and a2. Ganglia XXI, XXII, of each somite, in a2. Ganglion XXIII in a1 and a2. Ganglion XXIV in a1. Ganglion XXV in XXIV b6 and XXV a1. Ganglion XXVI in b5 and b6 of XXV. Posterior ganglionic mass in a1–a3 of XXVI.

Eyes in three pairs, first pair dorsally on anterior margin of III, second and third pairs dorsolaterally on posterior margin of V (a1 + a2) (Fig. 3A). Nephridiopores in 17 pairs, one each situated ventrally at posterior margin of a1 of each somite in VIII–XXIV (Fig. 3B, E). Papillae numerous, minute, hardly visible, one row on every annulus.

Pharynx agnathous, euthylaematous, reaching to XIV a1/a2 (Fig. 3G). Crop tubular, reaching to XIX b5/b6 (Fig. 3G). Gastropore conspicuous, ventral in middle of XIII a1 (Fig. 3E, F). Gastroporal duct bulbous, slightly winding at junction with

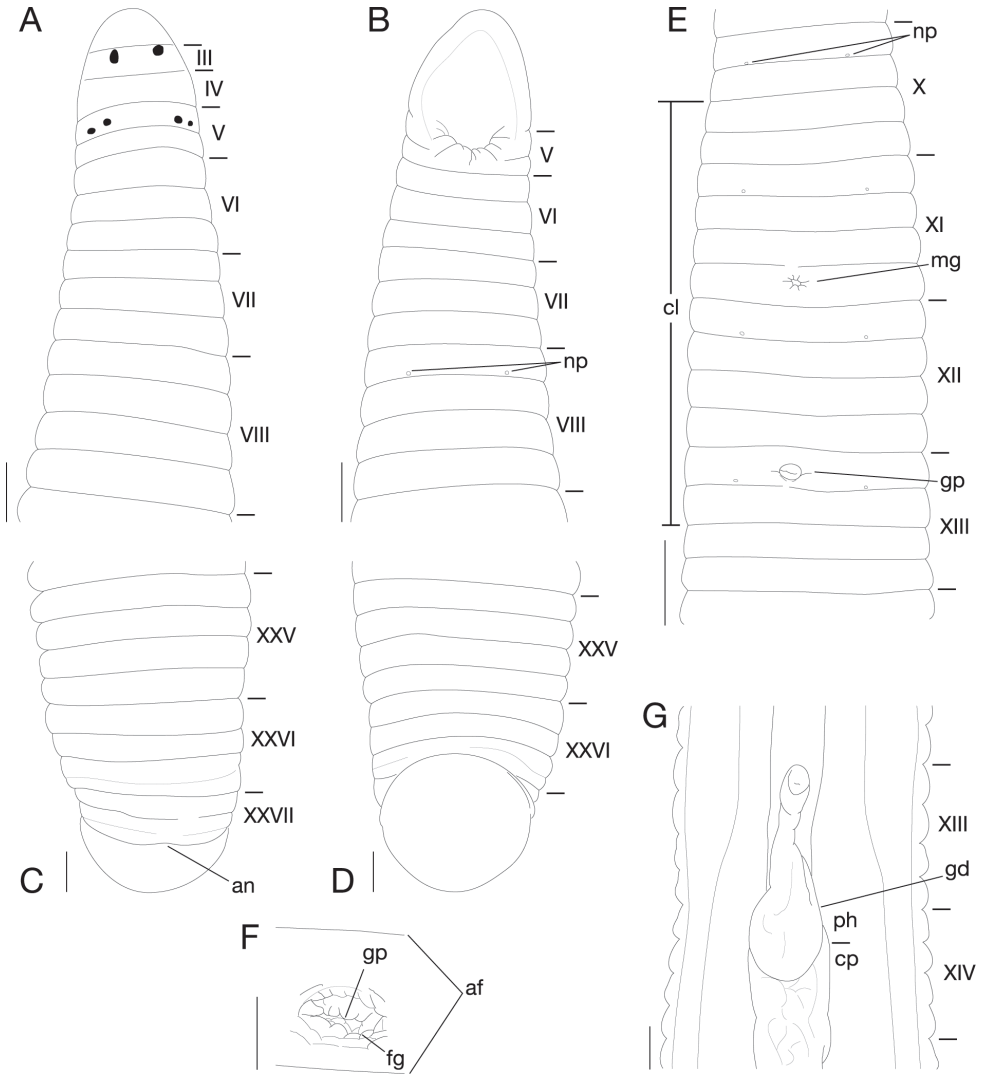

Figure 3. *Orobdella masaakikuroiwai* sp. n., holotype, KUZ Z694. **A** Dorsal and **B** ventral views of somites I–VIII. **C** Dorsal and **D** ventral views of somites XXV–XXVII and caudal sucker. **E** Ventral view of somites X–XIII. **F** Ventral view of gastropore and female gonopore. G Ventral view of gastroporal duct. Scale bars, 1 mm (**E**), 0.5 mm (**A**–**D**, **G**) and 0.25 mm (**F**). Abbreviations: af, annular furrow; an, anus; cl, clitellum; cp, crop; fg, female gonopore; gd, gastroporal duct; gp, gastropore; mg, male gonopore; np, nephridiopore; and ph, pharynx.

gastropore, joining with crop in XIV b5 (Fig. 3G). Intestine tubular, acecate, reaching to XXIV a1/a2. Rectum tubular, thin-walled, descending to anus.

Male gonopore in middle of XI b6 (Fig. 3E). Female gonopore in middle of XIII a1, inconspicuous, located posterior to gastropore (Fig. 3G). Gonopores separated by 1/2 + 4 + 1/2 annuli (Fig. 3E). Testisacs multiple, one or two on each side in each an-

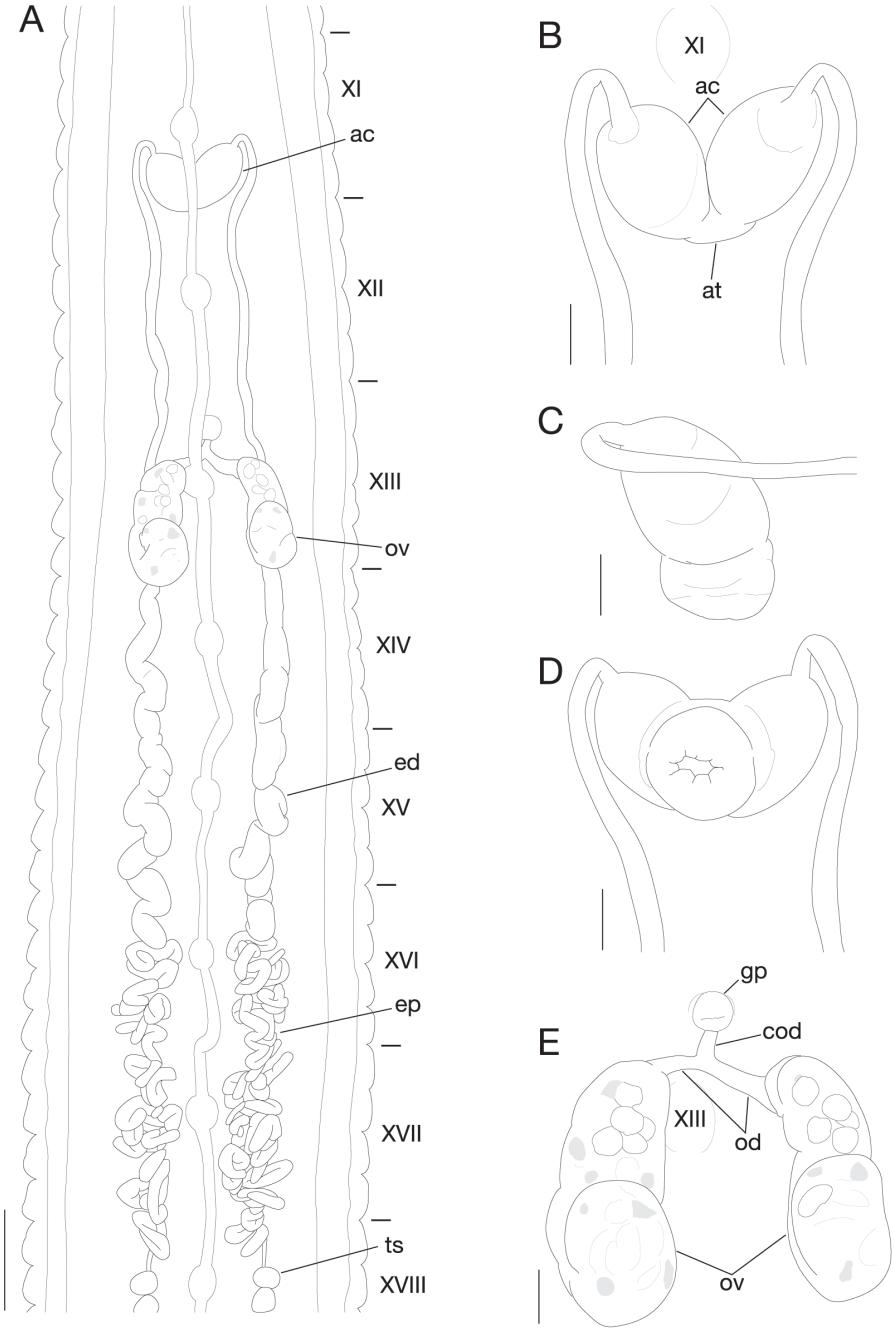

Figure 4. *Orobdella masaakikuroiwai* sp. n., holotype, KUZ Z694. **A** Dorsal view of reproductive system including ventral nervous system. **B** Dorsal, **C** lateral, and **D** ventral views of male atrium: **B** including position of ganglion XI. **E** Dorsal view of female reproductive system including position of ganglion III. Scale bars, 1 mm (**A**) and 0.25 mm (**B**–**E**). Abbreviations: ac, atrial cornua; at, atrium; cod, common oviduct; ed, ejaculatory duct; ep, epididymis; gp, gastropore; od, oviduct; ov, ovisac; and ts, testisacs.

nulus, in XVIII a2 to XXV a1 (Fig. 4A). Paired epididymides in XVI a2 to XVIII a1, occupying 8 annuli (Fig. 4A). Ejaculatory bulbs absent. Paired ejaculatory ducts in XI a2/b5 to XVI a2, coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then loosely curved in position anterior to ovisacs; each widening from respective junction with epididymis, narrowing at junction with atrial cornua, then turning sharply inward toward atrial cornua with pre-atrial loop reaching to anterior margin of XI b5 (Fig. 4A). Pair of muscular atrial cornua ovate, in XI b5 and b6 (Fig. 4A–D). Atrium short, muscular, globular in XI b6 (Fig. 4B–D). Penis sheath and penis absent. Paired ovisacs elongated globular, one each in XIII a2–b6 (Fig. 4A, E). Oviducts thin-walled, left oviduct crossing ventrally beneath nerve cord; both oviducts converging into common oviduct in XIII a1/a2 (Fig. 4A, E). Common oviduct thin-walled, short, directly descending to female gonopore (Fig. 4E).

Variation. BL 22.4 (KUZ Z686) –35.2 (KUZ Z684) mm, BW 2.3 (KUZ Z691) –3.5 (KUZ Z684) mm, CL 1.1 (KUZ Z686)–1.7 (KUZ Z693) mm, CW 1.1 (KUZ Z686)–2.1 (KUZ Z689) mm. Somites III, IV uniannulate, each with slight dorsal furrow (KUZ Z695). Somite XXVI variable; often dorsally quadrannulate, ventrally triannulate, rarely with slight ventral furrow in a3; KUZ Z699 with quadrannulate; KUZ Z698, Z691 with triannulate with slight dorsal furrow in a3; KUZ Z689 with triannulate. Somite XXVII biannulate, or uniannulate with slight dorsal furrow. Eyes in three pairs; KUZ Z699 with one eye dorsoleft on posterior margin of III. Pharynx reaching to XIII/XIV–XIV a2/b5. Crop reaching to XIX b5/b6–XX a1. Gastropore occasionally slightly posterior to middle of XIII a1. Gastroporal duct joining with crop in XIV a1/a2–XIV b6; KUZ Z687 with thick, tubular duct. Intestine reaching to XXIII/XXIV–XXV a2. Male gonopore rarely slightly anterior to middle of XI b6, or slightly posterior to middle of XI b6. Female gonopore occasionally slightly posterior to middle of XIII a1. Testisacs in XVII b6–XIX a1 to XXIV b5–XXV a2. Epididymides in XV/XVI–XVI b5/b6 to XVII b5/b6–XVIII/XIX; occupying 7–10 annuli. Atrial cornua generally ovate; KUZ Z696 ellipsoid; KUZ Z687 fusiform. Pre-atrial loop absent, or reaching to middle of XI b5 (KUZ Z693, Z697). Ovisacs often in XIII a2–b6; KUZ Z687, Z699 in XIII a2, b5; KUZ Z696 right one in XIII a2–XIV a1/a2, left one in XIII a2–XIV a1. Right or left oviduct crossing ventrally beneath nerve cord; KUZ Z684, Z693 both oviducts converging into common oviduct in XIII a2.

Coloration. In life, dorsal surface ochre (Fig. 5), whitish brown, or brown, ventral surface grayish white or yellowish white; individuals from Shizuoka Pref. (KUZ Z687, Z688), dorsal surface whitish yellow. Colour faded in preservative, rarely with one dorsal black line from VII a3–IX a2 to XIX b5–XXVI b6 (KUZ Z691, Z693, Z694, Z698).

Figure 5. *Orobdella masaakikuroiwai* sp. n., paratype, KUZ Z690. **A** Dorsal view of live animal. **B** Live animal found curled up under a stone at the type locality: scale bar, 2 mm.

Distribution (see Fig. 1 for the locality numbers). This species was primarily collected from localities in Nagano Prefecture: the east-central part (locality number 3), and the southeastern part along the Inadani Basin (locality numbers 4–7). This species was also found in the western mountainous part of the Metropolitan Tokyo area (locality number 1), as well as in the Amagi Mountain Range in the central part of the Izu Peninsula, Shizuoka Prefecture (locality number 2). The locality data for this species suggested that *O. masaakikuroiwai* sp. n. would be widely distributed in mountainous regions such as the southwestern part of the Kanto Region and the southeastern part of the Chubu Region, Honshu, Japan. The lowest elevation among the localities was 230 m above sea level (a.s.l.) (locality number 1), and the highest was ca. 1860 m a.s.l. (locality number 3).

Natural history. This species was generally found curled up under rocks or fallen leaves in moist mountainous habitats (Fig. 5B). Soil was sometimes observed in the digestive tract during specimen dissection. This species is therefore considered an earthworm-feeder as are the other known *Orobdella* leeches.

Mature leeches with an obvious clitellum were collected on 20 July (KUZ Z690, Z691, Z693, Z694) and 10 August (KUZ Z697) at two sites in Nagano Prefecture (locality numbers 4 and 7, elevation ca. 875 m and 1098 m, respectively). These findings indicate that the reproductive season of this species may begin in mid-to-late July.

Remarks. Although the leech specimens examined in this study were small (up to 35 mm), several individuals, including the holotype, were determined to be mature due to the possession of an obvious clitellum and developed testisacs. Specimen KUZ Z687 possessed a tubular gastroporal duct and fusiform atrial cornua. Immature leeches may have these characteristics, because the sperm ducts and testisacs of specimen KUZ Z687 are undeveloped and barely detectable.

The new species unambiguously belongs to the genus *Orobdella* as it has all the generic diagnostic characteristics (see Nakano et al. (2012) for the generic diagnosis): post-anal annulus absent; pharynx agnathous, euthylaematous; gastropore in XIII; gastroporal duct lying on female organ; gonopores separated by more than one full somite; testisacs multiple; male atrium in XI without penis sheath and penis; ovisacs globular in XIII; female median reproductive system essentially lacking.

According to previous taxonomic studies (Nakano 2010, 2011a, 2012b, Nakano and Gongalsky 2014, Nakano and Lai 2012, Nakano and Seo 2012, 2014), *O. masaakikuroiwai* sp. n. differs from the six other quadrannulate species (i.e., *O. esulcata* Nakano, 2010, *O. kawakatsuorum*, *O. ketagalan* Nakano & Lai, 2012, *O. koikei*, *O. tsushimensis* Nakano, 2011a, and *O. whitmani* Oka, 1895) by the following combination of characteristics (Table 3): body length less than 4 cm, IV uniannulate, gonopores separated by 1/2 + 4 + 1/2, XXV quadrannulate, gastroporal duct bulbous, epididymides in XVI to XVIII, atrial cornua ovate. Among the six above-listed quadrannulate species, only *O. whitmani* is present in Honshu. Both *O. masaakikuroiwai* sp. n. and *O. whitmani* possess 1/2 + 4 + 1/2 annuli between the gonopores, a bulbous gastroporal duct, and epididymides in XVI–XVIII. Thus, it is difficult to distinguish these two species using these diagnostic features. However, *O. whitmani* is a large species and

Table 3. Comparison of morphological characters between *Orobdella masaakikuroiwai* sp. n. and six quadrannulate congeneric species.

Character	*O. masaakikuroiwai* sp. n.	*O. esulcata* Nakano 2010	*O. kawakatsuorum* Richardson, 1975	*O. ketagalan* Nakano & Lai, 2012	*O. koikei* Nakano, 2012b	*O. tsushimensis* Nakano, 2011a	*O. whitmani* Oka, 1895
Body length of mature individual	less than 4 cm	up to ca 10 cm	up to ca 10 cm	up to ca 10 cm	less than 4 cm	up to ca 10 cm	up to ca 10 cm
Annulation of IV	uniannulate	uniannulate	biannulate	uniannulate	uniannulate	uniannulate	uni- or biannulate
Number of annuli between gonopores	1/2 + 4 + 1/2	2/3 + 4 + 1/3	6	1/2 + 4 + 1/2	1/2 + 4 + 1/2	1/2 + 5	1/2 + 4 +1/2
Annulation of XXV	quadrannulate	quadrannulate	quadrannulate	quadrannulate	triannulate	quadrannulate	quadrannulate
Gastroporal duct	bulbous	tubular, but bulbous at junction with gastropore	simple tubular	simple tubular	bulbous	bulbous	bulbous
Epididymides	XVI to XVIII	XVI to XX	XVI to XVII	absent	XV to XX	XVII to XIX	XVI to XVIII
Atrial cornua	ovate	ovate	undeveloped	undeveloped	ovate	ovate	ovate

grows up to ca. 10 cm (Nakano 2010, Oka 1895). Therefore, *O. masaakikuroiwai* sp. n. clearly differs from mature individuals of *O. whitmani* in body length. However, distinguishing the new species from a small juvenile of *O. whitmani* can be complex. Because immature individuals of *O. whitmani* possess a tubular gastroporal duct (Nakano, unpublished observation) and mature individuals of *O. masaakikuroiwai* sp. n. possess a bulbous gastroporal duct, the characteristics of the duct could be used to distinguish between the two. However, insofar as immature leeches of both species have a tubular gastroporal duct, this characteristic is not useful for discriminating between immature individuals of *O. masaakikuroiwai* sp. n. and *O. whitmani*. DNA data might be useful for identification, similar to the DNA barcoding of freshwater leeches (e.g. Oceguera-Figueroa et al. (2010)). In addition to DNA data, interbreeding experiments or karyological studies may be crucial for definitive clarification between *O. masaakikuroiwai* sp. n. and *O. whitmani* as is the case with the species of *Hirudo* Linnaeus, 1758 in Europe (Petrauskiene et al. 2009, Utevsky et al. 2009).

The quadrannulate *O. masaakikuroiwai* sp. n. is unequivocally distinguishable from the four species *O. dolichopharynx* Nakano, 2011b, *O. ijimai* Oka, 1895, *O. mononoke* Nakano, 2012a and *O. shimadae* Nakano, 2011b, due to their sexannulate mid-body somites, as well as *O. octonaria*, which possesses octannulate mid-body somites.

Molecular phylogenies and genetic distances

The ML tree (ln L = –23350.60) (Fig. 6) for estimating the phylogenetic position of the new species had an identical topology to the BI tree (not shown). The monophyly of the genus *Orobdella* was confirmed (BS = 99%, BPP = 100%) The genus was divided into two lineages (hereafter lineages A and B). Lineage A consisted of *O. kawakatsuorum* and *O. koikei* (BS = 99%, BPP = 100%). Monophyletic lineage B (BS = 97%, BPP = 100%) included the remaining 10 species (including the new species), and was divided into two sub-lineages (hereafter lineages B1 and B2). The monophyly of lineage B1, which consisted of six species, was not well supported by the ML analysis (BS = 50%, BPP = 99%). Lineage B2 included four species, but the monophyly of this lineage was also not well supported by the ML analysis (BS = 57%, BPP = 99%). The new species, *O. masaakikuroiwai* sp. n., was part of lineage B2, and was a sister taxon of *O. whitmani* within this lineage. However, this relationship was not fully supported by the ML analysis (BS = 57%, BPP = 99%).

The ML tree (ln L = –8756.30) (Fig. 7) for reconstructing the phylogenetic relationships of the new species had an identical topology to the BI tree (not shown). The monophyly of the specimens identified as *Orobdella masaakikuroiwai* sp. n. was well supported (BS = 99%, BPP = 100%). This clade was divided into two subclades (hereafter lineages 1 and 2). Monophyletic lineage 1 (BS = 99%, BPP = 100%) consisted of two specimens, KUZ Z684 (locality number 1; Tokyo Metropolis), and Z687 (locality number 2; Shizuoka Prefecture). The monophyly of lineage 2 was well supported (BS = 99%, BPP = 100%). Lineage 2 contained five specimens from Nagano Prefecture including the holotype, and consisted of two subclades (hereafter lineages 2' and 2"). The

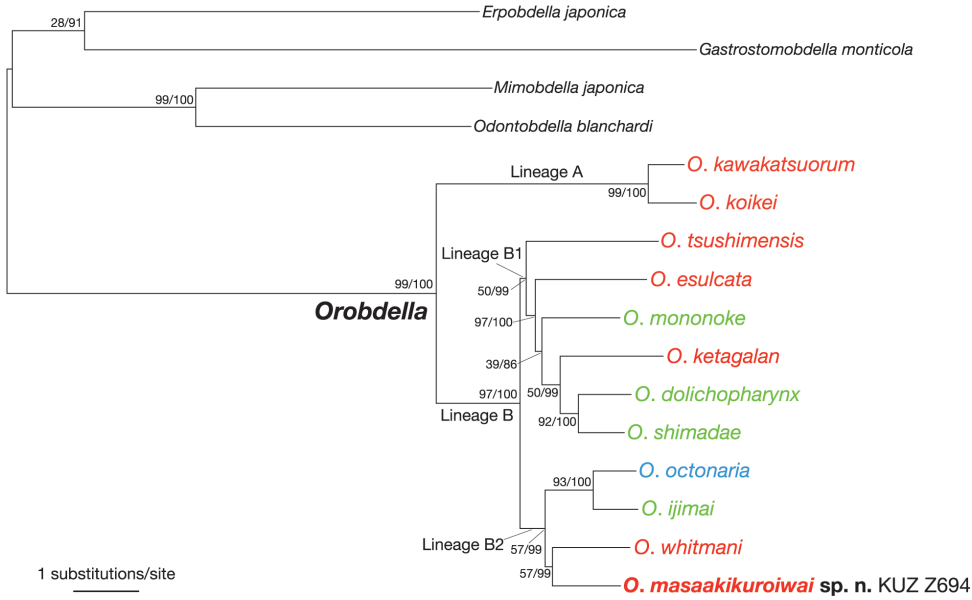

Figure 6. The ML tree (ln *L* = –23350.60) for 5,124 bp of nuclear 18S rDNA and histone H3, and mitochondrial COI, tRNACys, tRNAMet, 12S rDNA, tRNAVal, 16S rDNA, and ND1 markers. A species name of *Orobdella* in red indicates a quadrannulate species; in green, sexannulate; and in blue, octannulate. The numbers associated with the nodes represent the bootstrap values for ML (BS)/and Bayesian posterior probabilities (BPPs).

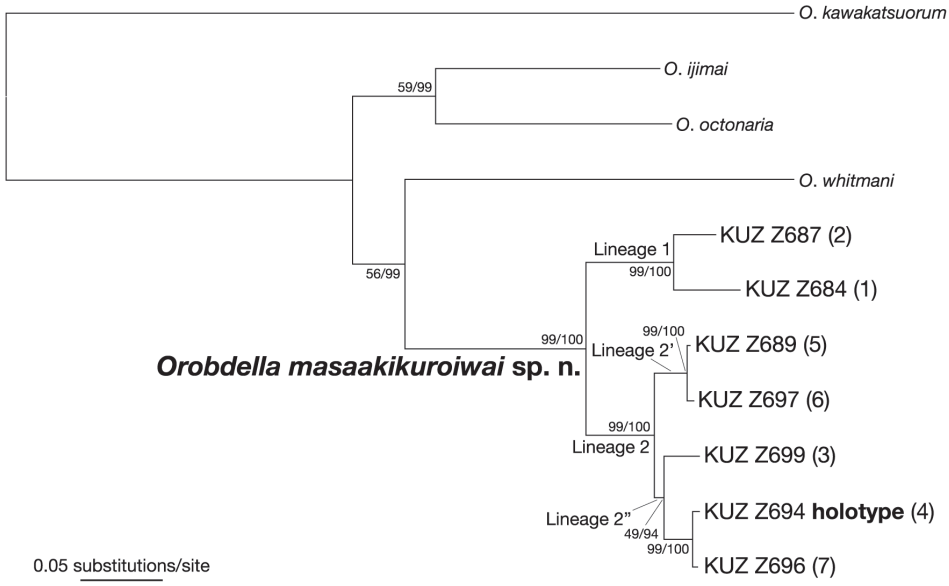

Figure 7. The ML tree (ln *L* = –8756.30) for 2,901 bp of mitochondrial COI, tRNACys, tRNAMet, 12S rDNA, tRNAVal, 16S rDNA, and ND1 markers. Voucher numbers of the specimens of *Orobdella masaakikuroiwai* sp. n. are accompanied by the collection locality numbers (see Fig. 1). The numbers associated with the nodes represent the bootstrap values for ML (BS)/and Bayesian posterior probabilities (BPPs).

monophyly of lineage 2' was well supported (BS = 99%, BPP = 100%). This lineage included two specimens, KUZ Z689 (locality number 5) and Z697 (locality number 6), observed in the southern part of the prefecture. The monophyly of lineage 2" was not well supported in the ML analysis (BS = 49%, BPP = 94%). Lineage 2" contained three specimens, KUZ Z694 (holotype; locality number 4), Z696 (locality number 7), and Z699 (locality number 3) collected from the east-central and mid-southern parts of Nagano Prefecture. KUZ Z694 and Z696 formed a monophyletic lineage (BS = 99%, BPP = 100%) within lineage 2".

The COI K2P distance within *O. masaakikuroiwai* sp. n. was 0.5–6.7% (mean = 4.4%) (Table 4). The genetic divergence between lineages 1 and 2 was 5.8–6.7% (mean = 6.3%), and that between lineages 2' and 2" was 2.7–3.5% (mean = 3.2%). The COI K2P distance between *O. masaakikuroiwai* sp. n. and *O. whitmani* (KUZ Z45, topotype) was 10.4–11.7% (mean = 11.0%).

Table 4. Kimura-2-parameter distances for the 1266 bp for the COI sequences of *Orobdella masaakikuroiwai* sp. n. specimens, with associated collection locality numbers (see Fig. 1 and Table 1).

Specimen (locality number)	1	2	3	4	5	6	7
1: KUZ Z684 (1)							
2: KUZ Z687 (2)	0.046						
3: KUZ Z689 (5)	0.065	0.061					
4: KUZ Z694 (4)	0.065	0.058	0.033				
5: KUZ Z696 (7)	0.063	0.059	0.035	0.005			
6: KUZ Z697 (6)	0.066	0.067	0.006	0.034	0.034		
7: KUZ Z699 (3)	0.063	0.059	0.027	0.023	0.023	0.027	

Discussion

The current molecular phylogenies showed that the specimens morphologically identified as the new species form a monophyletic group with strong support values. In addition, the K2P genetic distance of the COI sequences detected within the specimens was 0.5–6.7% (mean = 4.4%). Nakano (2012b) stated that the COI K2P distance between the sister species of *Orobdella*, *O. kawakatsuorum* and *O. koikei*, was 8.1–9.9% (mean = 9.0%). Therefore, the present genetic analyses support the taxonomic designation of the specimens examined in this study as belonging to the new species, *O. masaakikuroiwai* sp. n.

Orobdella masaakikuroiwai sp. n. was divided into two lineages (lineages 1 and 2) according to the molecular phylogenetic analyses. Lineage 1 consists of the individuals inhabiting the Kanto Region (KUZ Z684, locality number 1) and the Izu Peninsula (KUZ Z687, locality number 2). The Izu Peninsula is located on the Philippine Sea Plate and collided with Honshu island around 1 million years ago (Kitazato 1997).

Therefore, *O. masaakikuroiwai* sp. n. likely migrated into the peninsula after this collision event. In addition to lineages 1 and 2 composed of specimens from the mountainous region of Nagano Prefecture, the individuals of *O. masaakikuroiwai* sp. n. were sub-divided into central (lineage 2"; locality numbers 3, 4, 7) and southern (lineage 2'; locality numbers 5, 6) phylogroups. The Ina Basin is located in the southern part of Nagano Prefecture along the Tenryu River. Mountain districts are present to the east (including locality numbers 4–6) and west (containing locality number 7) along this basin. The specimen from Shiojidaira is the closest to the holotype from Mt. Mitsugaisan even though the Ina Basin separates the mountainous regions. In addition, the COI divergence between the two specimens from Shiojidaira (KUZ Z696) and Mt. Mitsugaisan (KUZ Z694) was low (0.5%). This may indicate that *O. masaakikuroiwai* sp. n. leeches in this area have recently dispersed. The same low genetic distance (0.6%) was detected between the specimens collected from the southern part of Nagano Prefecture (KUZ Z689, locality number 5, and KUZ Z697, locality number 6).

Orobdella masaakikuroiwai sp. n. is the second known species in which the body length of a mature individual is less than 4 cm. *Orobdella masaakikuroiwai* sp. n. is syntopic with *O. octonaria* in the Izu Peninsula (locality number 2), and the distribution of this new species partly overlaps with that of the latter species (Nakano, unpublished data). In addition, both *O. koikei* and *O. kawakatsuorum* are present in Hokkaido (Nakano 2012b). Therefore, a difference in the body size of mature individuals may allow different species of *Orobdella* to coexist in the same region. The phylogeny indicates that the small size of mature leeches likely evolved in parallel within *Orobdella*. *Orobdella whitmani* is the sister species of *O. masaakikuroiwai* sp. n. and grows to ca. 10 cm. In addition, *O. ijimai* and *O. octonaria* are close congeners of *O. masaakikuroiwai* sp. n. and *O. whitmani*, and they grow to ca. 10 cm and ca. 20 cm, respectively. Therefore, the intermediate size of mature individuals may be a plesiomorphic characteristic of the clade consisting of these four species. However, several undescribed species of *Orobdella* are known including small-sized species (Nakano, unpublished observation). Further faunal and systematic studies will help to elucidate the evolutionary and biogeographical history of the predaceous genus *Orobdella*.

Acknowledgments

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Erythraeid mites (Prostigmata, Erythraeidae) from Saudi Arabia, description of three new species and a new record

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Abstract

Three erythraeid genera *Balaustium* von Heyden, *Charletonia* Oudemans, and *Erythraeus* Latreille (Trombidiformes: Prostigmata) are reported for first time from Saudi Arabia based on three new larval species, *B. yousifi* **sp. n.**, *C. bahaensis* **sp. n.**, and *E.* (*Erythraeus*) *uhadi* **sp. n.** and one new record *Erythraeus* (*Zaracarus*) *lancifer* Southcott. All the three new species are described and illustrated from larvae.

Keywords

Balaustium, *Charletonia*, *Erythraeus*, Riyadh

Introduction

Mites of the family Erythraeidae (Trombidiformes: Prostigmata) are generally predators at postlarval stages, feeding upon various arthropods. However larvae of most erythraeids are parasites of different arthropods including insects e.g. bugs, grasshoppers, flies, aphids, etc. (Southcott 1961, 1991; Goldarazena et al. 2000; Gerson et al. 2003; Saboori and Cobanoglu 2010).

The genus *Erythraeus* Latreille comprises two subgenera, *Erythraeus* Latreille, 1806 and *Zaracarus* Southcott, 1995. The subgenus *Erythraeus* includes 93 species. Among

these, 45 species are known from larvae (Khanjani et al. 2012; Mąkol and Wohltmann 2012, 2013). The subgenus *Zaracarus* includes 27 species that all have been described from larvae (Mąkol and Wohltmann 2012, 2013). More than 50% of all larval species of subgenus *Erythraeus* have been recorded as parasites on Heteroptera, Thysanoptera, Neuroptera and other insects whereas others were captured free living on herbaceous plants (Haitlinger 2012; Khanjani et al. 2010, 2012; Kamran et al. 2013; Stroiński et al. 2013).

The genus *Charletonia* Oudemans comprises 117 species: two species described from both larvae and post larval stages; 92 species described only from larvae, and 23 species known only from post larval stages (Haitlinger 2007, Beron 2008; Mąkol and Wohltmann 2012, 2013). Most larval species of this genus were recorded as parasites on Orthoptera and Heteroptera (Haitlinger 2004a; Mayoral and Barranco 2011; Saboori et al. 2012; Haitlinger et al. 2014), however some larval species were recorded free living on herbaceous plants (Haitlinger 2004a, b; Hakimitabar and Saboori 2011). The free living larvae might be collected at early larval period while searching different hosts on herbaceous plants.

The genus *Balaustium* von Heyden widespread in the world, comprises 36 nominal species: 5 species described from both larval and post-larval stages, 17 described only from post larval stages, and 14 species based only on larvae (Mąkol et al. 2012; Mąkol and Wohltmann 2012). Larvae of *Balaustium* were generally collected from plants (Mayoral and Barranco 2009; Mąkol et al. 2012). Only *B. wratislaviensis* Haitlinger, 1996 was collected from different vertebrates species (Passeriformes: Paridae) (Haitlinger 1996). Family Erythraeidae is very poorly known in Saudi Arabia. Previously only *Leptus tammuzi* Haitlinger, 1994 was reported from this country (Haitlinger 1994). In this study, three genera, *Balaustium*, *Charletonia* and *Erythraeus* are reported for the first time from Saudi Arabia with three new species viz. *B. yousifi* sp. n., *C. bahaensis* sp. n. and *E.* (*E.*) *uhadi* sp. n. and one new record *E.* (*Z.*) *lancifer* Southcott.

Materials and methods

Three regions of Saudi Arabia, Al-Riyadh, Al-Madina and Baha, were surveyed for the collection of erythraeid mites during the years 2012-2013. Two collection methods were used: i) different plant parts were shaken over pieces of white paper and the mites were transferred using camel hair brush into 70% alcohol; ii) Tullgren funnels were used to extract mites from plant material brought to the laboratory. Mites parasitic on different insects were collected and preserved along with their hosts. Later, the mites were detached from their hosts under the stereomicroscope (Olympus®, SZX10, Japan). The collected mite specimens were cleared in Nesbitt's fluid for 10–12 h. Subsequently, the specimens were mounted on slides in Hoyer's medium, and dried in oven at 40 °C for one week. The mounted specimens were examined under a phase-contrast microscope (DM2500, Leica®, Germany). Template illustrations were either drawn

with pencil by using a drawing tube (Olympus®, Japan) attached to the microscope, or different body parts of mites were pictured with an Auto-montage Software System (SYNCROSCOPY®, Cambridge, UK) attached to the microscope. Final processing of drawings was done in Adobe Illustrator (Adobe Systems Incorporated, USA). The terminology used in this study follows that of Haitlinger and Saboori (1996). All measurements are given in micrometers. The measurements in description refer to the holotype followed by as a range of paratypes in parenthesis.

Results and discussion

Family Erythraeidae Robineau-Desvoidy
Subfamily Erythraeinae Robineau-Desvoidy

Genus *Erythraeus* Latreille

Type species. *Acarus phalangoides* (de Geer), by original designation.

***Erythraeus* (*Erythraeus*) *uhadi* sp. n.**
http://zoobank.org/D69C9E7F-7869-4556-9ABE-8485E7F66DEF
Figs 1–13

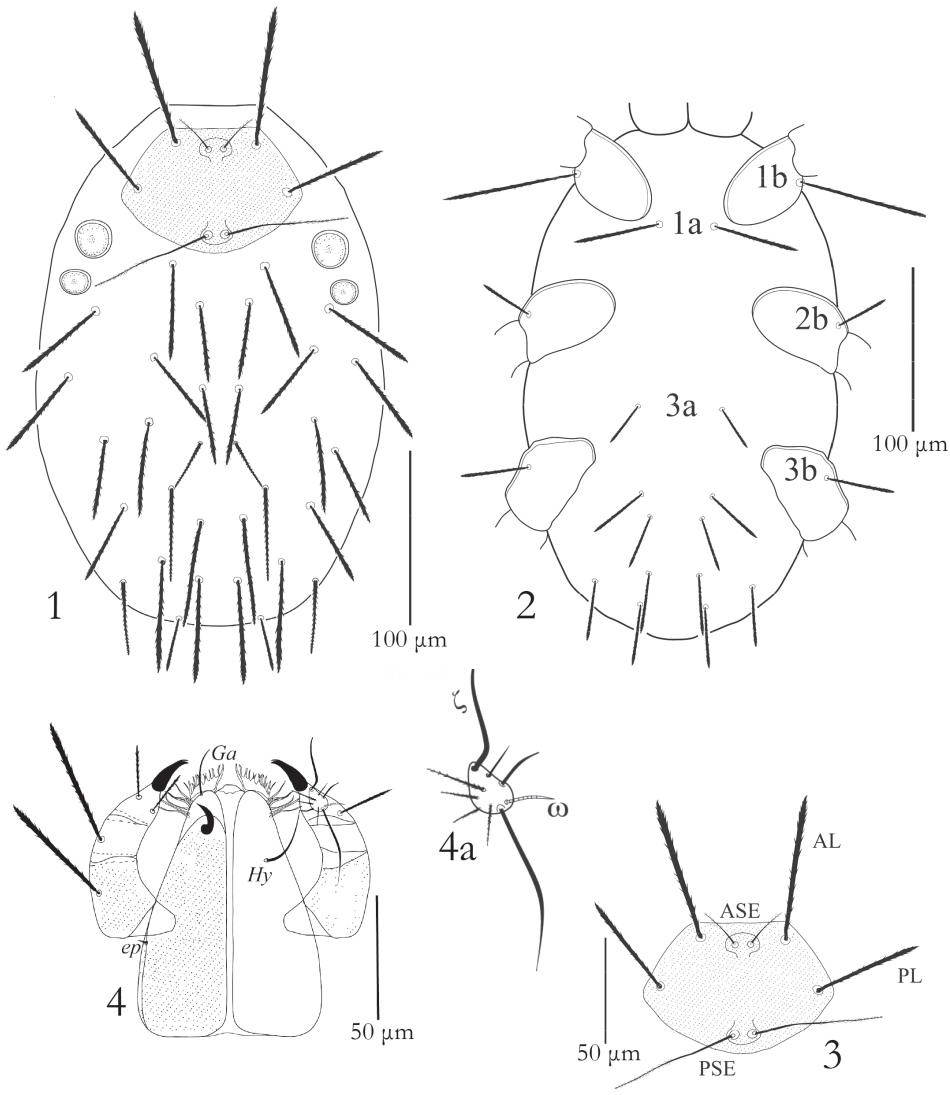
Diagnosis (n=6). fn Bfe 3-3-3, IP 2519–2597, fnTi 14-15-15, fD 32, fV 10, AL 90-97, AP 32–35, PSE 80–87, Ti III 279-289, Ti II 180-196, Genu III 143-149.

Description. (Holotype larva):

Dorsum: Prodorsal scutum with two pairs of sensilla (ASE and PSE) and two pairs of setae (AL and PL). AL located slightly anterior to ASE bases, PSE present at posterior pole of scutum, Posterior pair of sensilla (PSE) more than three times longer than anterior pair ASE, both finely ciliated on their distal halves. Cuticular lines surround both sensilla. AL longer than PL, both with long dense barbs on their entire lengths. Prodorsal scutum almost pentagonal in shape, straight anteriorly, round posteriorly, widest at the level of PL setae (Fig 3). Two pairs of eyes present at the level of posterior end of scutum dorsolaterally on idiosoma, anterior pair 24 (22–24) across, posterior pair 14 (13–14) across. Dorsal setae on idiosoma, 16 pairs (fD = 32), barbed and ranging in lengths from 29–61 (28–64)(Fig. 1).

Venter: Idiosoma ventrally bears setae *1a* between coxae I, setae *3a* slightly anterior to the area between coxae III; *1a* 50 (48-54), 3a 28 (28–32) long; opisthogaster behind the coxae III with 10 setae (fV=10). All ventral setae with dense barbs. NDV = 32+10 = 42 (Fig. 1B). Coxae I-III each with one coxalae; all coxalae barbed. Coxalae *1b* three times longer than *2b* (Fig. 2).

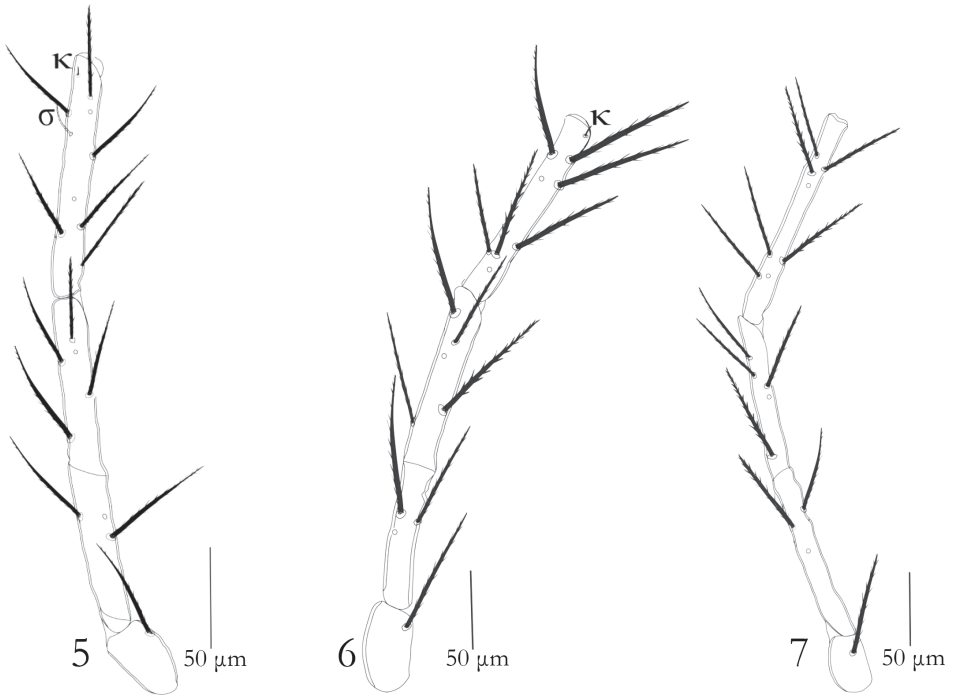
Gnathosoma: Infracapitulum with one pair of nude hypostomal setae (*Hy*) 30 (30–34) and nude galealae (*Ga*) 23 (21-24), supracoxalae present, very small, peg-like.



Figures 1–4. *Erythraeus* (*Erythraeus*) *uhadi* sp. n., (Larva): **1** Dorsum **2** Venter **3** Scutum **4** Gnathosoma (left dorsal view, right ventral view) **4a** Palptarsus.

Palp five segmented, palpfemur and genu each with one barbed seta, palptibia with three barbed setae, tibial claw bifurcate. Palptarsus with one eupathidium, one solenidion, two smooth and four barbed setae including one long seta (Figs 4, 4a). Palp setal formula: fPp: 0-B-B-BBB$_2$- NNBBBBζω.

Legs: Legs seven segmented with divided femora, all legs longer than body length; leg III the longest one, Tarsi terminate into two lateral claws and a claw like empodium. Chaetotaxy of leg segments: coxae 1-1-1; trochanters 1-1-1; basifemora 3-3-3;



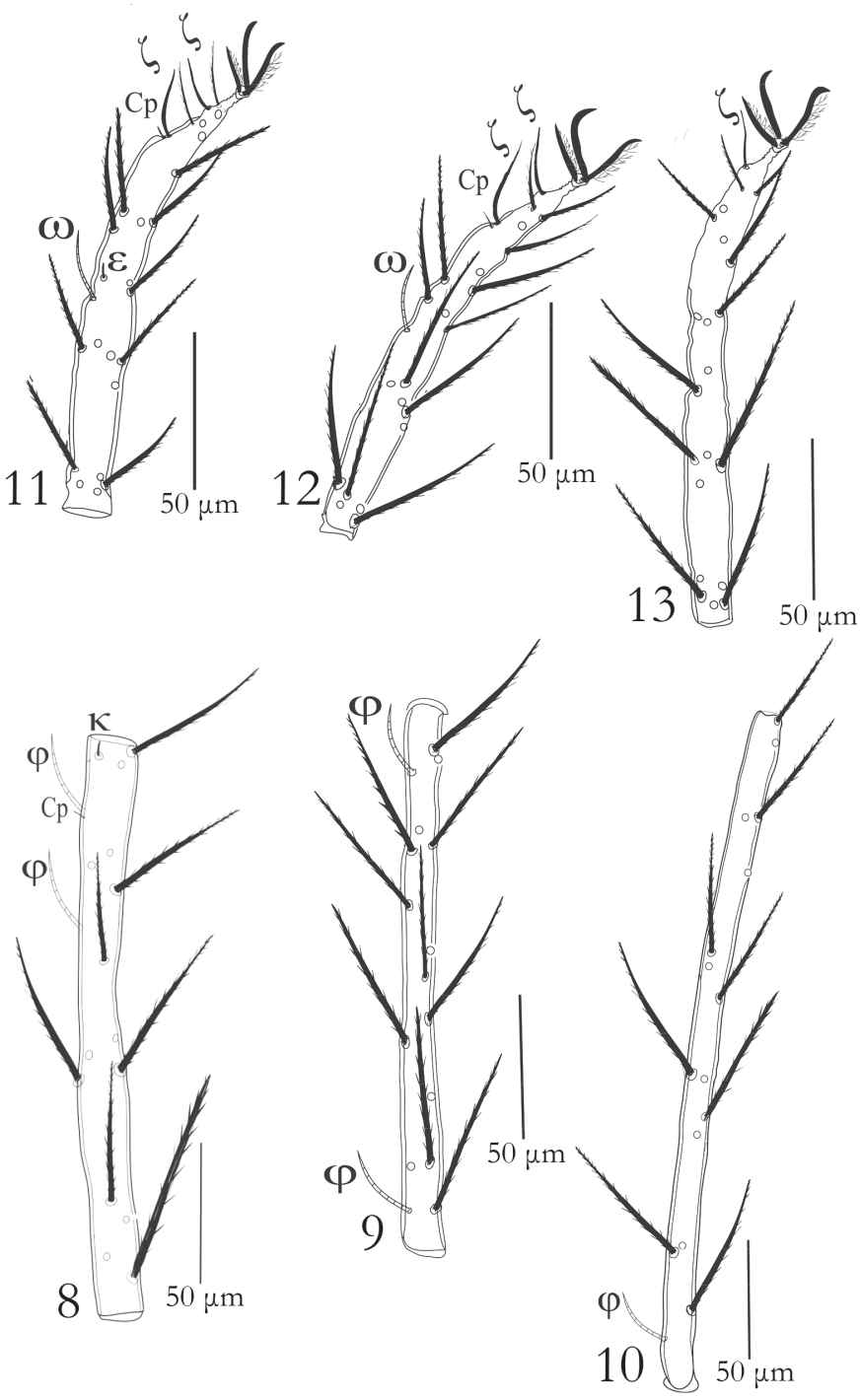
Figures 5–7. *Erythraeus* (*Erythraeus*) *uhadi* sp. n., (Larva): **5** Trochanter, femur & genu I **6** Trochanter, femur & genu II **7** Trochanter, femur & genu III.

telofemora 5-5-5; genua 8+1σ+1κ – 8+1κ – 8; tibiae 14 + 2φ+ 1*Cp* + 1κ – 15 + 2φ – 15+1φ; tarsi 22 **+** 1ω + 1ε + 1*Cp* + 2ζ – 20+ 1ω + 1*Cp* + 2ζ – 20 + 1ζ (Figs 5–13).

Etymology. The specific epithet is derived from the name of famous mountain "Uhad", where holotype larva was collected.

Type material. Holotype larva was collected from the mountain “Uhad”, Al-Madina, Saudi Arabia, 24°30.086'N, 39°36.41'E, on 23 February, 2013, coll. M. Kamran), parasitizing tamarix leafhopper, *Opseius* sp. (Hemiptera: Cicadellidae), from *Tamarix* sp. (Tamaricaceae). Paratypes 4 larvae, collection data same as holotype, while one paratype was collected from Wadi-e-Hanifa near Arqa over bridge, Riyadh, Saudi Arabia, 24°41.354'N, 46°37.042'E, on 14 April, 2013, from *Tamarix* sp. in association with the same host, coll. M. Kamran. Holotype and 4 paratypes (P2, P3, P4, P5) are deposited in the King Saud University Museum of Arthropods (KSMA) and Acarology Laboratory, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University. One paratype (P1- accession no. Acy: 14/47) has been deposited at the Agriculture Research Council, Plant Protection Research Institute, Biosystematics Division, Pretoria (ARC-PPRI), South Africa.

Remarks. *Erythraeus* (*E.*) *uhadi* sp. n. belongs to a group of species of subgenus *Erythraeus* that share the following combination of characters: basifemoral setal



Figures 8–13. *Erythraeus* (*Erythraeus*) *uhadi* sp. n., (Larva): **8** Tibia I **9** Tibia II **10** Tibia III **11** Tarsus I **12** Tarsus II **13** Tarsus III.

formula 3-3-3, tibia I with 14 normal setae, Ti III 270-334, Ti II 170-210, genu III 120-200. This group includes 7 species: *E.* (*E.*) *flavopictus* Kawashima, 1961; *E.* (*E.*) *sabrinae* Haitlinger & Saboori, 1996; *E.* (*E.*) *southcotti* Goldarazena & Zhang, 1998; *E.* (*E.*) *ankaraicus* Saboori et al., 2004; *E.* (*E.*) *zhangi* Haitlinger, 2006; *E.* (*E.*) *hilarae* Haitlinger, 2010, *E.* (*E.*) *chrysoperlae* Khanjani et al., 2012 (Kawashima 1961; Haitlinger and Saboori 1996; Goldarazena and Zhang 1998; Saboori et al. 2004; Haitlinger 2006a, 2010; Khanjani et al. 2012). *Erythraeus* (*E.*) *uhadi* sp. n. differs from *E.* (*E.*) *flavopictus* by shorter ASE (22-25 vs. 55), shorter W (99-108 vs. 153), shorter IP (2519-2597 vs. 2944), shorter AP (32-35 vs. 59), fD (32 vs. 42); from *E.* (*E.*) *sabrinae* by shorter AP (32-35 vs. 52), fD (32 vs. 62), fV (10 vs. 28), shorter W (99-105 vs. 132), shorter AW (44-47 vs. 60), shorter PW (81-85 vs. 110); from *E.* (*E.*) *southcotti* by shorter AP (32-35 vs. 48-50), longer PaScGed (50-54 vs. 25-30); fD (32 vs. 46), fV (10 vs. 16), fnTa (21-20-20 vs. 26-23-24); from *E.* (*E.*) *zhangi* by shorter L (69-81 vs. 96-128), shorter W (99-108 vs. 126-148); shorter GL (106-111 vs. 140-166), shorter IP (2519-2597 vs. 2622-3198), fD (32 vs. 86), fV (10 vs. 20); *E.* (*E.*) *ankaraicus* by fnTa (21-20-20 vs 25-22-24), fD (32 vs. 41), fV (10 vs. 18), AL (90-97 vs. 65-78), AP (32-35 vs. 41-48); from *E.* (*E.*) *hilarae* by shorter L (69-81 vs. 110), shorter W (99-108 vs. 128), shorter ISD (49-53 vs. 68), shorter GL (106-111 vs. 130), fV (10 vs. 16), fnTi (14-15-15 vs. 14-14-14) and from *E.* (*E.*) *chrysoperlae* by fV (10 vs. 14), fnTa (21-20-20 vs. 27-23-24), longer AL (90-97 vs. 70), shorter AP (32-35 vs. 50), shorter GL (106-111 vs. 150).

Table 1. Metric data of *Erythraeus* (*E.*) *uhadi* sp. n. larva (holotype and 5 paratypes).

Ch.	H	P1	P2	P3	P4	P5	Ch.	H	P-1	P2	P3	P4	P5
IL	302	300	305	307	298	297	Ta I(H)	16	15	16	15	16	16
IW	195	197	195	200	194	199	Ti I	205	206	205	210	211	207
L	71	73	70	74	69	81	Ge I	185	183	185	190	193	186
W	105	103	102	108	106	99	Tfe I	113	111	115	112	116	110
AW	44	45	44	48	46	47	Bfe I	105	106	103	107	110	104
PW	81	83	82	85	81	85	Tr I	44	45	46	43	47	44
AA	11	11	11	12	11	12	Cx I	35	34	36	34	36	35
SB	13	13	13	14	13	14	Leg I	829	828	834	843	853	826
ISD	50	52	49	53	53	51	Ta II(L)	136	138	135	139	141	134
AP	34	33	35	35	32	35	TaII(H)	15	15	15	14	15	15
AL	92	90	93	97	91	95	Ti II	189	187	189	180	196	192
PL	63	61	62	60	65	60	Ge II	126	127	129	124	131	122
ASE	23	24	25	22	23	22	Tfe II	110	108	113	107	113	110
PSE	81	80	82	87	81	84	Bfe II	95	97	96	98	94	94
DS	29–61	29–62	28–61	30–64	30–63	29–62	Tr II	50	52	50	48	54	53
PDS	29–61	29–62	29–61	29–64	29–63	29–62	Cx II	63	65	63	60	61	61
1a	50	52	53	54	48	50	Leg II	769	774	775	756	790	766

Ch.	H	P1	P2	P3	P4	P5	Ch.	H	P-1	P2	P3	P4	P5
3a	28	29	28	32	30	31	TaIII (L)	154	152	156	150	157	153
1b	100	99	102	105	100	103	TaIII(H)	15	15	15	14	15	15
2b	33	32	30	35	32	34	Ti III	286	287	279	287	289	283
3b	38	37	36	40	39	38	Ge III	148	149	146	146	143	144
Hy	30	31	30	34	32	30	Tfe	113	114	110	112	116	113
Ga	23	22	21	24	23	22	Bfe	123	123	125	126	128	122
G L	107	110	108	111	106	107	Tr III	50	53	52	50	53	51
PaScFed	50	52	51	54	51	49	Cx III	66	67	65	66	68	67
PaScGed	52	54	52	56	50	53	LegIII	940	945	933	937	962	933
Ta I(L)	142	143	144	147	140	140	IP	2538	2547	2542	2519	2597	2525

Ch = Character, H = Holotype, P = Paratype

Subgenus *Zaracarus* Southcott

Erythraeus (*Zaracarus*) *lancifer* Southcott

Erythraeus (*Z.*) *lancifer* Southcott, 1995: 223.

Material examined. Six larvae, Baha, Saudi Arabia, 20°7.918'N, 41°24'69'E on 24 April, 2013, coll. M. Kamran, parasitizing tamarix leafhopper, *Opseius* sp. (Hemiptera: Cicadellidae); two larvae were collected as free living on *Setaria viridis* L. (Poaceae) from the same locality and date.

Remarks. The type specimens were collected from a fly (Diptera, Dolichopodidae) Nr Pina, Zaragoza Province, Spain (Southcott 1995). This species has been hitherto only recorded from Spain. Present samples constitute a new record for Asia.

Table 2. Metric data of *Erythraeus* (*Z.*) *lancifer* larva (measurements of 4 specimens in range).

Ch.		Ch.		Ch.		Ch.	
IL	344–355	PSE	73–79	Ti I	228–234	Tr II	62–66
IW	230–238	DS	55–72	Ge I	164–167	Cx II	67–72
L	91–97	*1a*	41–44	Tfe I	110–115	Leg II	851–891
W	145–151	*3a*	30–34	Bfe I	112–116	TaIII (L)	156–163
AW	41–45	*1b*	88–94	Tr I	54–56	TaIII(H)	16
PW	110–115	*2b*	29–32	Cx I	63–67	Ti III	329–334
AA	20–21	*3b*	34–37	Leg I	893–923	Ge III	156–160
SB	15–15	*Hy*	30–33	Ta II(L)	137–143	Tfe	135–140
ISD	62–65	*Ga*	23–26	Ta II(H)	16–17	Bfe	129–133
AP	50–53	PaScFed	54–58	Ti II	229–236	Tr III	52–55
AL	186–197	PaScGev	67–71	Ge II	129–137	Cx III	68–72
PL	74–79	Ta I(L)	162–168	Tfe II	122–127	LegIII	1025–1057
ASE	28–30	Ta I(H)	17–18	Bfe II	105–110	IP	2769–2871

Subfamily Callidosomatinae Southcott
Genus *Charletonia* Oudemans

***Charletonia bahaensis* sp. n.**
http://zoobank.org/BEBA76A2-2E8F-4102-8BAD-714C99FE6F2A
Figs 14–23

Diagnosis (n=7). fnTi 18-18-18, fD 121-123, fV 60-61, with two hypostomalae, posterior hypostomalae barbed, galeala nude, GL 157-164, fnGe 12-12-12, four setae between coxae II & III, solenidion on genu I located distally.

Description of holotype larva. (Metric data of holotype followed by as a range of six paratypes in parenthesis).

Dorsum: Prodorsal scutum punctate entirely, with two pairs of sensillae (ASE, PSE) and three pairs of normal setae (AL, PL, PL). Posterior sensilla (PSE) longer than anterior ones (ASE), both finely barbed at distal halves. All three scutalae AL, ML and PL densely barbed and blunt ended, (Fig 16). Dorsum with 123 (121–123) barbed setae (fD = 123 (121–123) with blunt tips, ranging in lengths from 45 (42–56). A pair of eyes located laterally on idiosoma posterolateral to scutum, 21 (21–23) across (Fig. 14).

Venter: Venter with intercoxal setae (*1a*) between coxae I, one pair of intercoxal setae (*2a*) between coxae II, four setae in the area between coxae II & III, 57 (56–57) setae present on opisthogaster behind the coxae III (fV = 61 (60–61). All ventral setae barbed with pointed tips except postero-marginal setae on venter which are blunt-ended (Fig. 15).

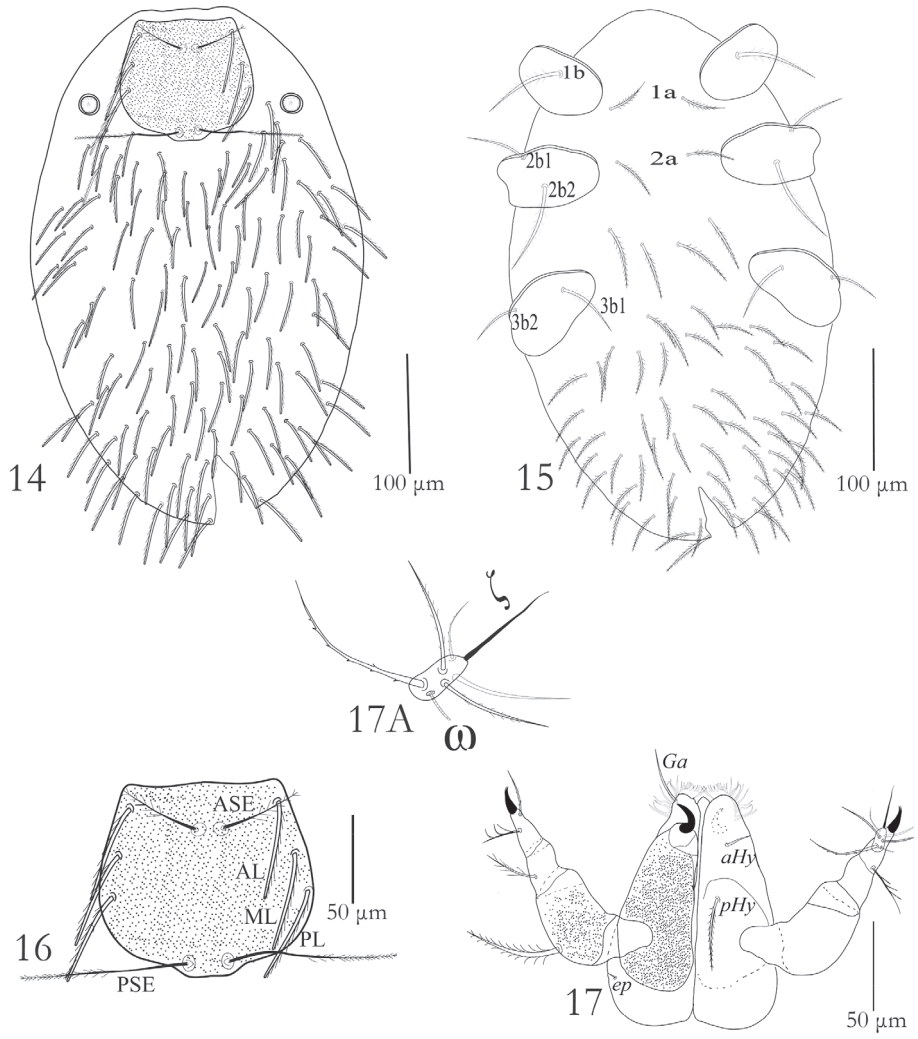
Gnathosoma: Subcapitulum with one pair of nude, spiniform galealae (*Ga*) 33 (30–34), two pairs of hypostomalae, anterior pair (*aHy*) nude, 16 (15–17), posterior pair (*pHy*) with long barbs, 45 (42–47). Chelicerae 114 (113–116), cheliceral blade 19 (18–19). Supracoxalae present, very small, peg-like. Palpfemur and genu each with one barbed seta, palptibia with three barbed setae and bifurcated claw (Fig. 17), palptarsus with one eupathidium, one solenidion, one nude and four barbed setae including long basal seta (Fig. 17A), eupathidium 25 (23–25), solenidion 7 (6–7) and long basal seta, 39 (35–40) long. Palp setal formula: 0-B-B-BBB_2–4BNωζ.

Legs: Legs seven segmented with divided femora, all longer than body length. Tarsi I–III terminate in two lateral claws and claw like empodium.

Leg setal formula: Cx: 1-2-2; Tr: 1-1-1; Bfe: 4-4-2; Tfe: 5-5-5; Ge: 12+1σ+1κ – 12+ 1κ – 12; Ti: 18+2φ + 1Cp+ 1κ – 18+ 2φ –18 + 1φ; Ta: 27+ 1ω + 1ε + 1Cp + 2ζ – 26 + 1ω + 1ζ – 27 + 1ζ (Figs 18–23).

Etymology. The specific epithet is derived from the city name "Baha" (in Saudi Arabia) where it was collected.

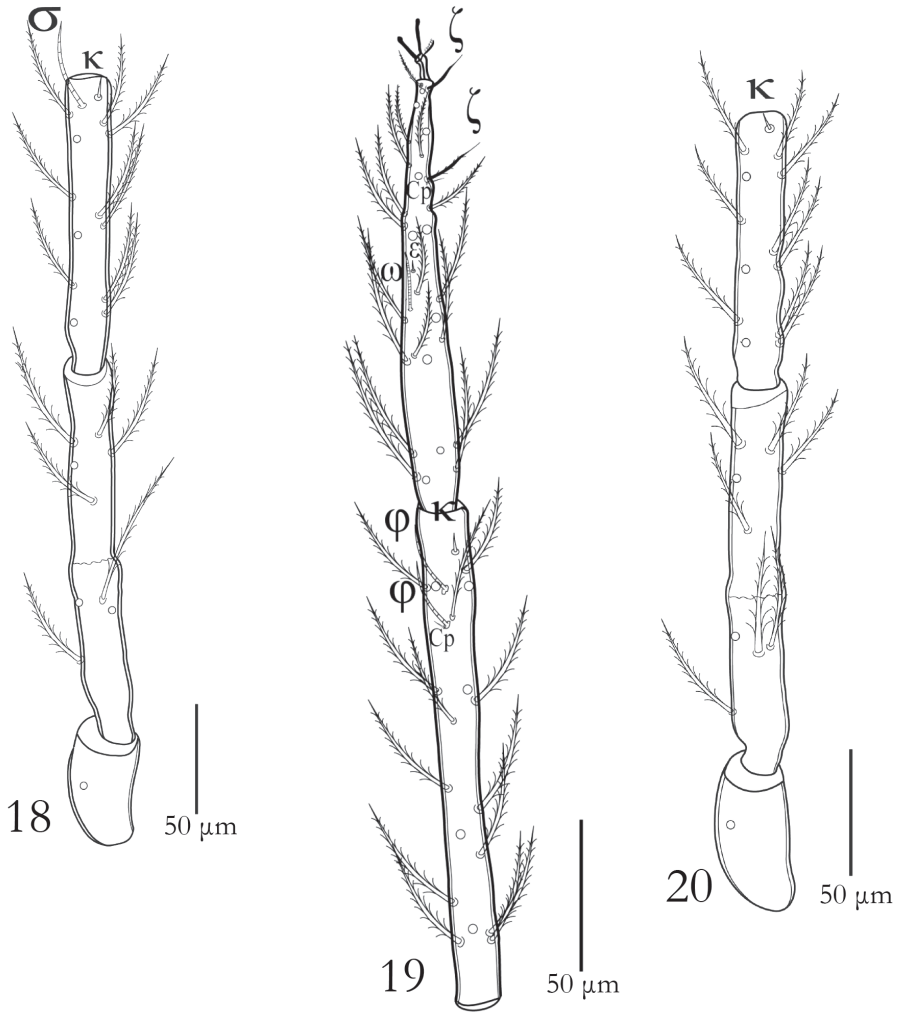
Type material. Holotype and 6 paratype larvae, from blue alfalfa aphid, *Acyrthosiphon kondoi* Shinji (Hemiptera: Aphididae), infesting alfalfa plants, *Medicago sativa* L., Baha, Saudi Arabia, 19°59.807'N, 41°25.715'E, on 25 April, 2013, coll. M. Kamran. Holotype and 5 paratypes (P2, P3, P4, P5, P6) are deposited in the King Saud



Figures 14–17. *Charletonia bahaensis* sp. n. (Larva): **14** Dorsum **15** Venter **16** Scutum **17** Gnathosoma (left dorsal view, right ventral view) **17A** Palptarsus.

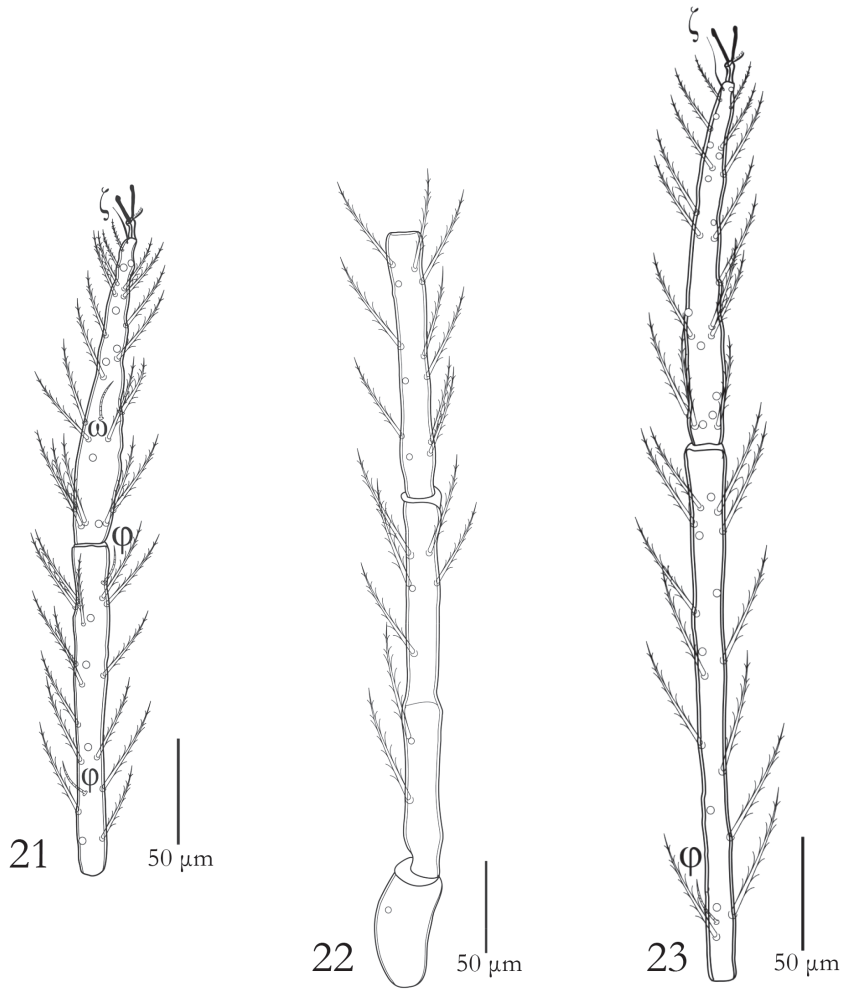
University Museum of Arthropods (KSMA) and Acarology Laboratory, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University. One paratype (P1- accession no. Acy: 14/46) has been deposited at the Agriculture Research Council, Plant Protection Research Institute, Biosystematics Division, Pretoria (ARC-PPRI), South Africa.

Remarks. *Charletonia bahaensis* sp. n**.** belongs to the species group of genus *Charletonia* with four setae between coxae II & III, solenidion placed distally on genu I, fn



Figures 18–20. *Charletonia bahaensis* sp. n. (Larva): **18** Trochanter, femur & genu I **19** Tibia & Tarsus I **20** Trochanter, femur & genu II.

Ge 12-12-12, Ti III 200-260 and two hypostomalae. This group includes 11 species: *C. areolata* (Trägårdh, 1908); *C. froggatti* Oudemans, 1910; *C. feideri* Southcott, 1966; *C. rageaui* Southcott, 1966; *C. paolii* Southcott, 1966; *C. banksi* Southcott, 1966; *C. enghoffi* Southcott, 1991*; C. hunanensis* Zheng, 1996; *C. lombokensis* Haitlinger, 2006; *C. grandpopensis* Haitlinger, 2007 and *C. salazari* Mayoral & Barranco, 2011 (Southcott 1966, Southcott 1991, Zheng 1996, Haitlinger 2006b, 2007, Mayoral and Barranco 2011). The new species differs from *C. areolata* by fD (121-123 vs. 97), fV (60-61 vs. 42), setae on Ti III (18 vs. 19), Ti III (231-242 vs. 259), Ti I (175-183 vs. 199), Ge I



Figures 21–23. *Charletonia bahaensis* sp. n. (Larva): **21** Tibia & Tarsus II **22** Trochanter, femur & genu III **23** Tibia & Tarsus III.

(127-135 vs. 157), Galealae (nude vs. ciliated); from *C. froggatti* by fD (123 vs. 64), fV (60-61 vs. 37), fnTi (18-18-18 vs. 14-14-18); from *C. feideri* by fD (121-123 vs. 86), fV (61 vs. 44), setae on Ti III (18 vs. 19), Ti I (173-184 vs. 138-159), Ge III (140-148 vs. 121), Ge I (127-135 vs. 112-125), Ta I (158-166 vs. 129-140); from *C. rageaui* by fD (121-123 vs. 94), fV (61 vs. 54), fnTi (18-18-18 vs. 18-18-19), Ta I (158-166 vs. 142-149); from *C. paolii* by fD (121-123 vs. 98), setae on Ti III 18 vs. 19), posterior hypostomalae (barbed vs. nude), W (114-118 vs. 98), PL (49-55 vs. 36-43), Ta I (158-166 vs. 137), galealae (nude vs. barbed), Ta III (165-177 vs. 133); from *C. banksi* by fD (121-123 vs. 97), fV (60-61 vs. 46), setae on Ti III (18 vs. 19), Ge III (140-148

vs. 125), galealae (nude vs. barbed), leg I (741-781 vs. 725), leg II (694-716 vs. 660), leg III (869-911 vs. 790); from *C. enghoffi* by fD (121-123 vs. 52), fV (60-61 vs. 40), setae on Ti I (18 vs. 17), posterior hypostomalae (barbed vs. nude), PSE (87-95 vs. 116-129), ASE (48-51 vs. 70-75); *C. hunanensis* by fD (121-123 vs. 73), fV (60-61 vs. 47), setae on Ti II (18 vs. 21), Ge III (140-148 vs. 125), setae on Tfe (5 vs. 6); from *C. lombokensis* by fD (121-123 vs. 74), fV (60-61 vs. 40), setae on Ti II (18 vs. 17), fnBfe (4-4-2 vs. 3-3-2), PW (106-113 vs. 50), ASE (48-54 vs. 22), PSE (87-95 vs. 36); from *C. grandpopensis* by fD (121-123 vs. 60), fV (60-61 vs. 43), setae on Ti II (18 vs. 17), setae on Ti III (18 vs. 17), ASE (ciliated vs. nude), DS (42-56 vs. 68-72), Ta I (158-166 vs. 130-134), GL (155-164 vs. 96-108), galealae (nude vs. barbed); from *C. salazari* by fD (121-123 vs. 76), fV (60-61 vs. 28), fnTi (18-18-18 vs. 15-16-16), ISD (71-78 vs. 54-63), AL (50-56 vs. 67-72), AP (48-52 vs. 68-72). In brief the new species can be differentiated from all other species of this group by having fD 123, fV 61 and fn Ti 18-18-18. All other species of this group have dorsal setae less than 100.

Table 3. Metric data of *Charletonia bahaensis* sp. n. larva, holotype and 6 paratypes (in range).

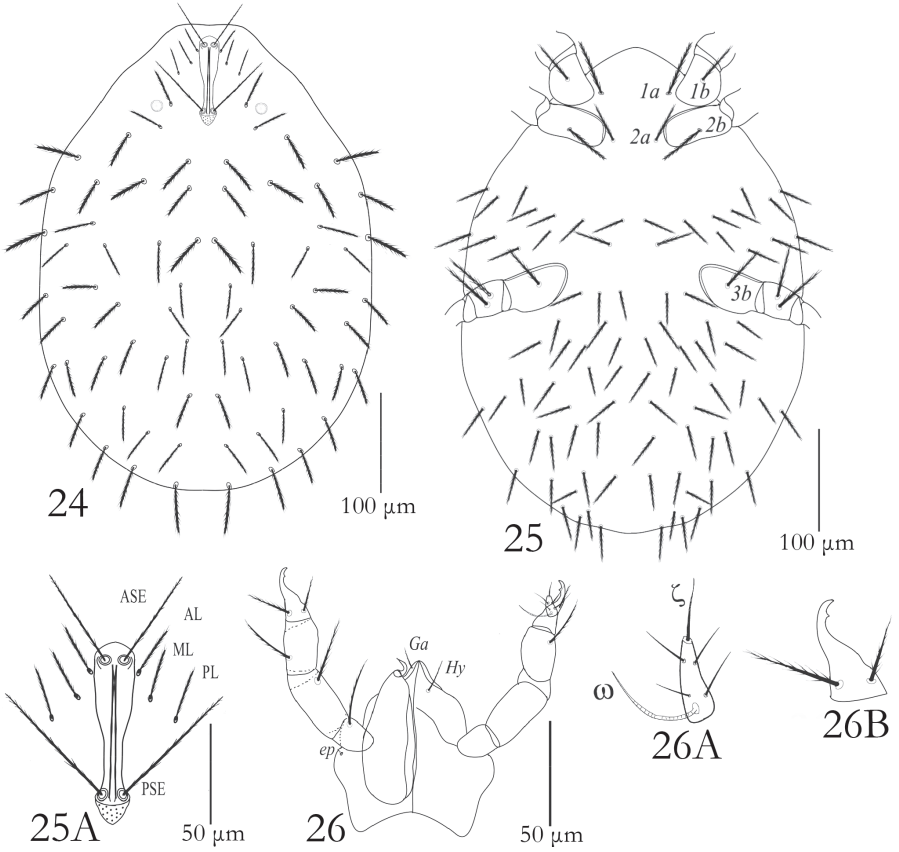
Ch.	H	P1	P2	P3	P4	P5	P6	Ch.	H	P1	P2	P3	P4	P5	P6
IL	441	436	439	435	430	442	441	PaScFed	58	55	57	58	57	55	59
IW	280	285	275	272	276	278	282	PaScGev	32	30	30	29	33	29	33
L	110	112	109	108	110	106	113	Ta I(L)	164	160	158	166	165	159	165
W	116	117	118	114	116	115	117	Ta I(H)	16	15	17	16	16	16	17
AW	84	81	86	81	86	84	85	Ti I	181	180	178	183	175	173	184
MW	98	94	100	97	101	93	98	Ge I	132	133	127	135	130	129	135
PW	110	112	109	108	112	113	106	Tfe I	88	85	89	90	90	86	91
AA	10	10	11	10	11	10	10	Bfe I	88	86	89	90	85	84	91
SB	20	19	20	10	19	21	18	Tr I	47	49	46	47	46	46	47
ISD	75	71	78	72	77	75	71	Cx I	66	65	67	67	63	64	68
AP	49	50	52	47	50	48	49	Leg I	766	758	754	778	754	741	781
AL	54	52	51	50	54	55	56	Ta II(L)	152	146	150	154	154	150	155
ML	54	55	52	53	57	57	58	Ta II(H)	15	15	16	15	16	15	16
PL	52	51	49	50	55	53	55	Ti II	156	159	153	153	151	150	155
ASE	49	50	51	48	54	50	49	Ge II	113	111	110	114	115	110	116
PSE	93	91	90	87	95	89	95	Tfe II	78	85	77	80	75	76	81
DS	45–54	44–55	43–54	42–53	45–55	44–54	45–56	Bfe II	79	78	80	82	77	80	83
PDS	45–54	44–55	43–54	42–53	45–55	44–54	45–56	Tr II	59	60	62	58	56	57	61
1a	44	45	42	40	45	44	46	Cx II	74	71	73	75	70	71	74
2a	57	55	54	54	60	58	59	Leg II	711	710	705	716	698	694	725
1b	71	69	68	67	73	73	72	Ta III (L)	172	170	166	177	165	168	175
2b1	71	69	73	67	78	77	73	Ta III (H)	16	15	16	16	15	15	16
2b2	55	53	56	52	56	57	54	Ti III	237	239	233	231	242	230	241
3b1	55	52	57	52	57	56	53	Ge III	146	144	148	148	140	141	147
3b 2	46	44	47	42	48	45	42	Tfe	113	111	115	109	112	110	115
GL	161	158	163	155	164	159	157	Bfe	89	88	90	87	**90**	87	90
pHy	45	44	42	43	47	46	47	Tr III	59	60	56	58	59	56	60
aHy	16	17	16	16	17	17	15	Cx III	80	81	78	77	80	77	83
Ga	33	34	32	31	34	33	30	LegIII	895	893	886	887	903	869	911
								IP	2372	2361	2345	2381	2355	2304	2417

Subfamily Balaustiinae Grandjean
Genus *Balaustium* von Heyden

***Balaustium yousifi* sp. n.**
http://zoobank.org/71EF1ABE-54D9-430E-9D44-40E5E1A3B5F1
Figs 24–29

Diagnosis (n=7). Scutum present, three pairs of scutalae present off the scutum, fnTr 3-3-2, fnBfe 4-4-3, fnTi 11-11-11, PSE 66-75, IP 1294-1363, ISD 65-69, fV 60 and fD 74.

Description of holotype larva. Dorsum: Idiosoma oval in shape, scutum elongate, 92 (88–95) long, 23 (21–25) wide, carries two pairs of sensilla (ASE & PSE), ASE located on anterior while PSE on posterior part of scutum, both sensilla finely barbed on their entire lengths. Crista present on scutum. Three pairs of scutalae (AL, ML, PL) present on the lateral sides of scutum, no scutalae located on scutum. AL located slightly posterior to the bases of ASE, ML lies slightly anterior to the middle of scutum and PL slightly posterior to the middle of scutum. One pair of eyes present on postero-lateral



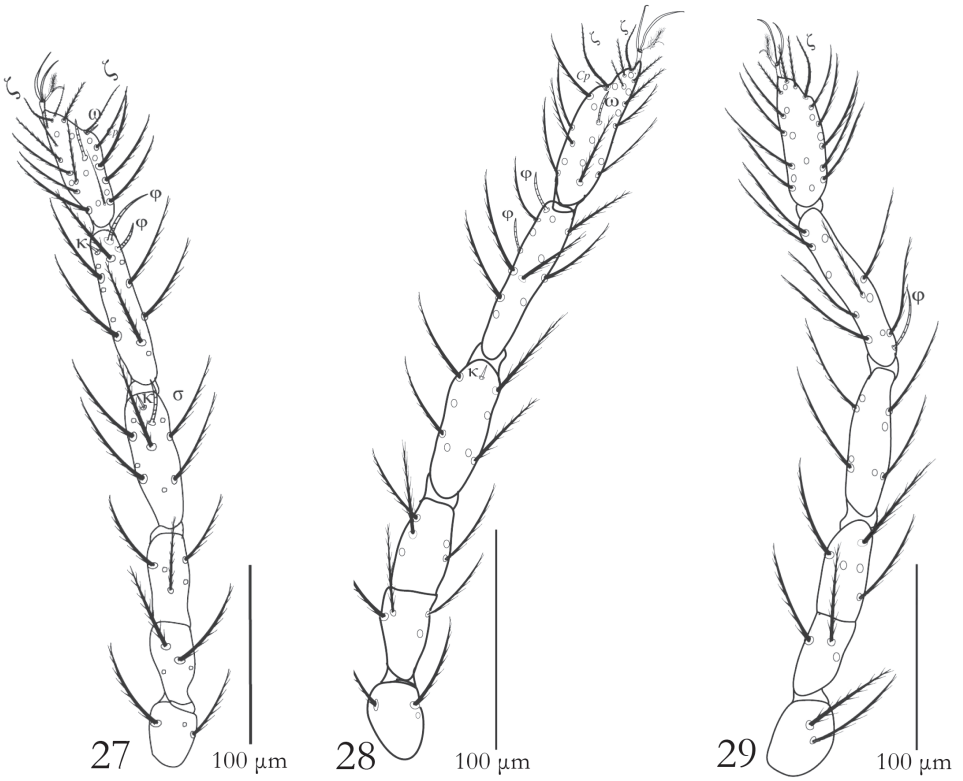
Figures 24–26. *Balaustium yousifi* sp. n. (Larva): **24** Dorsum **25** Venter **25A** dorsal scutum **26** Gnathosoma (left dorsal view, right ventral view) **26A** Palptarsus, **26B** Palptibia.

side of scutum at the level of PSE on the idiosoma, cornea of each eye 14 (13–14) in diameter. Dorsal setae on idiosoma 37 pairs, all barbed. fD = 74 (Fig. 24).

Venter: Idiosoma ventrally with one pair of sternalae *1a* between coxae I, 56 (52–57) long, one pair of setae *2a* between coxae II, 42 (41–47) long, 26 setae present in the area between coxae II & III, 60 (59–60) setae present between and behind the coxae III (fV = 86 (84–86). All ventral setae barbed (Fig. 25).

Gnathosoma: Gnathosoma with one pair of hypostomalae (*Hy*) 16 (15–17) and one pair of galealae (*Ga*) 10 (9–10), both barbed, supracoxalae present, very small, peg-like. Chelicerae 52–55 long, cheliceral blade 9 (9–10). Palp trochanter and palpfemur each with one barbed setae, palpgenu with two barbed setae (Fig. 26); palptibia with-three setae, palptarsus with four nude setae, one eupathidium and one solenidion(Fig. 26A). Palptibial claw entire with a median tooth (Fig. 26B). Eupathidium 7 (7), solenidion 16 (14–16). (Fig. 26). Palp setal formula: fPp: B-B-BB- BBN- NNNNωζ.

Legs: Legs seven segmented with divided femora, tarsi I–III terminated with two claws and claw-like empodium, empodium with pilose (pulvilliform) structure. Leg setal formula: leg I: Ta-ω, 2ζ , 1 Cp, 22B; Ti- 2φ,1κ, 11B; Ge- 1σ,1κ, 9B; Tfe- 5B;



Figures 27–29. *Balaustium yousifi* sp. n. (Larva): **27** Leg I **28** Leg II **29** Leg III.

Bfe- 4B; Tr- 3B; Cx- 1B (Fig. 27). Leg II: Ta- ω, 1ζ , 20B; Ti- 2φ, 11B; Ge- 1κ, 8B; Tfe- 5B; Bfe- 4B; Tr- 3B; Cx- 1B (Fig. 28). Leg III: Ta- 20B; Ti- 1φ, 11B; Ge- 8B; Tfe- 5B; Bfe- 3B; Tr- 2B; Cx- 1B (Fig. 29).

Etymology. The new species is named on the name of Professor Dr. Yousif Al-Duraihim.

Type. Holotype larva was collected from 5 Km Taif road, Baha, Saudi Arabia, 20°7.918'N, 41°24.69'E, 24 April, 2013 (Coll. M. Kamran), from foxtail grass, *Setaria viridis* L. Paratypes six larvae, collection data same. Holotype and 6 paratypes (P1, P2, P3, P4, P5, P6) are deposited in the King Saud University Museum of Arthropods (KSMA) and Acarology Laboratory, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University. One paratype (P1- accession no. Acy: 14/45) has been deposited at the Agriculture Research Council, Plant Protection Research Institute, Biosystematics Division, Pretoria (ARC-PPRI), South Africa.

Remarks. *Balaustium yousifi* sp. n. closely resembles with *Balaustium florale* Grandjean. However it differes from *B. florale*. by length of PSE (66-75 vs. 40-48); IP (1294-1363 vs. 850-988); ISD (64-69 vs. 42-48); fD (74 vs. 82). The new species can be distinguished from *B. bisculatae* Mayoral & Barranco by shorter ISD (65-69 vs. 56), fD (74 vs. 95), longer AL (28-32 vs. 24), longer TiIII (89-97 vs. 72-75), longer IP 1294-1348 vs. 1014-1042.

Table 4. Metric data of *Balaustium yousifi* sp. n. larva (holotype and 6 paratypes).

Ch.	H	P-1	P2	P3	P4	P5	P6	Ch.	H	P1	P2	P3	P4	P5	P6
IL	471	478	466	475	459	465	460	Ta I (H)	23	22	22	22	23	24	22
IW	345	336	355	349	332	340	342	Ti I	89	92	88	86	94	93	86
L	92	95	89	88	89	95	91	Ge I	92	88	89	90	93	93	86
W	23	22	24	23	24	25	21	Tfe I	54	50	55	53	56	49	54
AW	28	28	29	30	27	30	28	Bfe I	59	60	61	58	62	58	55
MW	39	37	40	39	36	41	41	Tr I	32	31	33	30	34	34	30
PW	64	66	61	62	60	63	65	Cx I	65	62	66	64	60	61	60
SBa	12	12	11	12	12	12	12	Leg I	479	473	479	463	484	470	462
SBp	16	15	16	15	16	15	16	Ta II(L)	79	82	76	75	83	79	81
ISD	68	66	69	65	64	66	68	Ta II(H)	22	23	22	22	23	21	21
AL	30	28	30	32	29	32	31	Ti II	77	75	79	76	76	77	76
ML	30	30	29	30	28	29	32	Ge II	71	72	73	68	74	69	68
PL	34	35	36	34	33	34	32	Tfe II	44	41	39	40	42	45	46
ASE	53	50	55	52	50	56	51	Bfe II	38	37	34	35	37	39	40
PSE	72	69	74	66	70	75	71	Tr II	36	38	39	39	42	43	35
DS	28–42	27–43	29–43	28–40	26–40	28–44	30–42	Cx II	60	58	60	60	65	63	64
PDS	33–42	34–43	33–43	31–40	30–40	29–44	34–42	Leg II	405	403	400	393	419	415	410
1a	56	54	52	56	52	57	57	Ta III (L)	82	81	79	79	83	85	78
1b	45	42	45	46	41	47	41	Ta III (H)	19	19	20	19	20	19	20
2b	49	44	50	48	44	46	46	Ti III	94	96	92	89	97	92	91
3b	47	47	46	47	45	45	48	Ge III	78	75	79	74	77	79	77
GL	88	90	88	85	85	92	82	Tfe III	51	51	55	55	54	56	50
PaScFed	33	34	35	33	31	35	31	Bfe III	51	49	54	49	55	56	54

Ch.	H	P-1	P2	P3	P4	P5	P6	Ch.	H	P1	P2	P3	P4	P5	P6
PaScFev	22	21	20	23	20	23	22	Tr III	35	34	36	33	37	36	33
PaScGed	24	25	23	24	22	26	22	Cx III	61	64	58	59	57	59	60
PaScGev	18	17	18	19	17	20	18	Leg III	452	450	453	438	460	463	443
Ta I (L)	88	90	87	82	85	91	91	IP	1336	1326	1332	1294	1363	1348	1315

Acknowledments

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On the validity of *Epeorella* Ulmer, 1939 (Ephemeroptera, Heptageniidae) with general considerations on the Heptageniidae of the Sunda Islands

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Abstract

The type material of *Epeorella borneonia* Ulmer, 1939, the sole species of the genus *Epeorella* Ulmer, 1939 is reinvestigated and a lectotype (male imago) is designated. Based on several morphological structures, the synonymy with *Epeorus* Eaton, 1881 (Rhithrogeninae) is rejected. *Epeorella* stat. prop., known only at the winged stages, belongs to the subfamily Ecdyonurinae, and is a probable endemic of the island of Borneo. The newly erected genus *Darthus* Webb & McCafferty, 2007, also endemic to Borneo and only known by one species at the nymphal stage, is shown to be a junior subjective synonym of *Epeorella*. The new combination *Epeorella vadora* (Webb & McCafferty, 2007) is proposed for the species. The distribution of known heptageniid species from the Sunda Islands is discussed.

Keywords

Epeorella borneonia, *Darthus vadorus*, Borneo, lectotype, new synonym, new status, new combination

Introduction

In his major work devoted to the Ephemeroptera of the Sunda Islands, Ulmer (1939) described three new genera in the family Heptageniidae. He gave them names which recalled names of those allied genera he assumed were closely related: *Compsoneuriella* Ulmer, 1939 and *Compsoneuria* Eaton, 1881; *Rhithrogeniella* Ulmer, 1939 and *Rhithrogena* Eaton, 1881; *Epeorella* Ulmer, 1939 and *Epeorus* Eaton, 1881.

The Afrotropical genus *Notonurus* Crass, 1947 was put in synonymy with *Compsoneuriella* (type species *C. thienemanni* Ulmer, 1939, known from winged and nymphal stages) by Gillies (1963; 1984), which in turn was put in synonymy with *Compsoneuria* (Braasch and Soldán 1986b; Webb et al. 2006). Recent studies, however, have shown the three genera constitute monophyletic clades supported by synapomorphies (Sartori 2014b; Vuataz et al. 2013).

The genus *Rhithrogeniella* (type species *Rh. ornata* Ulmer, 1939, based on winged stages only) had an enigmatic position for a long time, until another species from Vietnam (*Rh. tonkinensis* Soldán & Braasch, 1986) was described, with the first reference to the nymphal stage. Based on these descriptions, Wang and McCafferty (2004) indicated that *Rhithrogeniella* was a synonym of *Rhithrogena* and *Rh. ornata* was a species of *Rhithrogena*; the species *Rhithrogeniella tonkinensis* was transferred to the genus *Ecdyonurus*. Recently, the nymph of *Rh. ornata* was described for the first time, and the generic status of *Rhithrogeniella* revalidated (Sartori 2014a) as a member of Ecdyonurinae.

The monotypic genus *Epeorella* (type species *E. borneonia* Ulmer, 1939, known only from the winged stages) was synonymized with *Epeorus* (Wang and McCafferty 2004) on the basis of similarities in several characters which will be discussed below.

The family Heptageniidae is now divided into three subfamilies which can be broadly characterized as following (Kluge 1989; Webb and McCafferty 2008):

- Rhithrogeninae: nymph with a row of setae on the ventral surface of maxillae, with dorsal process of the forefemora projected and narrower than the ventral process, some genera with vestigial paracercus; winged stages with the median depression of the mesothoracic furcasternum convergent anteriorly, and prosternum lacking transverse and longitudinal ridges.
- Heptageniinae: nymph with a row of setae on the ventral surface of maxillae, with forefemora without a dorsal projection; winged stages with the median depression of the mesothoracic furcasternum convergent anteriorly, and prosternum with distinct transverse and longitudinal ridges.
- Ecdyonurinae: nymph with scattered setae on the ventral side of maxillae; winged stages with the median depression of the mesothoracic furcasternum parallel sided or divergent anteriorly, and prosternum generally lacking transverse and longitudinal ridges.

This study concludes the re-investigation of Ulmer's Heptageniidae from Southeast Asia deposited in the Zoologisches Museum of Hamburg University (ZMH) (Sartori 2014a; b; c; d). The type material of *Epeorella borneonia* Ulmer, 1939 is described, some morphological structures are clarified, the subfamily position is established and a new synonymy is proposed.

Material and methods

The studied material is composed of three pinned specimens. The female imago was rehydrated in a solution of trisodic phosphate 0.35% and then put in alcohol. Pictures were taken with a Visionary Digital Passport II in ZMH, and plates were assembled in Adobe Photoshop CS6.

Results

Epeorella borneonia Ulmer, 1939

Epeorella borneonia: Ulmer 1939: 579 (male and female imago).
Epeorus borneonia: Wang and McCafferty 2004: 21.

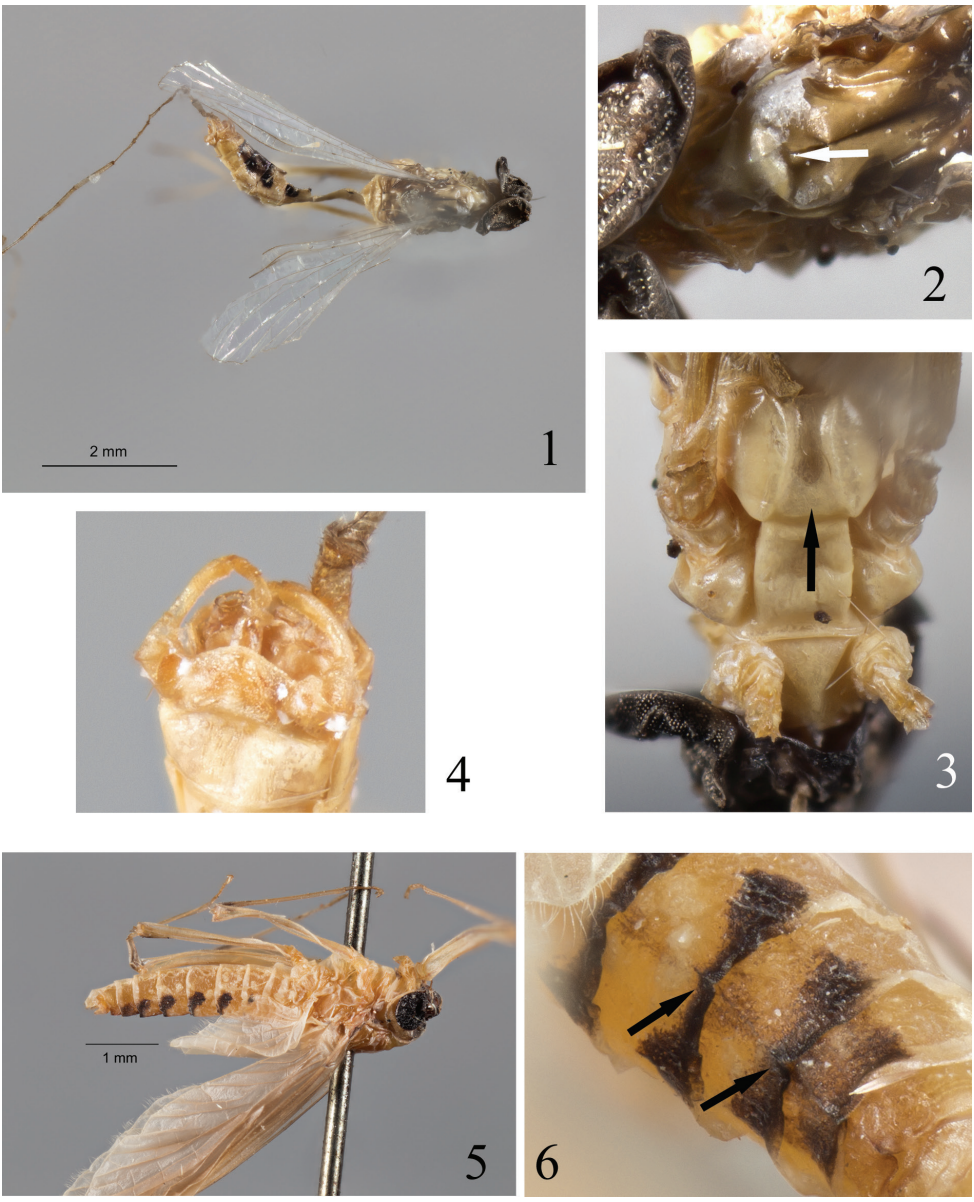
Material examined. One male imago, one female imago, one female subimago, all bearing the following labels: 1) Type [typewriting on red label], 2) Borneo, Nanga Serawei, 12–18.11.1924 3) Sammelreise Prof. Dr. H. Winckler, ded. 1924–1925 4) Z.M.H. Hamburg 5) G. Ulmer det. 1942 Vers. 13.9.1927. This last label is confusing, and according to Prof. H. Strümpel (in litt.) it is a probable mistake.

The male imago was wrongly mentioned as holotype by Weidner (1962). This terminology cannot be accepted because the "holotype" has not been designated by Ulmer and cannot be ascertained by the presence of a single specimen (see also Recommendation 73F. Avoidance of assumption of holotype, ICZN 1999).

The male imago is accordingly designated as LECTOTYPE of the species *E. borneonia* by present designation.

The three specimens have been adequately described by Ulmer (1939). Only significant morphological characters are mentioned here.

Male imago. Anterior margin of the head not protruding anteriorly (Fig. 1); median depression of mesothoracic furcasternum subparallel, not convergent anteriorly (Fig. 3); mesonotum with a transverse suture (Fig. 2); styliger plate strongly convex, penis lobes minute, rounded and closely tight together (Fig. 4), without apparent sclerites or titillators [a complete analysis of the genitalia will be presented later with the help of non-invasive techniques].



Figures 1–6. *Epeorella borneonia* Ulmer, 1939 **1** Lectotype male imago in dorsal view **2** Details of the mesonotum with transversal suture (arrow) **3** Detail of the mesothoracic furcasternum depression (arrow) **4** Detail of the genitalia in ventral view **5** Female subimago in lateral view **6** Detail of abdominal ridge (arrows) in dorsal view.

Female imago. Abdominal patterns similar to the male. Extracted eggs from the rehydrated specimen were unfortunately not in a satisfactory state for chorionic structure examination through SEM.

Female subimago. Similar to the female, except abdominal terga VI–VIII (IV–V to a lesser extent) present the remains of a longitudinal ridge (Figs 5–6).

Discussion

The synonymy of *Epeorella* with *Epeorus* was proposed by Wang and McCafferty (2004) based on the following assertions: i) male genitalia and forelegs are similar to those in *Epeorus*; ii) the vestiges associated with adults indicate that the larvae were two-tailed, and iii) the presence of median tubercles on abdominal segments VI–VIII can also be found in some *Epeorus* species.

The male genitalia greatly vary in shape among *Epeorus* species (see Webb and McCafferty 2008, figs 150–154) but are never as found in *Epeorella*; also, the forelegs of *Epeorella* are missing (see Ulmer 1939, p. 578) and therefore cannot be compared to *Epeorus*. The statement that, based on the vestigial paracercus, the nymph was two-tailed (hence comparable to *Epeorus*) is puzzling. "All Heptageniidae have the same vestigial paracercus, which does not allow to distinguish those with two-tailed and three-tailed larvae" (N. Kluge in litt.); the median ridge may be present on some *Epeorus* species which is true, but the genus *Epeorus* is absent from most of the Sunda Islands (Edmunds and Polhemus 1990, M. Sartori unpubl. data) as well as the Philippines. It is poorly diversified in Borneo where it is represented by a single species (*Epeorus boonsoongi* Braasch, 2011) with a large nymph of ca. 15 mm body length, and bifid tergal spines.

The male imago reinvestigated here presents all characteristics of the subfamily Ecdyonurinae, in peculiar the median depression of the mesothoracic furcasternum is not convergent anteriorly. Moreover, the presence of a clear transverse suture on the mesonotum, excludes it from the genus *Epeorus*. As already suggested by Braasch (2011) the synonymy proposed by Wang and McCafferty (2004) is incorrect and *Epeorella* is reinstated as *Epeorella* Ulmer, 1939, stat. prop.

One interesting character of Wang and McCafferty (2004) is the presence of remains of median tubercles visible at least on abdominal terga VI–VIII of the female subimago. It has already been demonstrated that the subimaginal stage may retain some nymphal structures, such as gill sockets (Sartori et al. 2008), which may help to link nymphal and winged stages. The presence of vestigial tubercles on the terga thus indicates that the nymph possesses a median single ridge on the abdomen. Among Ecdyonurinae, two genera are known to hold such structures, *Notacanthurus* Tshernova 1974 known from East Palaearctic, the Himalaya region and Southeast Asia, and *Darthus* Webb and McCafferty 2007, only known from Borneo (Figs 7–8). Male imagos of *Notacanthurus* possess the anterior margin of the head distinctly produced (Braasch 1986; Webb and McCafferty 2008), and genitalia possess clearly visible apical and lateral sclerites. *Darthus* is only known from the nymphal stage from the same island as *Epeorella*. Although distant by ca 500 km, the type locality of *Epeorella borneonia* and that of *Darthus vadorus* Webb & McCafferty, 2007 belong to the Dipterocarpaceae forest of lowland altitudes (Sartori et al. 2003). Moreover, the size of the mature nymphs of *D. vadorus* (5.5–8.5 mm) is compatible with the adult size of *E. borneonia* (5.0–5.5 mm) knowing that alate stages are generally smaller than mature nymphs. Therefore, it is likely that *Darthus* represents in fact the nymphal stage

Figures 7–8. *Epeorella vadora* (Webb & McCafferty, 2007), comb. n. **7** Nymph paratype in dorsal view **8** Nymph paratype in lateral view with median abdominal ridge (arrows).

of *Epeorella*, and is considered as a subjective junior synonym of *Epeorella* **syn. n.** The species *Epeorella vadora* **comb. n.** is retained as a valid species because it exhibits a different colour pattern of the abdomen.

General considerations on the Heptageniidae of the Sunda Islands

The three genera described by Ulmer (1939) have been put in synonymy by different authors but it is now demonstrated that they represent three groups of species deserving generic rank (Sartori 2014a; b, present study). They all belong to the subfamily Ecdyonurinae which is the most diversified in the studied area. Table 1 summarizes our current knowledge about the Heptageniidae of the Sunda Islands.

The Rhithrogeninae are known by only two species belonging to two widespread and speciose genera: *Rhithrogena* with more than 150 species and *Epeorus* with almost 100 species. Although mainly Holarctic, these two genera are present in the Oriental Region with 12 and 32 species respectively. The genus *Epeorus* is well represented in Indochina, where at least 13 species are known, but was unknown from the Sunda Islands until Braasch (2011) described *E. boonsoongi* from Borneo. The genus is not

Table 1. Current species of the family Heptageniidae known from the Sunda Islands.

Subfamily	Genus	species	Sumatra	Java	Bali	Borneo	Lombok	Sumbawa	Sulawesi	Reference
Rhithrogeninae	*Rhithrogena*	*sumatrana* (Ulmer, 1939)	X	X			X			Sartori 2014d
	Epeorus	*boonsoongi* Braasch, 2011				X				Braasch 2011
Heptageniinae	*Trichogenia*	*nasuta* (Ulmer, 1939)	X							Webb et al. 2006
		ulmeri Braasch & Webb, 2006	X			X				Webb et al. 2006
		hubleyi Webb & McCafferty, 2006							X	Webb et al. 2006
Ecdyonurinae	*Afronurus*	*javanicus* Ulmer, 1939		X						Ulmer 1939
		sarawakensis Braasch, 2011				X				Braasch 2011
		temburongensis Braasch, 2005				X				Braasch 2005
		webbi Braasch, 2011				X				Braasch 2011
	Asionurus	*ulmeri* Braasch & Soldán, 1986	X	X						Braasch and Soldán 1986a
	Atopopus	*edmundsi* Wang & McCafferty, 1995				X				Wang and McCafferty 1995
		tarsalis Eaton, 1881				X				Sartori et al. 2007
	Compsoneuria	*lieftincki* (Ulmer, 1939)		X						Sartori 2014b
		spectabilis Eaton, 1881	X	X						Sartori 2014b
		sp.							X	Sartori 2014b
	Compsoneuriella	*thienemanni* Ulmer, 1939	X	X						Sartori 2014b
		sp.							X	Sartori 2014b
	Epeorella	*borneonia* Ulmer, 1939				X				Present study
		vadora (Webb & McCafferty, 2007)				X				Present study
	Rhithrogeniella	*ornata* Ulmer, 1939	X	X						Sartori 2014a
	Thalerosphyrus	*determinatus* (Walker, 1853)		X	X			X		Sartori 2014c
		lamuriensis Sartori, 2014	X							Sartori 2014c
		sinuosus (Navás, 1933)	X	X						Sartori 2014c
		sp.				X				Braasch 2011, Sartori 2014c
			9	9	1	10	1	1	3	

recorded from Java, Sumatra and other Sunda Islands despite numerous samples over the last century. *Rhithrogena* exhibits an opposite trend in its distribution, being known from Sumatra, Java, Lombok, and possibly Bali, but seems to be absent from Borneo.

The only genus of the subfamily Heptageniinae present on the Sunda Islands is *Trichogenia* Braasch & Soldán, 1988, a Southeast Asian genus with one species in Sulawesi, *T. hubleyi* Webb & McCafferty, 2006 and two species in Sumatra, *T. nasuta* (Ulmer, 1939) and *T. ulmeri* Braasch & Webb, 2006, the latter also recorded from Borneo (Webb et al. 2006). There is a reasonable probability that *T. ulmeri*, known only at the nymphal stage, is a junior synonym of *T. nasuta*, known only at the winged stages, the distance between both type localities being less than 100 kilometres.

The subfamily Ecdyonurinae includes four times as many species as the two previous subfamilies combined. This is not surprising since Ecdyonurinae nymphs are among those which can tolerate slow flowing waters and high water temperatures; they have movable gills which is also an advantage when oxygen concentration is not optimal. The only two genera found in tropical Africa (*Afronurus* and *Notonurus*) also belong to the Ecdyonurinae. The genus *Afronurus* is the most diversified in the Oriental Region with 45 described species, but most of them are poorly known or badly described. It is probable that the concept of *Afronurus* in the Orient is paraphyletic; nevertheless, the genus seems present mainly on Borneo with three species; the species *Afronurus javanicus* Ulmer, 1939, is only known by adults collected on Java, which fit the current concept of *Afronurus* (M. Sartori, unpub. data). The genus is not reported from Sumatra, or Sulawesi, but seems present on Sumbawa and Sumba (M. Balke coll.). The two genera *Atopopus* and *Epeorella* are only found on Borneo, the former extending its range to the Philippines with two described species. The genera *Asionurus* and *Rhithrogeniella* have a distribution restricted to Indochina, extending to Java and Sumatra only. Finally Sulawesi is the most eastern island to have been colonized by Heptageniidae with *Compsoneuria*, *Compsoneuriella* and an undescribed genus (M. Sartori unpubl. data, M. Balke coll.). The family is not recorded from Moluccas, as well as Papua New Guinea, where the families Baetidae, Leptophlebiidae and Caenidae are eudominant.

More studies are needed, especially molecular phylogenies, to infer the timing and patterns of distribution of the genera and species in the area, particularly the relative importance of vicariance processes and dispersal events since the Miocene (Lohman et al. 2011).

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RESEARCH ARTICLE

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Two new species of the genus *Epuraea* Erichson, 1843 from China (Coleoptera, Nitidulidae, Epuraeinae)

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Abstract

Two new species belonging to the *consobrina*-group of the subgenus *Micruria* Reitter, 1875 (genus *Epuraea* Erichson, 1843), *E.* (*M.*) *lanuginosa* **sp. n.** and *E.* (*M.*) *pulliginis* **sp. n.**, found in Sichuan Province, China, are described. Pictures and details of structures important for diagnostics of the new species, including external characters and genitalia are given.

Keywords

Coleoptera, Nitidulidae, *Epuraea*, *Micruria*, new species, China, Sichuan

Introduction

The *Micruria* Reitter, 1875 is recognized as a taxon with a subgeneric status (Reitter 1884a, 1884b; Grouvelle 1908; Spornraft 1966; Jelínek 1978; Hisamatsu 1985; Kirejtshuk 1992, 1998). Reitter (1875) included in it the following species: *M. japonica* Reitter, 1875, *M. mandiularis* Reitter, 1873, *M. nitida* Reitter, 1875 and *M. macrophthalma* Reitter, 1875. Additional species attributed to *Micruria* were later described by Grouvelle (1892, 1894, 1897, 1902, 1908, 1914), Hisamatsu (1961),

Jelínek (1978) and Kirejtshuk (1992, 1997, 1998, 2005). According to Kirejtshuk (1998) the subgenus *Micruria* can be divided into five groups of species: *mandibularis*-group, *auripubens*-group, *melanocephala*-group, *grouvellei*-group, and *consobrina*-group. There are 55 known species spread in the Eastern Hemisphere, mainly in the Palaearchiarctic (East Chinese) Province and the Indo-Malayan Region, of which 16 were recorded from China (Kirejtshuk 1998). Here we add two new species, *E.* (*M.*) *lanuginosa* sp. n. and *E.* (*M.*) *pulliginis* sp. n., which are placed in the *consobrina*-group.

Most members of *Micruria* occur mainly in mountain forests. Some species have been found under bark with fermenting sap or oozing cambial tissue, in decomposing grass or leaves, and similar substrates of plant origin. However, adults of many representatives are associated with flowers of trees and bushes in nemoral forests (Kirejtshuk 1998).

Material and methods

The holotypes and paratypes of the new species are deposited in the collection of Northwest A&F University (NWUAF), Yangling and the Institute of Zoology, Chinese Academy of Sciences (IZCAS), Beijing.

All descriptions and measurements were made under an Olympus SZX 10 microscope. Figures were made using Leica ZOOM 2000 microscope with an O-image CCD. Images were produced using the software Synoptic Automontage.

Taxonomic treatment

Family Nitidulidae Latreille, 1802
Subfamily Epuraeinae Kirejtshuk, 1986
Tribe Epuraeini Kirejtshuk, 1986
Genus *Epuraea* Erichson, 1843

Subgenus *Micruria* Reitter, 1875

Type species. *Epuraea mandibularis* Reitter, 1873, designated by Kirejtshuk (1998).

***Epuraea* (*Micruria*) *lanuginosa* Zhao, Huang & Kirejtshuk, sp. n.**
http://zoobank.org/E3D2A67E-6AED-440F-B00F-3FEE2047E06F
Figs 1–8

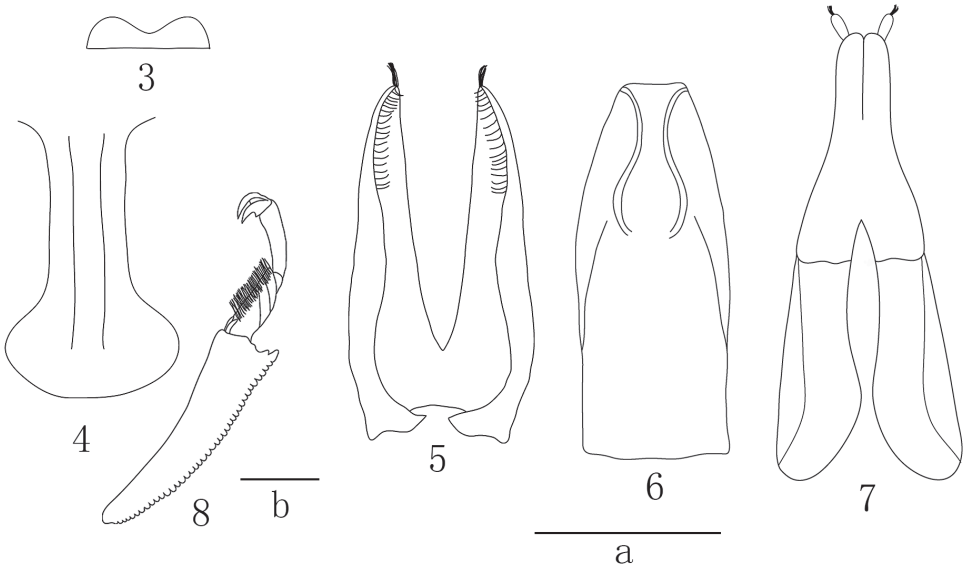
Type material. Holotype. ♂, China: Sichuan, Pingwu, Laohegou; 1800m, 7.VII.2013, Lingling Ren leg. (NWSUAF). **Paratypes.** (1♂, 3♀), same data as holotype (NWSUAF).

Figures 1, 2. *Epuraea* (*Micruria*) *lanuginosus* sp. n. male. **1** habitus, dorsal view **2** same, ventral view. Scale bar = 1 mm.

Description. *Body.* Length 3.7 mm, breadth 1.7 mm, height 0.9 mm. Oblong, moderately convex; dorsum dark brown and with bronze lustre, underside reddish-brown with appendages slightly lighter, pronotal and elytral margins light reddish to yellow; dorsum with long, strongly conspicuous and sparse silver yellowish hairs, which are three times longer than distance between their insertions (Figs 1, 2).

Integument. Head with irregular and indistinct punctures, surface between them microreticulated. Pronotum with finer punctures nearly as large as eye facets; interspaces between them greater than a puncture diameter and smoothly microreticulated. Scutellum triangular with shallow punctures smaller than punctures on pronotum and interspaces among them equal to a puncture diameter or greater. Elytra with punctures slightly smaller than those on scutellum, interspaces among them greater than a puncture diameter and microreticulated. Pygidial surface nearly as that of elytra, but with shallower punctures and denser pubescence. Abdominal ventrites with moderately distinct punctures slightly smaller than eye facets in diameter, interspaces among them smoothly microreticulated.

Head. Head slightly convex and eyes medium-sized. Labrum with a shallow median incision (Fig. 3). Antennal grooves start from hypostomal sinuses and are convergent posteriorly. Ultimate labial palpomere approximately 3 times as long as thick and somewhat narrowed at apex. Antennae slightly longer than head width, club approximately 2/5 of total length and about 1.5 times as long as wide. Pronotum moderately convex and 1.8 times as broad as long with apex emarginate, base lightly sinuate near posterior angles, sides arcuate with margins subexplanate and somewhat translucent, anterior angles square and posterior ones projecting slightly; widest at posterior angles, narrowed to both base and apex. Prosternal process curved along procoxae,



Figures 3–8. *Epuraea* (*Micruria*) *lanuginosus* sp. n. **3** labrum, dorsal view **4** prosternal process, ventral view **5** tegmen, ventral view **6** penis trunk, dorsal view **7** ovipositors, ventral view **8** protibia, dorsal view. Scale bars = 0.2 mm: **a** for Figs **3**–**7**, **b** for Fig. **8**.

widened apically (Fig. 4). Elytra much longer than their combined width (1.3:1), their sides arcuate and margins narrower than pronotum, with separately rounded apices, leaving uncovered the pygidium and part of preceding tergite. Pygidium triangular, apex of anal sclerite exposed from under pygidium. Distance between mesocoxae as great as width of antennal club and distance between metacoxae about three times as great as that between mesocoxae. Elytral epipleura at base as wide as antennal club. Metaventrite slightly convex with a distinct median depression.

Legs. All tibiae narrow and long; protibia with teeth gradually increasing in size along outer edge and two distinct larger teeth at apex. Mesotibia slightly curved inside near apex; tarsal claws with strong teeth at base (Fig. 8).

Aedeagus. Tegmen and penis trunk moderately sclerotized (Figs 5, 6).

Female. The apex of mesotibiae not curved. Ovipositor moderately long and weakly sclerotized (Fig. 7).

Etymology. The name derives from the conspicuously pubescent dorsum of the species ('*lanuginosus*' in Latin means 'woolly', 'downy').

Notes. Having moderately convex body, comparatively distinct dorsal punctation, subexplanate pronotal sides, simple mesotibiae, truncate apex of penis trunk the new species seems to belong to the *consobrina*-group which is hitherto known to comprise the following species: *E.* (*M.*) *bergeri* Sjöberg, 1939; *E.* (*M.*) *consobrina* Grouvelle, 1892; *E.* (*M.*) *kompantzevi* Kirejtshuk, 1999; *E.* (*M.*) *pulliginis* sp. n.; *E.* (*M.*) *reticulata* Grouvelle, 1892, *E.* (*M.*) *scapha* Kirejtshuk, 1999, *E.* (*M.*) *subita* Kirejtshuk, 1999 and *E.* (*M.*) *subreticulata* Grouvelle, 1892. It can be easily distinguished from all the members of the

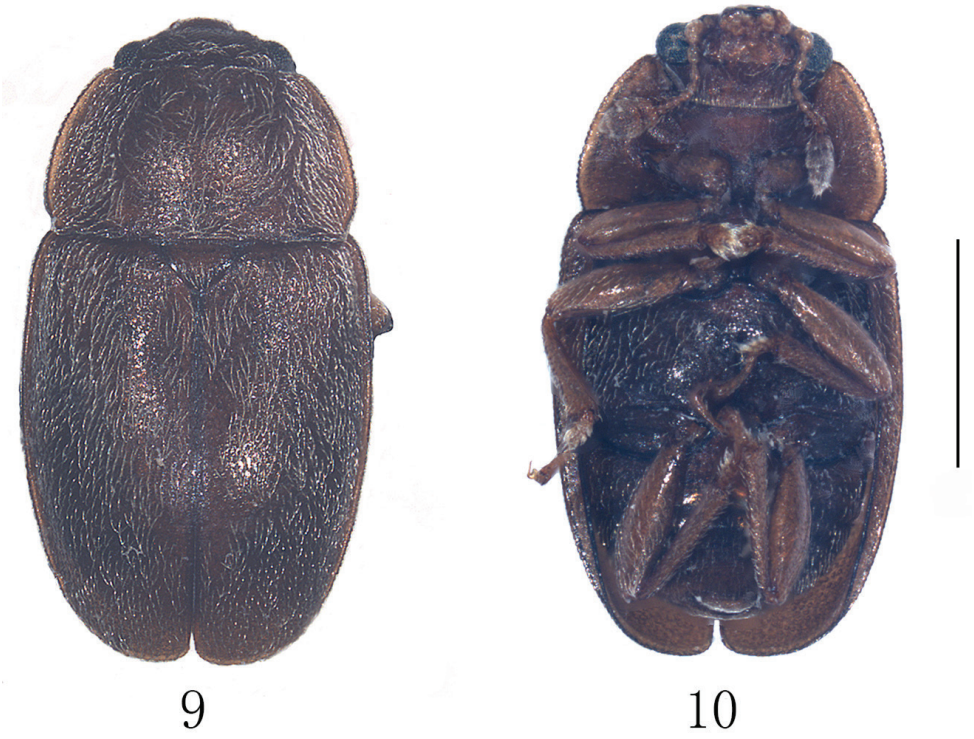
group in the bronze lustre on its rather dark dorsum, deep narrow depression along the middle of metaventrite and peculiar structure of aedeagus. Besides, it differs from:

– *E.* (*M.*) *bergeri* in the less convex pronotum narrowed at base and with more shallowly emarginate anterior edge and more clearly explanate and translucent sides, elytra more narrowing towards transversely oblique apices (not transverse), rounded apex of prosternal process, simple metafemur and metatibia, strong tooth at base of tarsal claws, ovipositor with wider base of coxites;
– *E.* (*M.*) *consobrina* in the subunicolorous disks of pronotum and elytra, coarser and deeper punctation (particularly on elytra), longer and denser silver pubescence, narrower explanate stripes of elytra, obliquely rounded elytral apices (not obliquely truncate), rounded apex of prosternal process, strong tooth at base of tarsal claws, narrower ovipositor with shorter coxites;
– *E.* (*M.*) *kompantzevi* in the more slender (not subovoid) body, denser and more clear dorsal punctation, pronotum narrowing at base, less gently sloping pronotal and elytral sides, subtruncate elytral apices (never forming a join curve), projecting subapical teeth of protibiae, lack of sexual dimorphism in elytral apices;
– *E.* (*M.*) *pulliginis* sp. n. in the much denser dorsal punctation, silver pubescence, elytra less narrowing towards subtruncate apices, more projecting subapical teeth on protibiae, narrower coxites of ovipositor;
– *E.* (*M.*) *reticulata* in the subunicolorous dorsum, much denser and more distinct dorsal punctation, denser and more conspicuous dorsal pubescence, widely rounded lobes of labrum, elytra more narrowing towards transversely oblique apices (not transverse), simple male metafemora, projecting subapical teeth of protibiae;
– *E.* (*M.*) *scapha* in the much more slender body, denser and more clear dorsal punctation, less gently sloping pronotal and elytral sides, subtruncate elytral apices (not forming a join curve), projecting subapical teeth of protibiae, simple male metafemora, meso- and metatibiae, and lack of sexual dimorphism in elytral apices, ovipositor with coxites shorter and narrower at base;
– *E.* (*M.*) *subita* in the less convex body and particularly pronotum with more clearly explanate and translucent sides, rounded apex of prosternal process, more elytra narrowing towards transversely oblique apices (not transverse), simple metafemur, strong tooth at base of tarsal claws, ovipositor with wider base of coxites.

***Epuraea* (*Micruria*) *pulliginis* Zhao, Huang & Kirejtshuk, sp. n.**
http://zoobank.org/77C35622-431E-4979-B47C-5AE642AEA2F6
Figs 9–16

Type material. Holotype. ♂, China, Sichuan, Wolong, 2200-2600m, 29.VII.1983, Xuezhong, Zhang leg., (IZCAS). **Paratypes.** 1♂,7♀, same data as holotype (IZCAS).

Description. *Body.* Length 3.2 mm, breadth 1.7 mm, hight 0.8 mm. Body oval, rather convex dorsally, dorsum nearly unicoloured chestnut brown with lighter

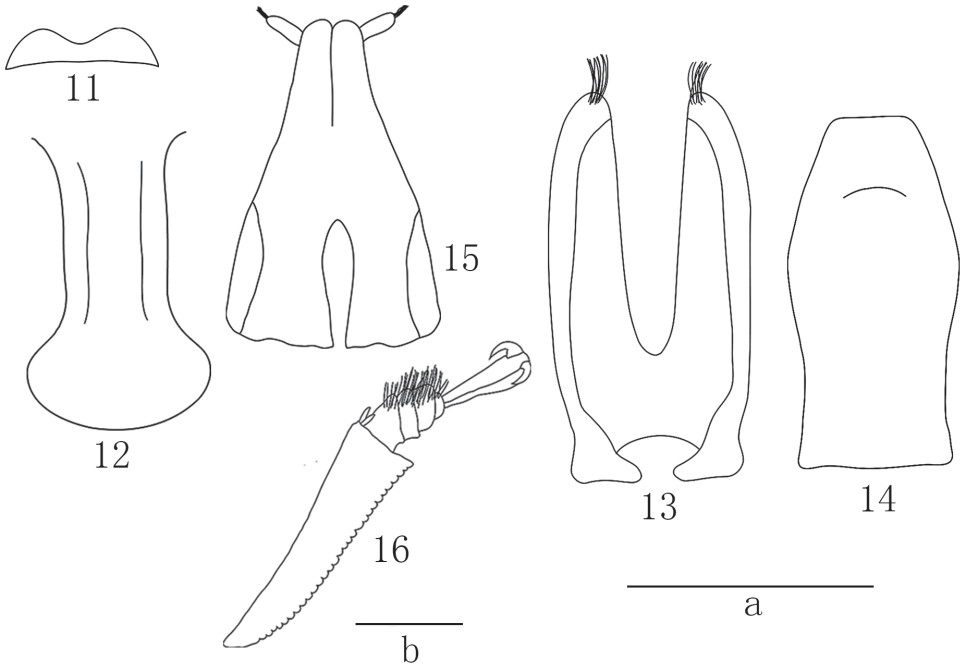


Figures 9, 10. *Epuraea* (*Micruria*) *pulliginis* sp. n., male. **9** habitus, dorsal view **10** same, ventral view. Scale bar = 1 mm.

pronotal sides, underside dark brown with brown appendages and prosternum. Pubescence silver, closely adpressed, and equal to or somewhat longer than distance between their insertions (Figs 9, 10).

Integument. Head with irregularly sized and spaced punctures. Pronotum with moderately deep punctation nearly as large as eye facets in diameter, interspaces between them slightly greater than one puncture diameter, surface microreticulated; elytra with slightly coarse punctures less than eye facets in diameter, interspaces between them approximately twice as great as a puncture diameter. Metaventrite and abdominal ventrites with indistinct punctures and microreticulated.

Head short, half as long as the distance between eyes (consisting of moderately fine facets with greater diameter than that of punctures). Labrum with shallow emargination in the middle (Fig. 11). Ultimate labial palpomere approximately three times as long as thick and somewhat narrower at apex. Antennae markedly longer than head breadth, antennal club oval, and composing approximately 1/3 of total length. Pronotum evenly convex, 1.8 times as wide as long, with apex transverse, base slightly sinuate near angles, sides arcuate and margins narrowly explanate, anterior angles projecting and posterior angles obtuse; widest just at posterior angles. Prosternal process moderately curved along



Figures 11–16. *Epuraea* (*Micruria*) *pulliginis* sp. n. **11** prosternal process, ventral view **12** labrum, dorsal view **13** tegmen, ventral view **14** penis trunk, dorsal view **15** apex of ovipositor, ventral view **16** protibia, left view. Scale bar = 0.2 mm; **a** for Figs **11**–**15**, **b** for Fig. **16**.

procoxae, moderately widened apically (Fig. 12). Elytra much longer than combined width, gradually narrowing to rounded apices, sides arcuate, and margins subexplanate and somewhat translucent. Pygidium not exposed from under elytral apices. Distance between procoxae subequal and that between matacoxae nearly three times more than between mesocoxae. Epipleura slightly narrower than antennal club.

Legs. All legs long and narrow. Protibia (Fig. 16) wider than meso- and metatibiae, with gradually increasing teeth along outer edges and two subapical long spurs. Protarsi 4/5 as wide as corresponding tibiae, meso- and metatarsi much narrower. Tarsal claws with strong teeth at base (Fig. 16).

*Aedeagus.*Tegmen well sclerotized, penis trunk moderately sclerotized (Figs 13, 14).

Female. Ovipositor simple, short and weakly sclerotized (Fig. 15).

Etymology. The specific epithet emphsizes the brown coloration of the species (Latin "*pulliginis*" – singular, genitive case from "*pulligo*" – brown, dark color).

Notes. This new species appears to be closely related to *E.* (*M.*) *kompantzevi* (*consobrina*-group) differing from it by having more slender (not subovoid) body, denser and more clear dorsal punctation, more conspicuous pubescence, less gently sloping pronotal and elytral sides, subtruncate elytral apices (not forming a join curve), projecting subapical teeth of protibiae, lack of sexual dimorphism in elytral apices and

peculiar structure of the aedeagus. Besides, in addition of characteristic structure of male genitalia of *E.* (*M.*) *pulliginis* sp. n. differs from:

– *E.* (*M.*) *bergeri* in the pronotum narrowed at base, elytra more narrowing towards transversely oblique apices (not transverse), rounded apex of prosternal process, simple metafemur and metatibia, strong tooth at base of tarsal claws, ovipositor with wider base of coxites;
– *E.* (*M.*) *consobrina* in the subunicolorous disks of pronotum and elytra, coarser and deeper punctation (particularly on elytra), longer and denser silver pubescence, narrower explanate stripes of elytra, obliquely rounded elytral apices (not obliquely truncate), rounded apex of prosternal process, strong tooth at base of tarsal claws, narrower ovipositor with shorter coxites;
– *E.* (*M.*) *pulliginis* sp. n. in the lighter coloration without bronze shine on dorsum, much sparser dorsal punctation, golden pubescence, elytra more narrowing towards subtruncate apices and completely covering abdomen, lack of deepened narrow nedian depression along the middle of metaventrite, less projecting subapical teeth on protibiae, wider coxites of ovipositor;
– *E.* (*M.*) *reticulata* in the subunicolorous dorsum, much denser and more distinct dorsal punctation, denser and more conspicuous dorsal pubescence, widely rounded lobes of labrum, elytra more narrowing towards transversely oblique apices (not transverse), simple male metafemora, projecting subapical teeth of protibiae;
– *E.* (*M.*) *scapha* in the much more slender body, denser and more clear dorsal punctation, less gently sloping pronotal and elytral sides, obliquely subtruncate elytral apices (not forming a join curve), projecting subapical teeth of protibiae, simple male metafemora, meso- and metatibiae, and lack of sexual dimorphism in elytral apices, ovipositor with coxites shorter and narrower at base;
– *E.* (*M.*) *subita* in the less convex body, rounded apex of prosternal process, elytra narrowing towards transversely oblique apices (not transverse), simple metafemur, strong tooth at base of tarsal claws, ovipositor with wider base of coxites.

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The larva of *Oecetis tripunctata* (Fabricius, 1793) (Trichoptera, Leptoceridae)

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Abstract

Oecetis tripunctata is a widely distributed leptocerid in Europe, ranging from the Iberian and Apennine peninsulas and the Central and Western European highlands to the plains of Eastern Europe. The long, single-bladed mandibles are indicative for a predacious lifestyle. This paper describes the previously unknown larva of *Oecetis tripunctata*. Information on the morphology of the 5th larval instar is given, and the most important diagnostic features are illustrated. A synoptic key for the European species of *Oecetis* is also provided. In the context of existing identification keys the larva of *O. tripunctata* keys together with *O. intima* and *O. notata*. *Oecetis tripunctata* is separated from the other two species by the fact that a double row of long setal fringes is lacking at the hind tibiae and that several long setae are present on the protrochantinus.

Keywords

Description, distribution, larva, identification, West Paleartic fauna

Introduction

From Europe, ten species of genus *Oecetis* McLachlan, 1877 are known (Malicky 2004, 2005). However, the larvae of only seven species were described up to now: *Oecetis furva* (Rambur), *O. intima* McLachlan, *O. lacustris* (Pictet), *O. notata* (Rambur), *O. ochracea* (Curtis), *O. struckii* Klapálek (unvalid synonym = *Paroecetis* Lestage)

and *O. testacea* (Curtis). References for the larval descriptions available are listed in Table 1. The larvae of *O. canariensis* Brauer, *O. grazalemae* Gonzalez & Iglesias and *O. tripunctata* (Fabricius) were unknown. Recently, however, W. Graf managed to collect larvae of *O. tripunctata* at the Thaya river in the northern part of Lower Austria. *Oecetis tripunctata* is a widely distributed leptocerid in Europe, ranging from the British Isles and the Iberian and the Apennine peninsulas and the Central and Western European highlands to the plains of Eastern Europe (Graf et al. 2008). Across Asia, this species ranges even to Bali, with many records in the countries between. *Oecetis tripuncatata* is probably the caddisfly species with the widest distribution range currently known worldwide (Malicky 2005, 2009). This taxon was described by Fabricius (1793) in the genus *Phryganea*, redescribed as *Setodes punctatella* (Rambur, 1842), as *Oecetis buitenzorgensis* (Ulmer, 1951) and as *O. alexanderi* (Kumanski, 1976). *Oecetis buitenzorgensis* was declared as synonym of *O. tripunctata* (Fabricius, 1793) by Malicky (2009b) whereas *O. alexanderi* was declared as synonym of *O. tripunctata* by Kumanski (1988) (Yang and Morse 2000; Morse 2014). With our description of its larva, proposed here, the identification of eight out of ten European *Oecetis* species is now possible even without an adult male as frequently practiced within caddisflies studies.

Material and methods

The larvae were sampled on 18 December 2012 by Wolfram Graf in the Thaya river at Hohenau, a short distance upstream of its confluence with the March river in Lower Austria. The catchment of the Thaya is situated within the granite and gneiss complex of the Bohemian highlands. Due to its low slope, the river meanders strongly and has created some scenic, deeply carved valleys descending up to 150 m steeply downwards from the plateau of the surrounding highlands. The watershed area of the Thaya is 13.319 km^2 with an average discharge of 43.9 $m^3\ s^{-1}$ and a Strahler stream order of seven. Some river stretches of the Thaya situated within the Czech Republic have been transformed in reservoirs used for irrigation, drinking water supply and hydroelectric power plants (Waringer 2003). A hand net was used to collect larvae of *Oecetis tripunctata* in the Thaya river at Hohenau in the northern part of the federal state of Lower Austria on 18 December 2012 (48°36'04"N, 16°56'10"E, 161 m above sea level). The material was preserved in 70% ethanol. A Nikon Labophot 2 microscope and a Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software for combining 8–42 frames in one focused image were used to study and photograph the larvae.

Species association was enabled by the fact that all other five *Oecetis* species reported from Austria (Malicky 2009a: *Oecetis furva*, *O. lacustris*, *O. notata*, *O. ochracea*, *O. testacea*) are well known in the larval stage (e.g., Wallace et al. 2003; Waringer and Graf 2011), and the new taxon is morphologically very different from the other species. Additionally, close to our larval collecting site more than 300 adults of *O. tripunctata* were sampled by mobile light traps.

The 5th instar larvae of *O. tripunctata* are deposited in the collection of J. Waringer (Vienna, Austria) and in the collection of W. Graf (Vienna, Austria). Comparative material of other *Oecetis* species included the following 5th instar larvae: *Oecetis furva* (n = 5), *O. lacustris* (n = 2), *O. notata* (n = 6), *O. ochracea* (n = 2), *O. struckii* (n = 3) and *O. testacea* (n = 3) (all taxa from the collection of J. Waringer, Vienna, Austria). We used the morphological terminology by Waringer and Graf (2011) and Wallace et al. (2003).

Results

***Oecetis tripunctata* (Fabricius, 1793)**
Description of the 5th instar larva

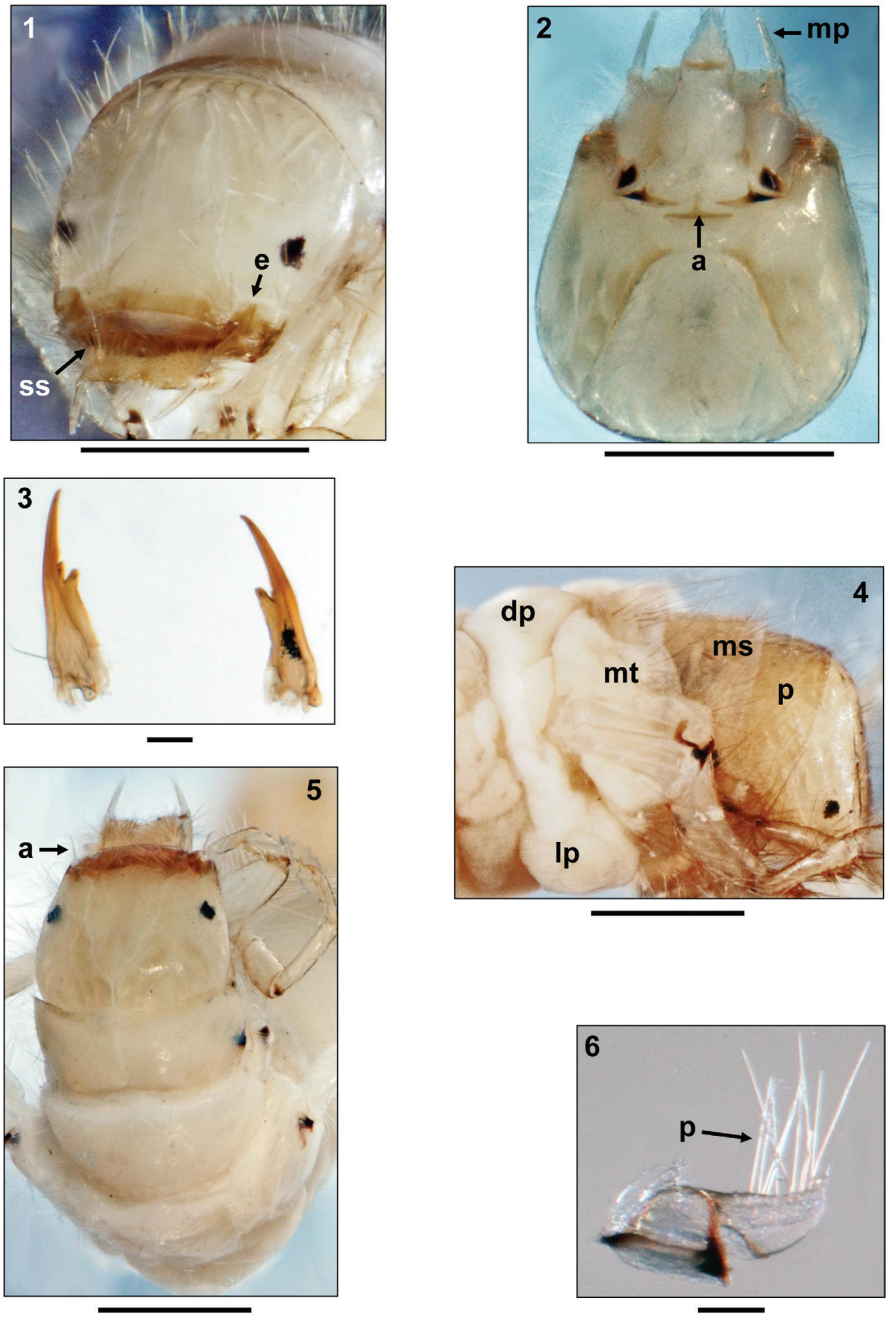
Diagnosis. Mandible sickle-shaped, with only one cutting edge; head capsule without distinct dark patches; hind tibiae without double row of long setal fringes; number of long setae on protrochantinus is > 1; basal setae on 2nd and 3rd tarsal claw rudimentary.

Biometry. Body length of 5th instar larvae ranging from 2.7 to 4.5 mm, head width from 0.66 to 0.70 mm (n = 15).

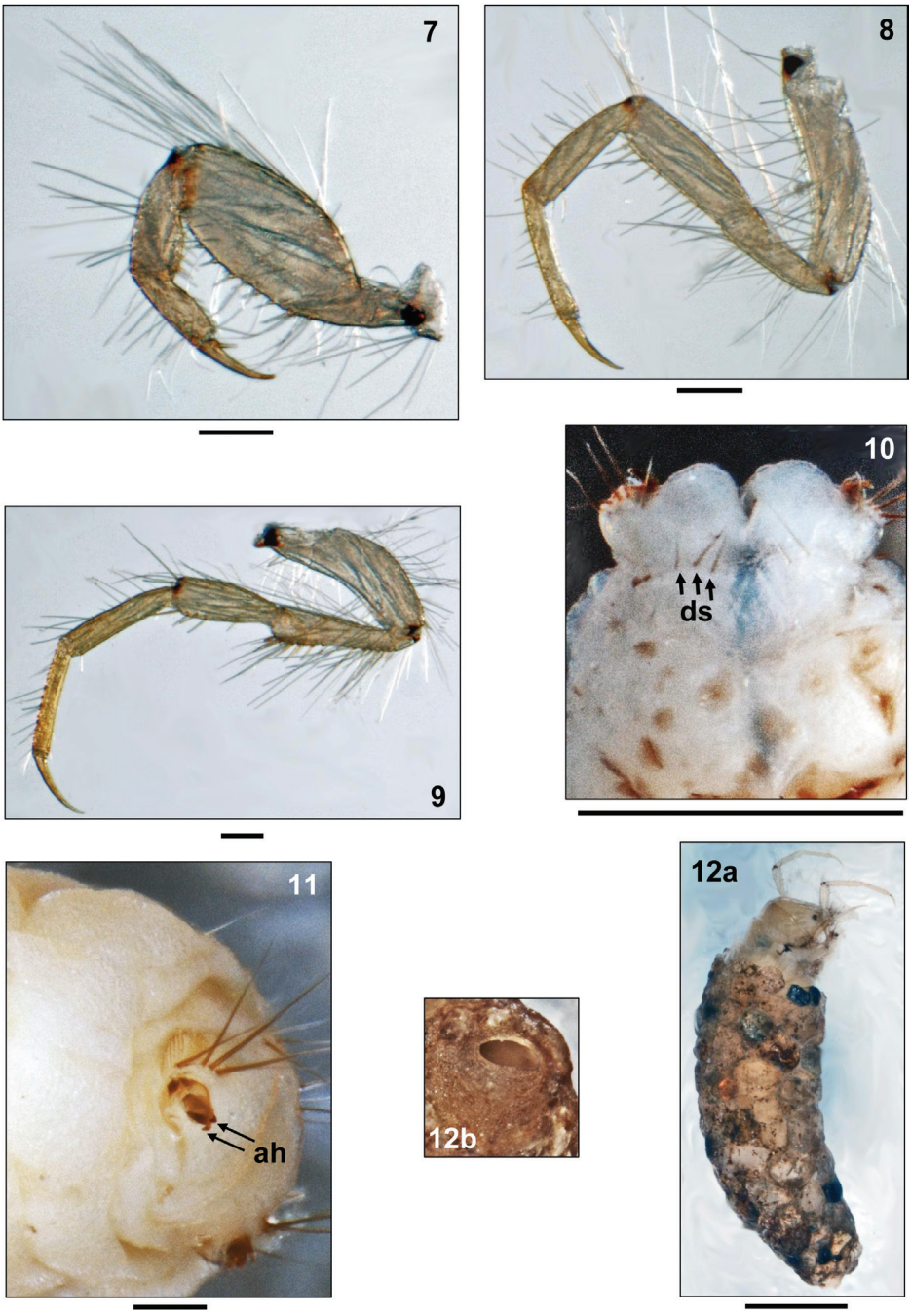
Head. Head capsule surface very smooth, roundish and hypognathous with pale yellow coloration. Light muscle attachment spots on frontoclypeus and parietalia very indistinct (Figs1–2). White ring present around eyes (Fig. 1). In addition to complete set of primary setae, head capsule densely covered by pale secondary setae, especially at anterolateral corners, dorsally of eyes and over frontoclypeus (Figs1, 4, 5). Frontoclypeus elongated, narrow, with very shallow central constriction at eye level (Figs1, 5). Subocular ecdysial line running from foramen occipitale to lateral section of parietalia, ventrally to the eyes. Anteriorly of the eyes the subocular ecdysial line bends dorsally, eventually meeting frontoclypeal suture (Fig. 1e). Antennae slender, approximately six times longer than its width at widest section, situated at extreme anterior end of parietalia and originating from a socket-like ridge (Fig. 5a). Labrum light brown, quadrangular, with anterior median notch (Fig. 1); in addition to 6 pairs of primary setae, with numerous secondary setae on dorsal surface (Fig. 1ss). Maxillary palps very long, distinctly protruding labrum (Fig. 2mp). Ventral apotome trapezoidal in shape (Fig. 2a), pale yellow with light brown anterior border; apotome not separating parietalia posteriorly. Mandibles single-bladed, sickle-shaped, with only 1 cutting edge; with sharp terminal tooth and 1–2 subapical secondary teeth (Fig. 3).

Thorax. Pronotum covering posterior section of head, light yellowish-brown, semitransparent, without distinct markings and muscle attachment spots (Fig. 5); dense continuous row of straight, pale setae along anterior border; pronotal surface densely covered by high number of pale setae (Figs1, 4). Pleural sclerites pale, semicircular, with thin, blackish-brown ventral margins; anteriorly, with pale, large, ear-like protrochantin bearing numerous pale setae (Fig. 6p). Prosternal horn absent.

Mesonotum completely covered by two sclerites, yellowish and paler than pronotum, with distinct markings and muscle attachment spots (Figs 4, 5); dense cover of



Figures 1–6. *Oecetis tripunctata* (Fabricius, 1793), 5th instar larva. **1** Head, dorsal view (e = subocular ecdysial line bending dorsally and meeting frontoclypeal suture; ss = secondary setae on labrum) **2** Head, ventral view (a = ventral apotome; mp = maxillary palp) **3** Mandibles, dorsal view **4** Head, thorax and 1st abdominal segment, right lateral view (p = pronotum; ms = mesonotum; mt = metanotum; dp = dorsal protuberance; lp = lateral protuberance) **5** Head and thorax, dorsal view (a = antenna) **6** Right protrochantin, lateral view (p = pale setae). Scale bars: 0.5 mm (except Figs **3**, **6**: 0.1 mm).



Figures 7–12. *Oecetis tripunctata* (Fabricius, 1793), 5th instar larva. **7** Left 1st leg, posterior view **8** Left 2nd leg, posterior view **9** Left 3rd leg, posterior view **10** Tip of abdomen, dorsal view (ds = dorsal setae) **11** Tip of abdomen, left posterolateral view (ah = 2 accessory hooks on anal claw) **12a** Larva and case, right lateral view **12b** Tip of larval case, posterior view. Scale bars: 0.1 mm (except Fig. **10**: 0.5 mm and Fig. **11**: 1 mm).

pale setae on the surface and along anterior border. Pleural sclerites pale, with thin, blackish-brown ventral margin (Fig. 4). Mesoventer without setae.

Metanotum without sclerotization except by pleural sclerites and with dense dorsal setal cover; pleural sclerites arrangement as on mesonotum (Fig. 4).

Legs yellowish, with very numerous setae, especially on coxae, trochanters and femora (Figs 7–9); tibiae and tarsi undivided and without central constrictions. Femur of foreleg much wider than those of mid- and hind legs. Claw of mid leg curved and not hook-shaped (Fig. 8). Long setal fringes for swimming lacking on hind legs; distal section of hind trochanter broadened and club-like (Fig. 9).

Abdomen. First abdominal segment with one dorsal (Fig. 4dp) and two lateral protuberances (Fig. 4lp), the latter with very pale and inconspicuous lateral sclerites. Lateral fringe present from segments 3–7, consisting of extremely short, pale hairs. Dorsum of 9th abdominal segment with 6–8 setae (Fig. 10ds). Anal prolegs medium brown, anal claws dark brown, each with two small dorsal accessory hooks (Fig. 11ah). Anal region without rows of spines and tooth-edges plates (Fig. 11).

Gills single-filamented; dorsal gills present at most from 2nd segment (presegmental position) to 3rd segment (presegmental position); ventral gills only at 3rd segment (presegmental); lateral gills absent.

Case. Larval case 3.0–3.7 mm long (n = 15), curved, tapered (width at anterior opening 1.2–1.5 mm and at posterior opening 0.6–0.7 mm), consisting of mix of mineral particles of unequal grain size (Fig. 12a). Posterior case opening partly closed by terminal silken membrane with oval foramen 0.2 mm wide and arranged transversally; ventral lip of membrane slightly protruding, creating an upward-directed twist of the foramen (Fig. 12b).

Synoptic key for the currently known European *Oecetis* larvae (final instars; Table 1)

Within genus *Oecetis*, *O. tripunctata* keys together with *O. intima* McLachlan, 1877 and *O. notata* (Rambur, 1842) (Table 1). *Oecetis tripunctata* is easily separated from the other two species by the fact that a double row of long setal fringes is lacking at the hind tibiae (Fig. 9) and that several long setae are present on the protrochantinus (Fig. 6).

Biological remarks

Our collection time of the larvae is in accordance with the reported spring to summer emergence and flight periods of the species; the emergence period is short and mostly limited to two months or less (Graf et al. 2008). In a light trap study from the nearby river March, we observed *O. tripunctata* to be on the wing only from June 27th to August 3rd (Waringer and Graf 2006).

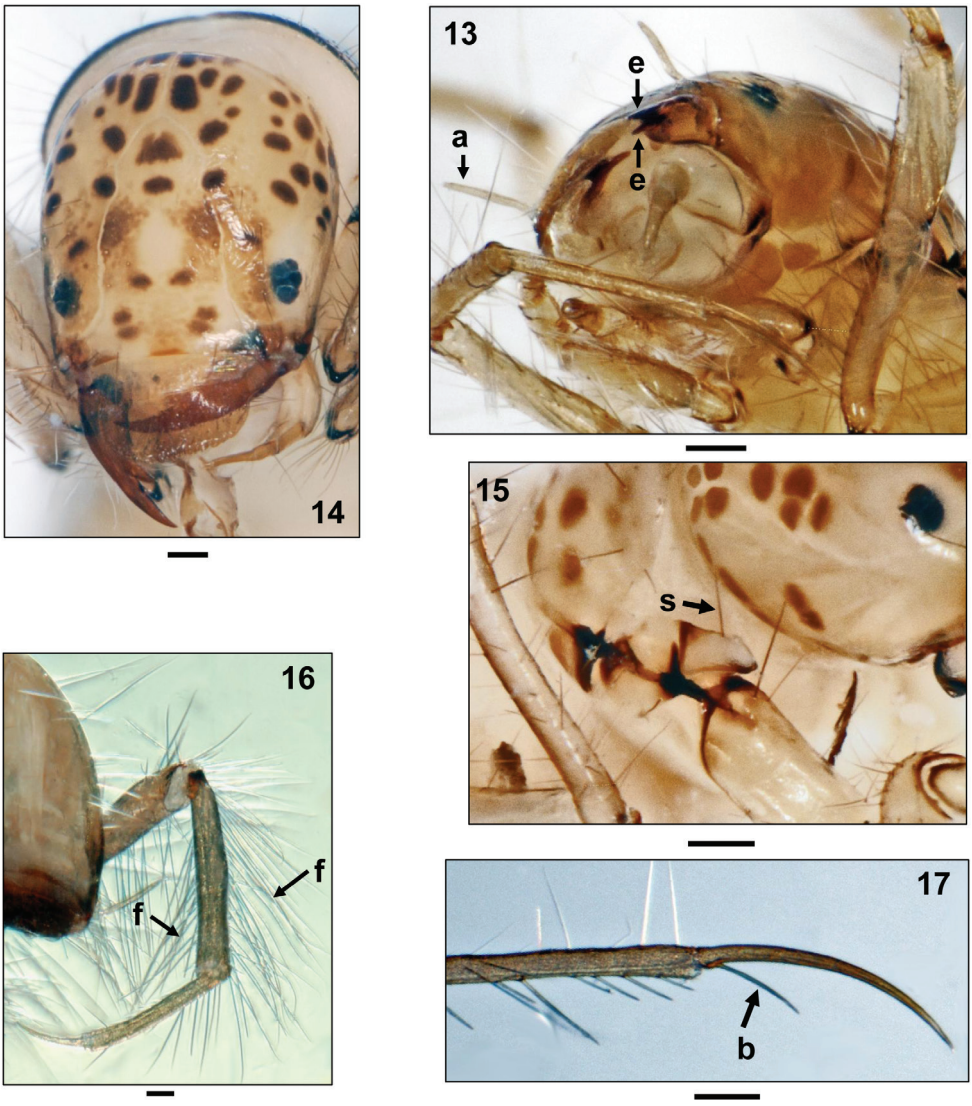
As pointed out by Wiggins (1996), *Oecetis* larvae are bottom-dwellers covering a wide range of habitats from lentic to lotic environments and may be even collected from brackish

Table 1. Synoptic key for the currently known European *Oecetis* (incl. *Paroecetis*) larvae (5th instars).

Species/character	Mandible with 2 cutting edges (Fig. 13)?	Mandible sickle-shaped, 1 cutting edge (Fig. 3)?	Head with distinct dark patches (Fig. 14)?	Hind tibiae with 2 long setal fringes (Fig. 16)?	Number of long setae on protrochantin (Figs 6, 15)	Basal seta of 2nd and 3rd tarsal claw rudimentary? (Figs 9, 17)	References
Oecetis furva [2]	no	yes	yes	no	1	no	Wallace et al. 2003, Waringer and Graf 2011
Oecetis intima	no	yes	no[1]	no	1	yes	Lepneva 1964
Oecetis lacustris	no	yes	yes	no	several	yes	Wallace et al. 2003, Waringer and Graf 2011
Oecetis notata	no	yes	no	yes	several	no	
Oecetis ochracea [2]	no	yes	yes	no	1	yes	
Oecetis struckii	yes	no	yes	no	1	no	Wiberg-Larsen and Waringer 1998
Oecetis testacea	no	yes	yes	yes	1	no	Wallace et al. 2003, Waringer and Graf 2011
Oecetis tripunctata	no	yes	no	no	several	yes	present paper

[1] Larvae in brackish water; very pale head pattern may be present in some larvae (Lepneva 1964). Southeastern species (Pontic province, eastern plains, Caucasus, Caspic depression, Asia Minor; Graf et al. 2008).

[2] In *O. ochracea* 1–4 setae are present at each side of the mesoventer; such setae are lacking in *O. furva*.



Figures 13–17. 13 *Oecetis struckii* Klapálek, 1903, 5th instar larva, left ventrolateral view (a = antenna, e = two cutting edges of the mandible) **14** *Oecetis testacea* (Curtis, 1834), 5th instar larva, head, frontal view **15** *Oecetis furva* (Rambur, 1842), 5th instar larva, right propleuron, lateral view (s = long seta on protrochantin) **16** *Oecetis notata* (Rambur, 1842), 5th instar larva, head and 3rd leg, frontolateral view (f = two long setal fringes on hind tibia) **17** *Oecetis furva* (Rambur, 1842), 5th instar larva, distal section of right 3rd tarsus and claw (b = basal seta). Scale bars: 0.1 mm.

waters (e.g., *O. intima*; Lepneva 1964). In Europe, the preferred habitats are lowland rivers with low current velocities, e.g. the Raab and the March systems in Austria.

The long, single-bladed predatory jaws of *O. tripunctata* and most other known *Oecetis* larvae are unusual among cased caddisflies; they are used for catching worms

and chironomid larvae which are ingested whole (Wallace et al. 2003). Interestingly, *O. struckii*, once attributed to genus *Paroecetis* Lestage, is unique in that the mandibles are double-edged (Fig. 13, arrows). Nevertheless, Wiberg-Larsen and Waringer (1998) reported also for this *Oecetis* species only animal remains in the foregut (Testacea, Hydrachnellae, Oribatei, Cladocera, *Asellus*, Chironomidae).

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