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New records of *Benthesicymus* Bate, 1881 (Dendrobranchiata, Penaeoidea, Benthesicymidae) from the abyssal depths of Taiwan

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Abstract

The deep-sea *Benthesicymus* shrimps generally inhabit waters deeper than 1000 m deep. Recent deep-sea cruises off Taiwan collected two species of *Benthesicymus* Bate, 1881 from the abyssal depths greater than 3,000 m. They are *B. crenatus* Bate, 1881 and *B. laciniatus* Rathbun, 1906. Both of them are new records for Taiwan, with *B. crenatus* also representing the deepest (5,314 m) marine animal so far known for the island. The major distinguishing characters of these two species are described and illustrated.

Keywords

deep-sea, new records, West Pacific

Introduction

The eastern and southern coasts of Taiwan are deep-sea areas including the abyssal zone. Knowledge on the deep-sea fauna of Taiwan, however, was rather limited only until recently. The ongoing Taiwan deep-sea cruises begun in 2000 have successfully sampled the deep-sea benthic fauna off the island and reported on several abyssal decapod crustaceans (Osawa et al. 2006, 2008a, b, Baba et al. 2009, Komai and Chan 2009).

Shrimps of the genus *Benthesicymus* Bate, 1881 are generally distributed in waters between 1000 and 2000 m depth (Crosnier 1986, Kikuchi and Nemoto 1991). Like other members of the family Benthesicymidae Wood-Mason & Alcock, 1891, they have a thin integument and short rostrum. The characteristics of this genus are the presence of podobranchia on the second maxilliped to third pereiopod, a telson with an acute tip and bearing at least three pairs of movable lateral spines, and dactyli of the fourth and fifth pereiopods not being subdivided (Kikuchi and Nemoto 1991, Pérez Farfante and Kensley 1997). Only one species of *Benthesicymus*, *B. investigatoris* Alcock & Anderson, 1899, has been listed from Taiwan but without further information (Lee et al. 1999). During recent deep-sea cruises off Taiwan, many specimens of *Benthesicymus* were collected and some of them were obtained from abyssal depths of more than 3000 m, including the deepest trawling station down to 5,314 m. Careful examination of the abyssal material of *Benthesicymus* revealed two species, namely *B. crenatus* Bate, 1881 and *B. laciniatus* Rathbun, 1906. Both are new records for Taiwan and with specimens of *B. crenatus* representing the deepest marine animals currently known in Taiwan. The present work reports upon these findings.

The specimens are deposited at the National Taiwan Ocean University (NTOU). The measurement given is carapace length (cl) measured dorsally from the postorbital margin to the posterior margin of the carapace. The synonymy provided is restricted to important works on the species, and the description given is based on the material from Taiwan.

Taxonomy

Family Benthesicymidae Wood-Mason & Alcock, 1891
Genus *Benthesicymus* Bate, 1881

***Benthesicymus crenatus* Bate, 1881**
Figs 1, 2

Benthesicymus crenatus Bate, 1881: 190 (type localities: central Pacific near Low Archipelago); Bate 1888: 329, pls. 54–55; Crosnier 1986: 851, figs. 6d-e, 7d-e, 8f-g; Kikuchi and Nemoto 1991: 67, figs. 2–3; Kim et al. 2000: 7, figs 2f, g, 8c.

Material examined. "TAIWAN 2005", stn OCP296, 22°15.08'N, 121°55.09'E, 4430–4455 m, 10 Aug 2005, 2 females cl 29.2–37.3 mm (NTOU M02182). "TAIWAN 2008", stn CP413, 22°15.06'N, 121°54.98'E, 4412–4446 m, 12 Jun 2008, 2 males cl 34.2–53.7 mm (NTOU M02183); stn CP414, 22°37.91'N, 122°32.72'E, 5011–4990 m, 13 Jun 2008, 3 males cl 25.2–37.7 mm, 1 female cl 20.2 mm (NTOU M02184); stn CP415, 22°26.16'N, 122°21.10'E, 4813–4807 m, 14 Jun 2008, 3 males cl 25.8–48.6 mm (NTOU M02185); stn CP416, 22°26.44'N, 122°21.18'E, 4824–4807 m, 15 Jun 2008, 1 male cl 25.7 mm, 1 female cl 27.1 mm (NTOU M02186).

Figure 1. *Benthesicymus crenatus* Bate, 1881, stn CP413, 4412–4446 m, male cl 34.2 mm (NTOU M02183).

"TAIWAN 2012", stn CP465, 22°37.56'N, 122°32.23'E, 5004–4996 m, 01 Jul 2012, 1 male cl 40.8 mm (NTOU M02187); stn CP466, 22°47.86'N, 122°29.72'E, 5226–5314 m, 02 Jun 2012, 2 males cl 28.1–33.3 mm, 3 females cl 20.1–37.3 mm (NTOU M02188); stn CP467, 22°48.01'N, 122°29.69'E, 5227–5154 m, 02 Jun 2012, 4 males cl 23.8–30.7 mm, 4 females cl 13.7–39.1 mm (NTOU M02189).

Description. Integument membranous and soft. Rostrum dorsally compressed and slightly elevated into a low crest, dorsal margin with three or rarely four (only in one specimen of the present material) teeth, ventral margin without teeth. Antennal spine minute but distinct. Hepatic spine present with deep cervical groove behind it. Hepatic and branchiocardiac carinae elevated (Fig. 2A). Fourth to sixth abdominal somites with posteromedian spines. Posterior margin of fourth abdominal tergite distinctly crenate; with 19–33 (usually 23–28) teeth, medial teeth larger but obtuse (except for median tooth which is largest and acute) while lateral teeth sometimes sharper but smaller (Fig. 2B). Telson with three pairs of movable lateral spines and one pair of terminal spines, distal pair of lateral spine adjacent to terminal spine (Fig. 2C). Third maxilliped and first pereiopod both with merus and ischium bearing a sharp distomesial spine (Figs. 2D–E). Distal segment of third maxilliped with sharp spine at tip (Fig. 2F). Thelycum lacking seminal receptacles but bearing median longitudinal carinae on fifth and sixth thoracic sternites. Seventh and eight thoracic sternites with large tubercle and sharp median spine, respectively (Fig. 2G). Petasma with lateral lobe generally wide and flat, except for a long strong and narrow submarginal fold at ventrolateral lobule, another shorter subdistal fold also present on dorsolateral lobule; median lobe

with distal margin minutely serrated (Fig. 2I), dorsomedian lobule strongly folded and densely covered with small hooked spines (Fig. 2H).

Coloration. Body entirely reddish to orangish red (Fig. 1). Antennular and antennal flagella orange. Scaphocerite and bascerite pinkish white to pale white. Cornea white.

Distribution. Northwest and Central Pacific, at depths of 3,530 to 6,350 m. There is one record of 0–5,700 m for this species (Kikuchi and Nemoto 1991) but it is very likely that the material was collected from more than 3500 m deep.

Remarks. The 27 specimens examined were collected from 4,412 to 5,134 m deep and most of them are damaged due to their fragile bodies even though their sizes are quite large. Nevertheless, they can be positively identified as *B. crenatus* by the characteristic comb-like crenation on the posterior margin of the fourth abdominal tergite. The Taiwanese material also generally fits well with previous descriptions of *B. crenatus* (Bate 1881, 1888, Crosnier 1986, Kikuchi and Nemoto 1991, Kim et al. 2000). The present report of *B. crenatus* from 5,324 m represents the deepest marine animal recorded from Taiwan. The previous deepest records from Taiwanese waters were the squat lobsters (Galatheidae Samouelle, 1819), *Munidopsis profunda* Baba, 2005 and *M. taiwanica* Osawa, Lin & Chan, 2008b from 5,011 m deep (Osawa et al. 2008b, Baba et al. 2009).

The 15 species known in *Benthesicymus* (De Grave and Fransen 2011) are often separated into two groups by the following characteristics: (1) branchiostegal spine not sharp and located at margin of carapace in group I vs. very sharp but situated behind the carapace margin in group II; (2) exopod of first maxilliped abruptly narrow to tip in group I vs. tapering to tip in group II; (3) merus of second maxilliped expanded in group I vs. unexpanded in group II; (4) dactylus of third maxilliped triangular with only one terminal spine in group I vs. subrectangular with more than one spine in group II; (5) exopods of all pereiopods small but visible in group I vs. very tiny in group II (Burkenroad 1936, Kikuchi and Nemoto 1991). Groups I and II consist of ten and five species respectively, and *B. crenatus* belongs in group I.

The closest species to *B. crenatus* is *B. laciniatus* (Crosnier 1986, Kikuchi and Nemoto 1991), which was also collected from Taiwan (see below). The two species can be readily separated by the following characters: (1) dorsal margin of rostrum armed with three or four teeth in *B. crenatus* (Fig. 2A) but with only two teeth in *B. laciniatus* (Fig. 3A); (2) hepatic spine present in *B. crenatus* (Fig. 2A) whereas absent in *B. laciniatus* (Fig. 3A); (3) cervical groove deep and with elevated branchiocardiac carina extending to posterior carapace in *B. crenatus* (Fig. 2A), carapace without deep groove nor distinct carina in *B. laciniatus* (Fig. 3A); (4) teeth on crenation of fourth abdominal pleuron more numerous (25–29 teeth) and rather blunt in *B. crenatus* (Fig. 2B), whereas fewer (13–19 teeth) and sharp in *B. laciniatus* (Fig. 3B); (6) posterior margin of fifth abdominal pleuron bearing distinct spine in *B. laciniatus* (Fig. 3B) whereas smooth in *B. crenatus* (Fig. 2B); (7) mesial margin of merus and ischium in third maxilliped and first pereiopod with a sharp spine in *B. crenatus* (Fig. 2D–E), whereas without spine in *B. laciniatus* (Fig. 3D); (8) thelycum with strong median spine on eighth thoracic sternite in *B. crenatus* (Fig. 2G), whereas without spine in *B. laciniatus* (Crosnier 1986: fig. 6c).

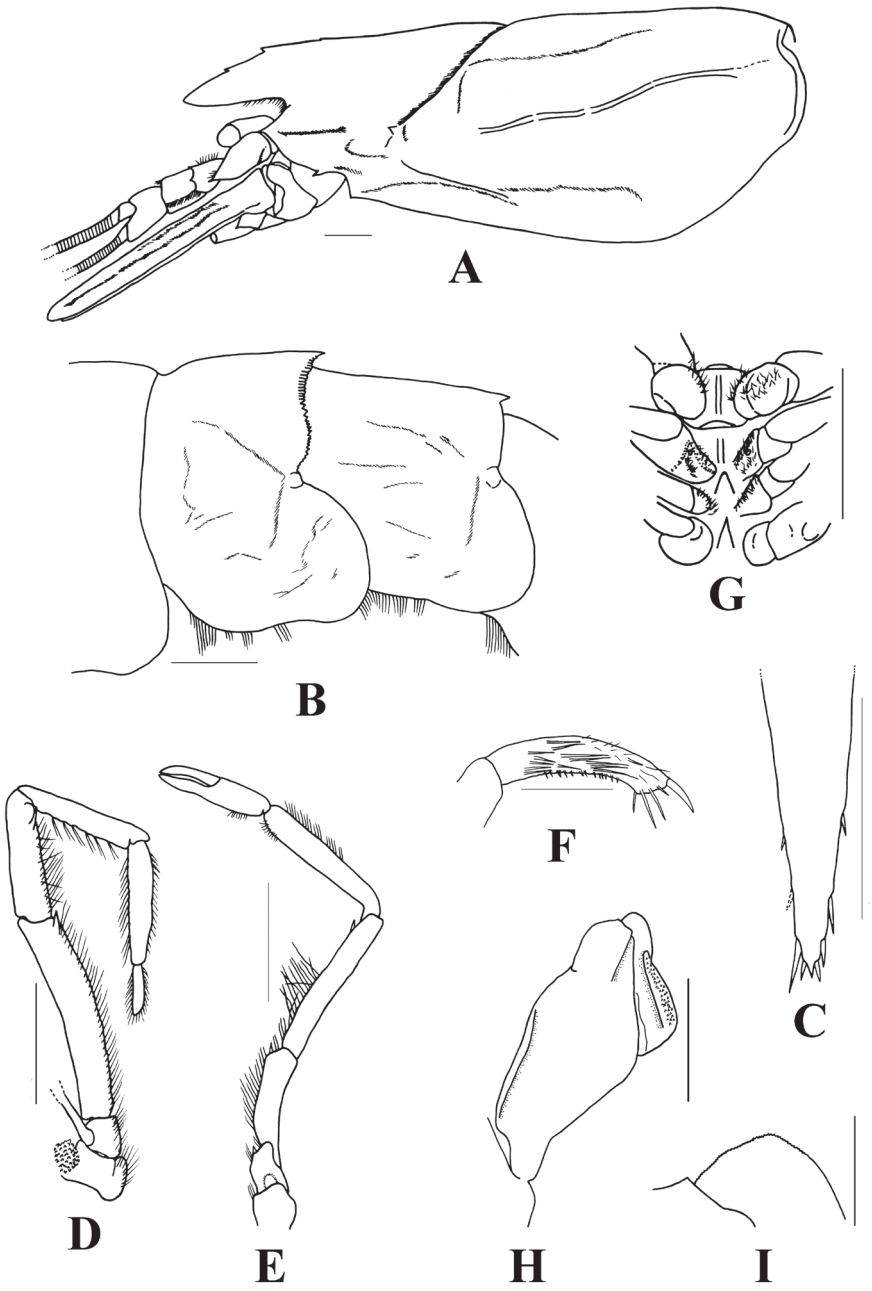

Figure 2. *Benthesicymus crenatus* Bate, 1881, **A, B, H, I** stn CP413, 4412–4446 m, male cl 53.7 mm (NTOU M02183) **C** stn CP466, 5226–5314 m, female cl 20.1 mm (NTOU M02188) **D–G** stn CP416, 4824–4807 m, female cl 27.1 mm (NTOU M02186) **A** carapace and anterior appendages, lateral view **B** abdominal somites III to V, lateral view **C** telson, dorsal view **D** left maxilliped III **E** left pereiopod I **F** dactylus of left maxilliped III **G** thoracic sternites V to VIII **H** left petasma, ventral view **I** tip of median lobe, ventral view. Scale bars: 5 mm (**A–E, G, H**); 1 mm (**F, I**).

***Benthesicymus laciniatus* Rathbun, 1906**
Fig. 3

Benthesicymus laciniatus Rathbun 1906: 906, fig. 59, pl. 19 fig. 3 (type locality: vicinity of Kauai Island, Hawaii); Burkenroad 1936: 26, fig. 1; Crosnier 1986: 851, figs. 6c, 7a-c, 8a-e; Kikuchi and Nemoto 1991: 65.

Benthesicymus Hjorti Sund 1920: 30, fig. 48, pl. 11 fig. 4 (type locality: south of Canary Islands).

Gennadas pectinatus Schmitt 1921: 25, fig. 12, pl. 11 fig. 1 (type locality: off Santa Catalina Island, California).

Material examined. Taiwan, "TAIWAN 2006", stn CP366, 22°02.87'N, 121°10.08'E, 1302–1301 m, 24 Aug 2006, 1 male cl 14.3 mm (NTOU M02190); stn CP369, 24°18.96'N, 122°04.20'E, 3030–3070 m, 25 Aug 2006, 1 male cl 25.0 mm (NTOU M02191).

Description. Integument moderately rigid. Rostrum rather straight, armed with two dorsal teeth. Carapace with surface rather smooth, lacking hepatic spine and without distinct grooves or carinae (Fig. 3A). Abdomen with fourth to sixth somites each armed with a distinct posteromedian spine; fourth tergite also with posterior margin distinctly crenate or serrated, bearing 17–19 sharp teeth that progressively become smaller laterally (Fig. 3B). Telson with three pairs of movable lateral spines and one pair of terminal spines, distalmost pair of lateral spines at some distance from terminal spines (Fig. 3C). No spine on both merus and ischium of third maxilliped and first pereiopod. Third maxilliped heavily setose and distal segment lacking spine (Fig. 3D). Petasma generally flat and simple, bilobed (Fig. 3E), distal margin of median lobe slightly serrated (Fig. 3F).

Coloration. Unknown.

Distribution. Worldwide distribution and reported from eastern Atlantic, eastern Pacific and Indo-West Pacific, at depths of approximately 1,325–4,000 m but generally from 1,500–3,000 m (Crosnier 1986).

Remarks. *Benthesicymus laciniatus* is recorded from Taiwan for the first time. As mentioned by Kim et al. (2000), *B. laciniatus* generally inhabits shallower waters than *B. crenatus* and only sometimes occurs in the abyssal zone. The two Taiwanese specimens have been collected from depths of 3,030–3,070 m and 1,301–1,302 m respectively. In *Benthesicymus* only two species have the posterior margin of the fourth abdominal tergite crenate (see Burkenroad 1936, Crosnier 1986). The distinguishing characters of these two species are given in the "Remarks" of *B. crenatus*.

The present two males collected from Taiwan generally agree with previous descriptions of the species except for the petasma with the distal margin of the median lobe not distinctly serrated (see Burkenroad 1936: fig. 1, Crosnier 1986: fig. 8a, b, e). Such difference may be due to the present males are much smaller (cl 14.3–25.0 mm vs. cl 33–36 mm for Burkenroad 1936: fig. 1; Crosnier 1986: fig. 8a, b, e) and probably juveniles.

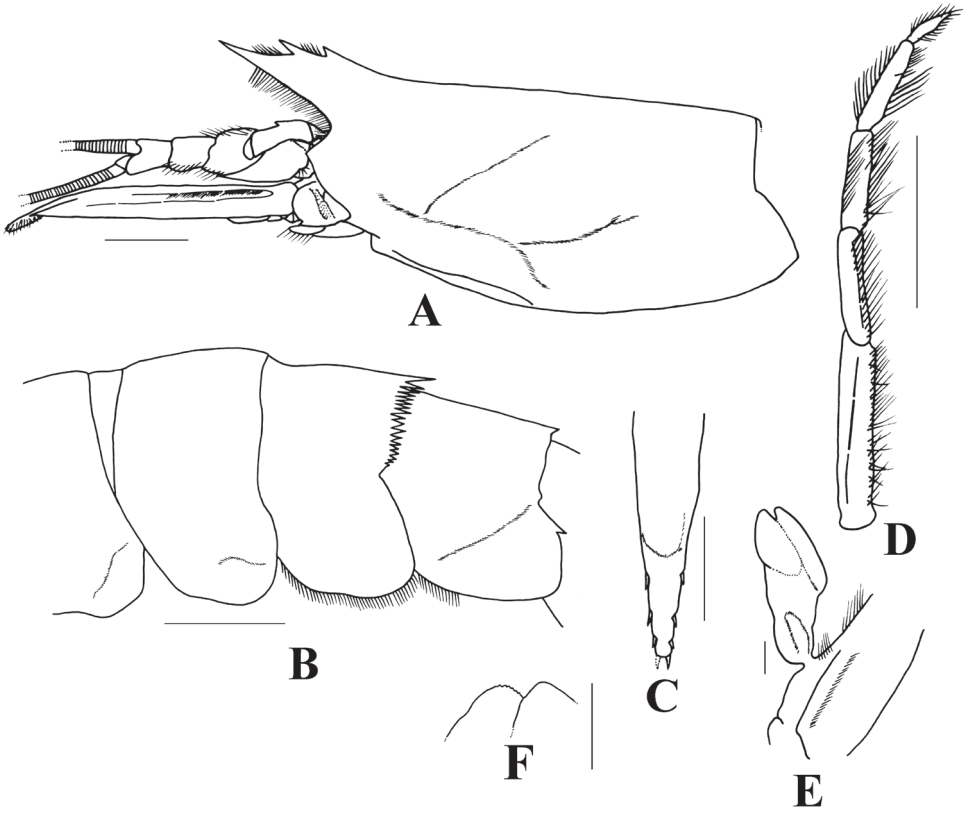

Figure 3. *Benthesicymus laciniatus* Rathbun, 1906, stn CP369, 3030–3070 m, male cl 25.0 mm (NTOU M02191) **A** carapace and anterior appendages, lateral view **B** abdominal somites IV to V, lateral view **C** telson, dorsal view **D** left maxilliped III **E** right petasma, ventral view **F** tip of median lobe, ventral view. Scale bars: 5 mm (**A–D**); 1 mm (**E, F**).

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Redescription and redefinition of the genus *Chiltana* Shakila-Mushtaq & Akbar, 1995 (Hemiptera, Fulgoromorpha, Dictyopharidae, Dictyopharini), with description of a new species from Pakistan

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Abstract

The genus *Chiltana* Shakila-Mushtaq & Akbar, 1995 is redescribed and redefined based on the types and new material from Pakistan. *Chiltana* includes two species, *C. acarinata* **sp. n.** and *C. baluchi* Shakila-Mushtaq & Akbar, 1995 (the type species), both from Chiltan, Balochistan, Pakistan. A key to the species of the genus is provided. Nomenclatorial remarks on original publication, author, and date of *Chiltana* are given.

Keywords

Fulgoroidea, morphology, taxonomy

Introduction

The dictyopharid planthopper genus *Chiltana* was firstly described by Shakila-Mushtaq in her Ph.D. thesis for a single species from Pakistan (Shakila-Mushtaq 1984). Shakila-Mushtaq (1994) listed this genus in her paper "Family Dictyopharidae (Fulgoroidea: Homoptera) from Pakistan" and stated that "*Chiltana*, a new monotypic genus has been described from Pakistan by Shakila-Mushtaq (1989 [sic]) to be added in the family Dic-

tyopharidae" (Shakila-Mushtaq 1994: 30). One year later, Shakila-Mushtaq and Akbar (1995) erected *Chiltana* as a new genus formally published in a peer-reviewed journal. Thus the original publication, author and date of *Chiltana* have been debatable.

Chiltana was placed in the tribe Dictyopharini (Emeljanov 2011). The morphological phylogeny of the world Dictyopharidae showed that *Chiltana* is quite unique in Dictyopharini and has many autapomorphies supporting its monophyly but affecting its phylogenetic assessment in the tribe Dictyopharini (Song et al. 2018).

Based on examination of the type specimens of *C. baluchi* and a critical review of the literature, *Chiltana* is here redescribed and redefined. The second *Chiltana* species, *C. acarinata* sp. n., is described and illustrated from Pakistan. Nomenclatorial remarks on original publication, author, and date of *Chiltana* are given.

Material and methods

The specimens studied in the course of this work are deposited in the following institutions, which are subsequently referred to by their acronyms: CAS, California Academy of Sciences, San Francisco, CA, USA; and ZMUK, Zoological Museum, University of Karachi, Karachi, Pakistan.

The post-abdomen of the specimens used for dissections were cleared in 10% KOH at room temperature for ca 6–12 hours, rinsed and examined in distilled water and then transferred to 10% glycerol and enclosed in microvials to be preserved with the specimens. Observations and measurements were conducted under a Zeiss Stemi SV II optical stereomicroscope, and photography was under Zeiss Discovery V12 stereomicroscope equipped with a Nikon D7000 digital camera in Institute of Zoology, Chinese Academy of Sciences, China. Some final images were compiled from multiple photographs using CombineZM image stacking software and improved with the Adobe Photoshop CS5 software.

The morphological terminology and measurements used in this study follow Song et al. (2016a, b, 2018) for most characters and Bourgoin et al. (2015) for the forewing.

Taxonomy

Family Dictyopharidae Spinola, 1839

Genus *Chiltana* Shakila-Mushtaq & Akbar, 1995

Chiltana Shakila-Mushtaq, 1984: 158, nomen nudum of *Chiltana* Shakila-Mushtaq & Akbar, 1995.

Chiltana Shakila-Mushtaq, 1994: 2 (in key), 30 (in catalogue), nomen nudum of *Chiltana* Shakila-Mushtaq & Akbar, 1995.

Chiltana Shakila-Mushtaq & Akbar, 1995: 374. Type species: *Chiltana baluchi* Shakila-Mushtaq & Akbar, 1995; by original designation and monotypy.

Diagnosis. *Chiltana* may be distinguished from other genera in the Dictyopharini by the following combination of characters: cephalic process absent due to anterior margin of vertex not reaching beyond anterior margin of eyes; vertex with lateral carinae weakly ridged and subparallel, anterior and posterior margins nearly straight, without median carina; frons without median and intermediate carinae; pronotum with anterior and posterior margins nearly straight and subparallel anteroposteriorly, lateral marginal areas distinctly convex, median carina complete but weak, without intermediate carinae; mesonotum distinctly arched, carina absent; fore and middle femora flattened and dilated, with several various sized spines on ventral margin; hind tibiae with eight apical teeth; phallobase with inflated membranous paired lobes, with numerous small superficial spines on apex of lobes.

Redescription. Head very short, cephalic process absent due to anterior margin of vertex not reaching beyond anterior margin of eyes, so anterior part of dorsal surface of head occupied by basal extension of frons in dorsal view (Fig. 2A). Vertex (Fig. 2A) moderately broad, basal width slightly wider than transverse diameter of eyes; anterior margin not reaching beyond anterior margin of eyes, just approaching apical fourth of eyes; posterior plane elevated above pronotum; lateral carinae weakly ridged and subparallel; anterior and posterior margins weakly ridged and nearly straight; median carina absent, with a lateral large pit on each side. Frons (Fig. 2C) with lateral carinae weakly ridged, nearly parallel; median and intermediate carinae absent; basal margin of frons projecting anteriad to apex of vertex, distinctly visible in dorsal view (Fig. 2A). Postclypeus and anteclypeus (Fig. 2C) convex medially, with distinct median carina. Rostrum moderately long, reaching base of hind femora; basal segment slightly longer than distal one. Compound eyes large and globose. Ocelli relatively large, reddish. Antennae with very small scape; pedicel large and subglobular, with more than 50 distinct sensory plaque organs distributed over entire surface; flagellum long, setuliform.

Pronotum (Fig. 2A) distinctly shorter than mesonotum at midline, anterior and posterior margins nearly straight and subparallel anteroposteriorly; lateral marginal areas distinctly convex and sloping down with two longitudinal carinae on each side; intermediate carinae absent; median carina complete but weak, with a lateral pit on each side. Mesonotum (Fig. 2A) distinctly arched, carina absent. Forewings (Fig. 2D) hyaline, venation with sparse transverse veins; MP bifurcating MP_{1+2} and MP_{3+4} near middle and beyond CuA; number of apical cells between R and CuA equal to 13; Pcu and A_1 veins fused into a long Pcu+A1 vein at apical 1/4 in clavus; pterostigmal area clear, with 4 or 5 cells. Legs (Fig. 3A–D) moderately long; fore femora strongly flattened and dilated, with about 10 various sized spines on ventral margin; middle femora flattened and dilated, with about six various sized spines on ventral margin; fore and middle tarsomeres I and II with several acutellae; hind tibiae with four lateral spines and eight apical teeth; hind tarsomeres I and II with about 14 apical teeth, respectively.

Male genitalia. Pygofer (Fig. 4A, B, D) in lateral view wider ventrally than dorsally, dorsal margin slightly excavated to accommodate segment X, dorsoposterior margins angular, produced into a distinct lobe which is short and broad. Gonostyles (Fig. 4B–E) symmetrical, with narrow base, expanded toward apex, broadest at api-

cal fourth; dorsal margin with a claw-like process directed dorsad, outer dorsal edge with a spiny hook-like process near middle directed ventrad. Aedeagus (Fig. 5A–F) with one pair of elongate endosomal processes extended from phallobase membranous, acute and sclerotised apically; phallobase sclerotised and pigmented basally, membranous and inflated apically, with paired lobes. Segment X (Fig. 4A, B) large, in dorsal view, with apex deeply excavated to accommodate anal style; anal style elongate and large.

Female genitalia. Gonapophyses VIII with anterior connective lamina large and sclerotized, with teeth of varying sizes and shapes. Gonoplacs with two lobes homologous; lateral lobe sclerotized, large and elongate, with a distinct sensory appendage on apex, a bunch of long setae on sensory appendage; the posterior lobe membranous, containing long sclerotized plate. Segment X large and broad, apex deeply excavated to accommodate anal style; anal style large and elongate.

Diversity and distribution. This genus contains two species restricted to Chiltan, Balochistan, Pakistan.

Remarks. In the original descriptions and illustrations of *Chiltana*, the frons and mesonotum were described as "tricarinate" (Shakila-Mushtaq and Akbar 1995). Actually, the carinae on the frons and mesonotum of *Chiltana* species are absent based on examination of the type specimens of *C. baluchi* and new species, although these corresponding positions show the different colored patterns, like some other dictyopharid species. In addition, the legs, female genitalia and other characters of *Chiltana* were not mentioned in the original paper. The original generic diagnosis of *Chiltana* is short and incomplete. Thus, *Chiltana* is here redescribed and redefined based on examination of the type specimens of *C. baluchi* and new species and a critical review of the literature.

Chiltana is similar to the genera *Afronersia* Fennah, 1958 and *Gilgitia* Shakita-Mushtaq, 1991 in various characters of head, venation and genitalia (Shakila-Mushtaq and Akbar 1995). In the tribe Dictyopharini, *Chiltana* has several diagnostic characters that serve to differentiate it from other genera. The smaller dimensions of the head, absence of carinae on the frons and mesonotum, and flattened and dilated fore and middle femora with variously sized spines on the ventral margin may easily distinguish *Chiltana* from remaining genera in the Dictyopharini.

Key to the species of *Chiltana*

1 Aedeagus with endosomal processes directed ventrad; dorsal apical process of gonostyles large and broad, directed dorsad; phallobase with a pair of dorsal lobes with a large and stout spine on apex of each lobe..... ***C. acarinata* sp. n.**

– Aedeagus with endosomal processes directed dorsad; dorsal apical process of gonostyles small, directed dorsocephalad; phallobase with two pairs of dorsal lobes, without spine on apex..... ***C. baluchi* Shakila-Mushtaq & Akbar**

***Chiltana acarinata* sp. n.**
http://zoobank.org/40014A57-8B37-4C06-832D-2599286E843D
Figures 1–5

Type material examined. Holotype ♂, Pakistan: Hazarganji, Chiltan National Park, 20 km SW Quetta, 3–6.vii.1989, W.J. Pulawski & W.A. Khan (CAS). Paratype, 1 ♂, Pakistan: same as holotype (CAS)

Description. Body length (from apex of head to tip of forewings): 11.5–11.7 mm; head length (from apex of head to base of eyes): 1.2 mm; head width (including eyes): 1.5 mm; forewing length: 9.6–9.7 mm.

Coloration. General color brownish ochraceous marked with ivory white, pale green and purplish red on head and thorax, and dark brown on abdomen in dorsal view (Fig. 1). Head excluding eyes ivory white, vertex ochraceous basally and yellowish green apically (Fig. 2A), frons yellowish green, areas along intermediate carinae purplish red (Fig. 2C). Compound eyes fuscous with posterior margin ochraceous red and ivory white, ocelli purplish red (Fig. 2B). Clypeus pale ochraceous basally and apically, and dark brown medially, with a pair of small black spots on anteclypeus (Fig. 2C). Pronotum entirely ivory white. Mesonotum purplish red to ochraceous brown, areas of median and lateral carinae and lateral marginal areas flavescent or greenish (Fig. 2A). Forewings membrane hyaline, veins ochraceous, pterostigmal area and a large sublunate streak on distal fourth dull ochraceous (Fig. 2D). Thorax yellowish ochraceous ventrolaterally with dark brown patches adjacent to base of fore coxae. Legs pale to dark brown, with numerous black small spots (Fig. 3A–D). Abdomen dorsally ochraceous to dark brown, with dark brown or pale ochraceous stripes of various sizes and shape, ventrally more or less uniformly yellowish ochraceous; male and female terminalia brown.

Structure. Head (Fig. 2A–C) very short, cephalic process absent. Vertex (Fig. 2A) wider than length, with ratio of length at midline to width between eyes 0.8:1. Frons with basal margin of frons projecting anteriad to apex of vertex, distinctly visible in dorsal view (Fig. 2A); in ventral view, frons with ratio of length at midline to maximum width 2.0:1; median and intermediate carinae absent (Fig. 2C). Forewings (Fig. 2D) hyaline, ratio of length to width about 3.2:1. Legs (Fig. 3A–D) moderately long; fore femora (Fig. 3A) strongly flattened and dilated, with about 10 various sized spines on ventral margin; middle femora (Fig. 3C) flattened and dilated, with about six variously sized spines on ventral margin; fore and middle tarsomeres I and II (Fig. 3B) with several acutellae; hind tibiae (Fig. 3D) with four lateral spines and eight apical teeth; hind tarsomeres I and II with about 14 apical teeth, respectively.

Male genitalia. Pygofer, in lateral view (Fig. 4B), with dorsoposterior margin forming a small and broad lobe; in ventral view (Fig. 4D) a little longer than in dorsal view (Fig. 4A) with ratio of ventral to dorsal width about 1.3:1. Gonostyles (Fig. 4B–E) elongate, relatively narrow in basal half, dorsal apical process large and broad, directed dorsad (Fig. 4E). Aedeagus (Fig. 5A–C) large and strongly inflated, endosomal pro-

Figure 1. Habitus of *Chiltana acarinata* sp. n. Scale bar: 2 mm.

cesses elongate and robust, extended from phallobase, curved dorsad and then ventrad, apex sclerotized, elongate and acute (Fig. 5B). Phallobase with three pairs of inflated membranous lobes: a pair of large and stout dorsal lobes, directed dorsad, with a large and stout spine on apex of each lobe (Fig. 5A, B, D); a pair of large, strongly inflated, rounded ventral lobes, directed laterad, covered with numerous minute superficial spines (Fig. 5B–D); and a pair of elongate thumb-like ventral lobes extended from dorsal side of rounded ventral lobes, their apices gradually convergent and tapering dorsad, muricate apically (Fig. 5B–D). Segment X, in dorsal view (Fig. 5A), oval and broadest medially, with ratio of length to maximum width 1.1:1; in lateral view (Fig. 5B), short and robust, with ventral margin gradually widening from base to apex; anal style large, beyond apical ventral margin of segment X.

Figure 2. *Chiltana acarinata* sp. n. **A** Head, pronotum and mesonotum, dorsal view **B** head and pronotum, lateral view **C** head and pronotum, ventral view **D** forewing. Scale bars: 1 mm.

Etymology. The specific epithet is borrowed from New Latin *acarinatus*, referring to the carinae on the frons and mesonotum being absent.

Distribution. So far only known from Chiltan, Balochistan, Pakistan.

Figure 3. *Chiltana acarinata* sp. n. **A** Fore leg **B** fore tarsomeres and pretarsus **C** middle leg **D** hind leg. Scale bars: 1 mm.

Remarks. The new species may be distinguished from the type species of *Chiltana*, *C. baluchi*, by the different male genitalia.

Chiltana baluchi Shakila-Mushtaq & Akbar, 1995

Chiltana baluchi Shakila-Mushtaq, 1984: 160, fig. 33A–I, nomen nudum of *Chiltana baluchi* Shakila-Mushtaq & Akbar, 1995.

Chiltana baluchi Shakila-Mushtaq, 1994: 30 (in catalogue), nomen nudum of *Chiltana baluchi* Shakila-Mushtaq & Akbar, 1995.

Chiltana baluchi Shakila-Mushtaq & Akbar, 1995: 374, figs 1–12.

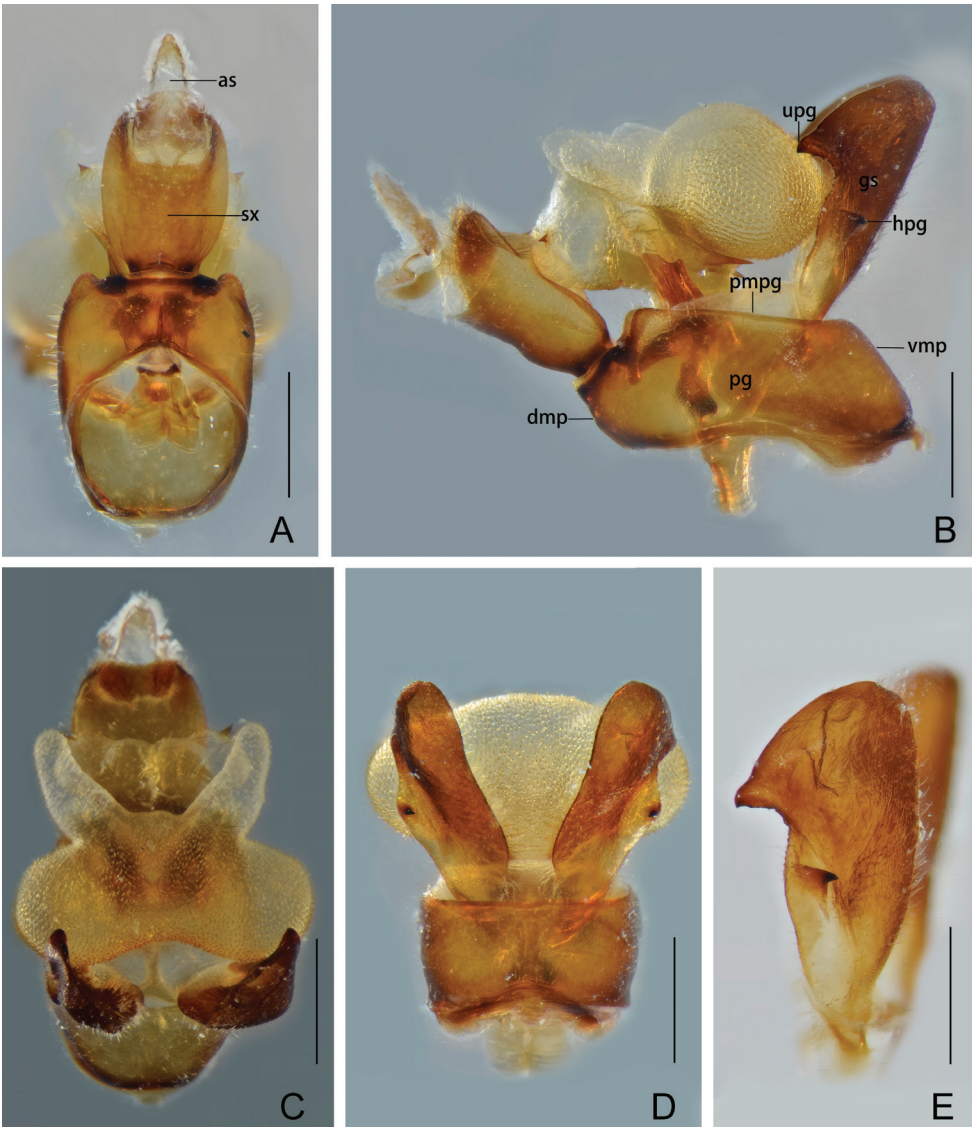

Figure 4. *Chiltana acarinata* sp. n. **A** Male segment X and pygofer, dorsal view **B** male pygofer, gonostyles, and segment X, lateral view **C** male pygofer, gonostyles, and segment X, caudal view **D** male pygofer and gonostyles, ventral view **E** gonostyle. Abbreviations: as, anal style; dmp, dorsal margin of pygofer in profile; gs, gonostyle; hpg, hook-like process of gonostyle; pg, pygofer; upg, upper process of gonostyle; sx, segment X; vmp, ventral margin of pygofer in profile. Scale bars: 0.5 mm.

Type material examined. Holotype ♂ of *Chiltana baluchi* Shakila-Mushtaq & Akbar, Pakistan: (1) [red round label]; (2) Loc. Chiltan Muslim, Host. wild mint, Date. 6.vii. [19]64, Coll. S.M. Khan (ZMUK). Allotype ♀ *Chiltana baluchi* Shakila-Mushtaq & Akbar, Pakistan: (1) Loc. Chiltan Muolnig, Host. wild mint, Date. 6.vii.[19]64, Coll. S.M. Khan; (2) Dictyophara chiltanii [red written label] (ZMUK).

Distribution. So far only known from Chiltan, Balochistan, Pakistan.

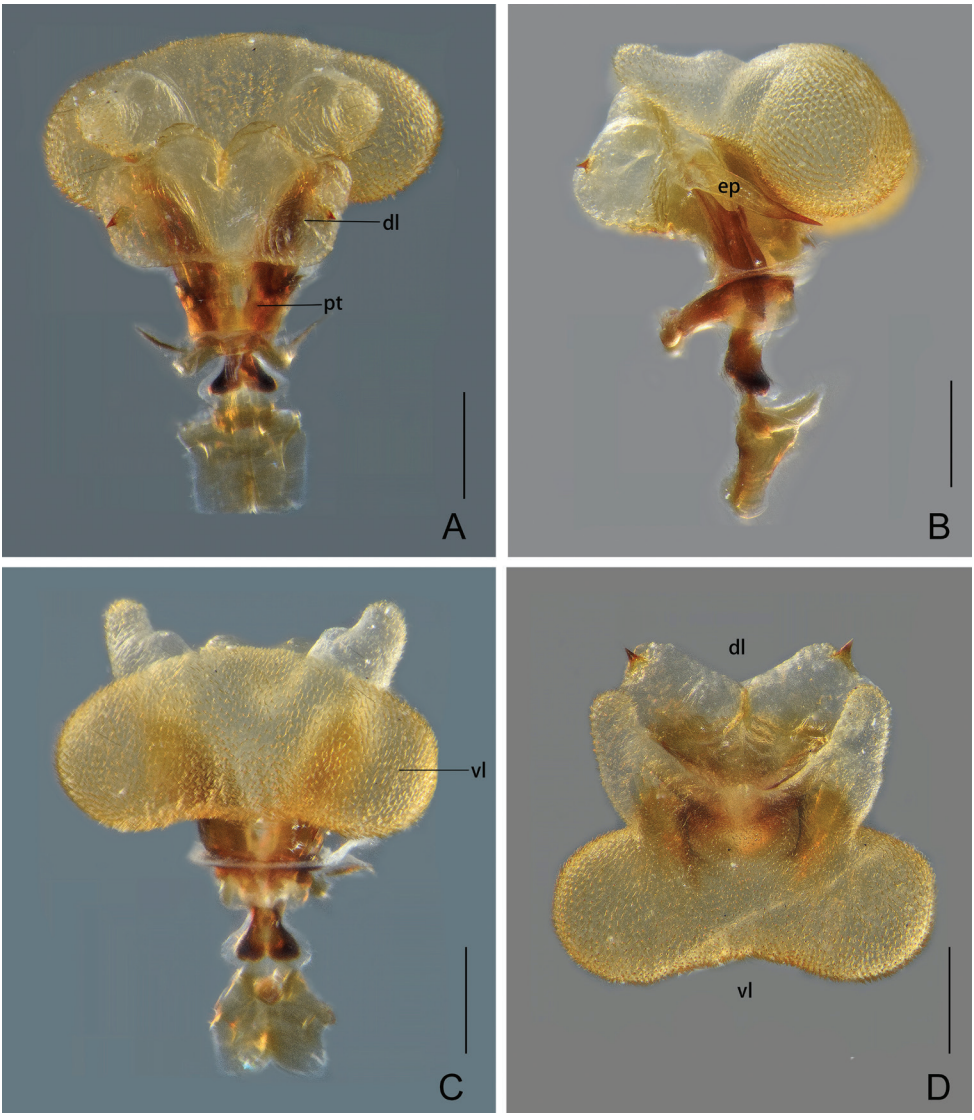

Figure 5. *Chiltana acarinata* sp. n. **A** Aedeagus, dorsal view **B** aedeagus, lateral view **C** aedeagus, ventral view **D** aedeagus, caudal view. Abbreviations: dl, dorsolateral lobe of phallotheca; ep, endosomal processes; pt, phallotheca; vl, ventral lobe of phallotheca. Scale bars: 0.2 mm.

Discussions

Chiltana was firstly described and illustrated as new genus by Shakila-Mushtaq in her Ph.D. thesis which was produced in 1984 (not 1989 as cited by Shakila-Mushtaq in her papers, e.g., Shakila-Mushtaq 1994, Shakila-Mushtaq and Akbar 1995). According to the printed fourth edition of the International Code of Zoological Nomenclature (ICZN 1999), the works, such as a Ph.D. thesis, could be regarded as published if they comply

with the requirements of Article 8 and are not excluded by the provisions of Article 9. Shakila-Mushtaq's thesis satisfied the criteria of Article 8.1, which it was issued for the purpose of providing a public and permanent scientific record (Article 8.1.1.), obtainable when first issued (Article 8.1.2.), and produced in an edition containing simultaneously obtainable copies by a method that assures numerous identical and durable copies (Article 8.1.3.). This thesis also did not provide a statement that the names and acts might be disclaimed (Articles 8.2. and 8.3.), and it was produced before 1986 by a printing method then conventional, i.e., printing on paper (Article 8.4.). This thesis might be considered a published work, and all the names and nomenclatural acts within it might be available under the framework of the Code (ICZN 1999).

However, the International Commission on Zoological Nomenclature (ICZN) has voted in favour of a revised version of the amendment to the Code that was proposed in 2008. The purpose of the amendment is to expand and refine the methods of publication allowed by the Code, particularly in relation to electronic publication. The revised version for the fourth edition of the Code, including the amendments to Articles 8, 9, 10, 21 and 78, with effect from 1 January 2012, has been available online until the fifth edition of the Code is published (ICZN online). A new Article 9.12. has been added in the online version of the Code, which says "facsimiles or reproductions obtained on demand of an unpublished work, even if previously deposited in a library or other archive" do not constitute published work (ICZN online). An example helps to explain this article: "A Ph.D. thesis that was distributed only to members of the student's thesis committee is listed for sale in the catalogue of a print-on-demand publisher. The print-on-demand work is a reproduction of the thesis. Because the thesis was an unpublished work in its original form, it remains unpublished" (ICZN online). Therefore, according to Article 9.12., we suggest that the Ph.D. thesis of Shakila-Mushtaq (1984) does not constitute published work, and the names in the thesis are regarded as nomina nuda.

Shakila-Mushtaq and Akbar (1995) later described and illustrated *Chiltana* in a published work. We herein suggest that the original authors of *Chiltana* are Shakila-Mushtaq and Akbar, and the date to be adopted is 1995 based on the published work of Shakila-Mushtaq and Akbar (1995).

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We wish to thank the late Dr Norman D. Penny (CAS) for loan of the specimens of *Chiltana acarinata* sp. n. and Dr Arshad Azmi and Mr. Munawwar (ZMUK) for access to dictyopharid collections in Zoological Museum, University of Karachi, Pakistan. We are grateful to two anonymous reviewers for their comments on this paper. Dr Mike Wilson is appreciated for his kind editorial help.

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RESEARCH ARTICLE

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Simopone fisheri sp. n., a new species of Dorylinae ants (Hymenoptera, Formicidae) from China, with an illustrated key to the *S. grandidieri*-group species

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Abstract

Simopone fisheri **sp. n.**, a new species of the subfamily Dorylinae, is described based on the worker caste. The new species is separated easily from the other named congeners by the longitudinally striate sculpture on the posterolateral portion of pronotum. An illustrated key is presented to species of the *S. grandidieri* group based on the worker caste.

Keywords

Simopone grandidieri group, new species, China

Introduction

The genus *Simopone* was established by Forel (1891) based on the type species *Simopone grandidieri* and assigned to Dorylinae by Emery (1895, 1901). Over the years it was considered as a member of the subfamily Ponerinae (Dalla Torre 1893; Forel

1893, 1917; Wheeler 1910, 1922; Emery 1911; Donisthorpe 1943; Brown 1975) or Cerapachyinae (Wheeler 1902; Bolton 1990, 1994, 2003). Brady et al. (2014) placed it into the subfamily Dorylinae again. The genus is an Old World lineage and 39 species have so far been described (Borowiec 2016; AntCat 2018). Brown (1975) first provided a key to Afrotropical species. Bolton and Fisher (2012) revised the genus globally, recognized 38 species, and proposed three species groups: *S. emeryi* group, *S. grandidieri* group, and *S. schoutedeni* group. Later, Chen et al. (2015) described one new species from Yunnan, China, which they assigned to the *S. grandidieri* group and included in a key to all known species of the *S. grandidieri* group. Other related taxonomic works were made by the following authors: Kutter (1976, 1977), Menozzi (1926), Taylor (1965, 1966), Emery (1899), Forel (1891, 1892), Santschi (1923), Arnold (1915, 1954), Radchenko (1993), and Weber (1949).

In the course of our recent survey of ants in Guangxi Daqingshan, southern China, we discovered a species that is clearly different from the known species of *Simopone*. We describe it as *S. fisheri* sp. n. and provide an updated key to the *S. grandidieri* group based on the worker caste.

Materials and methods

The examination of the specimens was carried out by Leica M205A stereomicroscope. High-quality multifocal montage images were produced with Leica DFC 450 digital imaging system and Leica Application Suite v. 4.3 software. Standard measurements and indices follow Bolton and Fisher (2012). All measurements are expressed in millimeters.

AIIL Abdominal Segment II (petiole) Length: The maximum length of abdominal segment II (petiole), measured in dorsal view and including longitudinal projections of the posterolateral corners where such occur.

AIIW Abdominal Segment II (petiole) Width: The maximum width of abdominal segment II (petiole), measured in dorsal view but omitting laterally projecting teeth when such occur at the posterolateral corners.

AIIIL Abdominal Segment III Length: The maximum length of abdominal segment III (postpetiole), measured in dorsal view.

AIIIW Abdominal Segment III Width: The maximum width of abdominal segment III (postpetiole), measured in dorsal view.

AIVL Abdominal Segment IV Length: The maximum length of the posttergite of abdominal segment IV (first gastral), measured in dorsal view, omitting the pretergite.

AIVW Abdominal Segment IV Width: The maximum width of abdominal segment IV (first gastral), measured in dorsal view.

CI Cephalic Index: HW divided by HL, × 100.

ED Eye Diameter: The maximum diameter of the eye.

EP Eye Position Ratio: In full-face view, the distance from a horizontal line that intersects the mid-point of the anterior clypeal margin, or from a line that spans the anterior-most points of the frontal lobes (depending on which projects farthest forward), to the level of a line that spans the anterior margins of the eyes, divided by the horizontal distance from a line that spans the posterior margins of the eyes to one that spans the posterior corners of the head.

HL Head Length: The length of the head capsule excluding the mandibles; measured in full-face view in a straight line from the mid-point of the anterior clypeal margin or from a line that spans the anteriormost points of the frontal lobes (depending on which projects farthest forward) to the level of a line that spans the posterior corners of the head capsule. In species with a strongly reflexed true anterior clypeal margin (i.e. the clypeo-labral junction) the measurement is taken from the midpoint of the apparent margin as seen in full-face view.

HW Head Width: The maximum width of the head immediately behind the eyes, measured in full-face view.

SI Scape Index: SL divided by HW, × 100.

TL Total Length: The total outstretched length of the individual, from the mandibular apex to the gastral apex.

SL Scape Length: The maximum straight-line length of the scape, excluding the basal constriction or neck that occurs just distal of the condylar bulb.

SW Scape Width: The maximum width of the scape, usually at its apex. FCW-Frontal Carina Width: The distance across the maximum separation of the frontal lobes or frontal carinae (whichever is greatest), measured in full-face view.

WL Weber's Length of Mesosoma (= Alitrunk Length): The diagonal length of the mesosoma in profile, from the angle at which the pronotal collar meets the neck to the posterior basal angle of the metapleuron.

The holotype worker and seven paratype workers are deposited in the Insect Collection of Guangxi Normal University (**GXNU**), Guilin, Guangxi, China, and one paratype worker will be deposited in the Insect Collection, Southwest Forestry University (**SWFU**), Kunming, Yunnan, China.

A list of *Simopone grandidieri*-group species

***S. bakeri* Menozzi, 1926**: 92. SINGAPORE.

[Non-type gyne images examined, CASENT0173045, photos by California Academy of Sciences, available on AntWeb.org].

***S. chapmani* Taylor, 1966**: 287. PHILIPPINES.

[Holotype worker images examined, CASENT0173044, photos by California Academy of Sciences, available on AntWeb.org].

***S. elegans* Bolton & Fisher, 2012:** 48. MADAGASCAR.

[Holotype worker images examined, AntWeb, CASENT0492213, photos by Shannon Hartman, available on AntWeb.org].

***Simopone fisheri* sp. n.** CHINA.

[Holotype worker and 8 paratype workers examined].

***S. grandidieri* Forel, 1891:** 141. MADAGASCAR.

[Holotype worker images examined, CASENT0101842, photos by April Nobile, available on AntWeb.org].

***S. gressitti* Taylor, 1965**: 3. NEW GUINEA.

[Holotype worker images examined, CASENT0249114, photos by Ryan Perry, available on AntWeb.org].

***S. laevissima* Arnold, 1954**: 291. UGANDA.

[No specimen and image examined].

***S. oculata* Radchenko, 1993:** 45. VIETNAM.

[Holotype worker images examined, CASENT0917355, photos by Kate Martynova, available on AntWeb.org].

***S. yunnanensis* Chen, Zhou & Liang, 2015**: 8. CHINA.

[Holotype worker examined].

Description

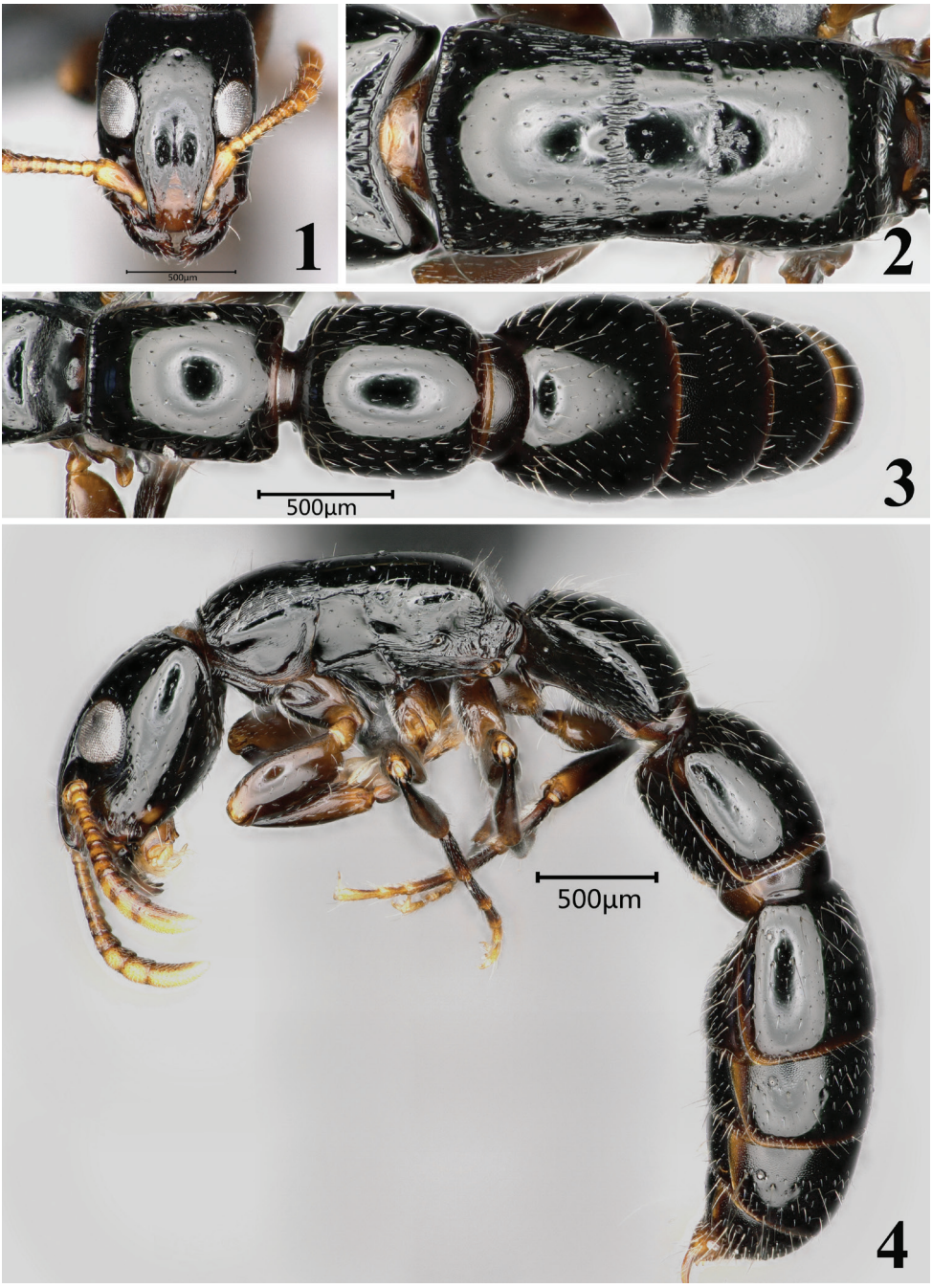
Tahonomy

***Simopone fisheri* sp. n.**
http://zoobank.org/0C2A62F4-CE26-4AA7-A135-3C27E763EDD4

Type material. Holotype worker: CHINA, Guangxi, Longzhou County, bingqiao Town, Daqingshan, 22.297° N, 106.695° E, 500 m alt., evergreen broad-leaved forest, nest in a twig, hand collecting, 21.V.2016, Zhilin Chen leg., No. G160312. Paratypes: 8 workers from the same colony as the holotype.

Holotype worker. (Figs 1–4). AIIL 0.80, AIIW 0.68, AIIIL 0.75, AIIIW 0.74, AIVL 0.85, AIVW 0.86, CI 76, ED 0.29, EP 86, HL 1.06, HW 0.81, SI 46, SL 0.28, SW 0.13, TL 6.06, WL 1.45, AIIW/AIIL 0.85, AIIIW/AIIIL 0.99.

Head in full-face view nearly rectangular, longer than broad (CI 76–78), broadest around the level of eye; sides broadly weakly convex, but shallowly concave anterior to eyes; posterior margin concave; posterolateral corner forming a blunt angle. Man-



Figures 1–4. *Simopone fisheri* sp. n., holotype worker **1** head in full-face view **2** mesosoma in dorsal view **3** petiole and gaster in dorsal view **4** body in lateral view.

dibles subtriangular, with masticatory margin finely dentate. Clypeus without median carina; anterior margin of median portion of clypeus broadly rounded. Frontal carinae horizontal, widely separated by broad frontal area; outer margins of frontal lobe divergent posteriad and extending beyond to the anterior margins of eyes. Antennae 11-segmented; scape short, clavate, not reaching to anterior margin of eye. Antennal scrobe extending from antennal socket to the anterior margin of the eye. Eyes large, occupying about 1/3 length of the side of head; the center point of eye posterior to the mid-length of head; outer margin of eye in full-face view not touching the lateral margin of head. Median and lateral ocelli present, minute and closely approximated to each other.

Mesosoma in lateral view weakly convex on pronotum, with a weak concavity between pronotum and mesonotum. Pronotal disc in dorsal view with anterodorsal margin carinate and convex anteriad; humeri narrowly round (not sharply angulate); lateral margins weakly convergent posteriad. Promesonotal suture in dorsal view recognized as a narrow and longitudinally rugose band, slightly convex anteriad. Dorsolateral borders of pronotum and mesonotum not forming longitudinal carina. Metanotal groove in dorsal view as a very narrow band, slightly convex posteriad. Dorsum and posterior slope of propodeum in lateral view forming a round corner, without a carina between the two faces.

Petiole (AII) longer than broad (AIIW/AIIL = 0.85), with anterodorsal carina strong and straight, in dorsal view with sides divergent posteriorly, with posterolateral corner narrowly round; dorsum in lateral view continuously convex; posteroventral corner of subpetiolar process produced as an acute hook or spine. Postpetiole (AIII) as broad as long, a little longer than high, in lateral view with sides almost parallel; dorsum in lateral view moderately convex. A conspicuous girdling constriction present between AIV and AV.

Head scattered with minute piligerous punctures, with spaces between punctures smooth and shining; mesosoma largely smooth and shining, with sparse minute piligerous punctures, longitudinally striate on posterolateral portion of dorsal face of pronotum, central portion of lateral face of pronotum and most part of metapleuron smooth and shining; waist segments and gaster largely smooth and shining, with sparse minute piligerous punctures, finely reticulate on anterior portions of AV, AVI and AVII.

Body scattered with short and decumbent background hairs; sides of head with one or two long setae; inner margin of each eye posteriorly with two long setae posteriorly; scape with several suberect setae; antennal funiculi with abundant setae; anterior portion of mesosoma scattered with long suberect setae; petiole, postpetiole, tergite of AIV, posterior edges of AV and AVI, pygidium and hypopygium with abundant setae.

Body color black; antenna, trochanter, spur, apical portion of tarsi yellowish brown.

Paratype wokers. AIIL 0.79–0.83, AIIW 0.66–0.69, AIIIL 0.72–0.77, AIIIW 0.72–0.75, AIVL 0.81–0.86, AIVW 0.85–0.87, CI 76–78, ED 0.29, EP 85–86, HL 1.04–1.06, HW 0.80–0.83, SI 44–46, SL 0.27–0.28, SW 0.12–0.13, TL 6.01–6.12,

WL 1.45–1.49, AIIW/AIIL 0.82–0.85, AIIIW/AIIIL 0.97–0.99. Similar to holotype, with the following exceptions. The metanotal suture of one paratype specimen well developed but incomplete and another one paratype specimen faintly marked.

Etymology. The new species is named in honor of Brian L. Fisher (California Academy of Sciences, United States of America) for his outstanding contributions to ant systematics.

Comparison notes. This new species is the ninth species of the *S. grandidieri* species group and is morphologically most similar to *S. oculata*, but is easily differentiated from it by dorsolateral borders of pronotum round and not forming longitudinal carina. The new species is also similar to *S. yunnanensis* but is easily differentiated from it by dorsolateral portion of pronotum longitudinally striate and metanotal groove present.

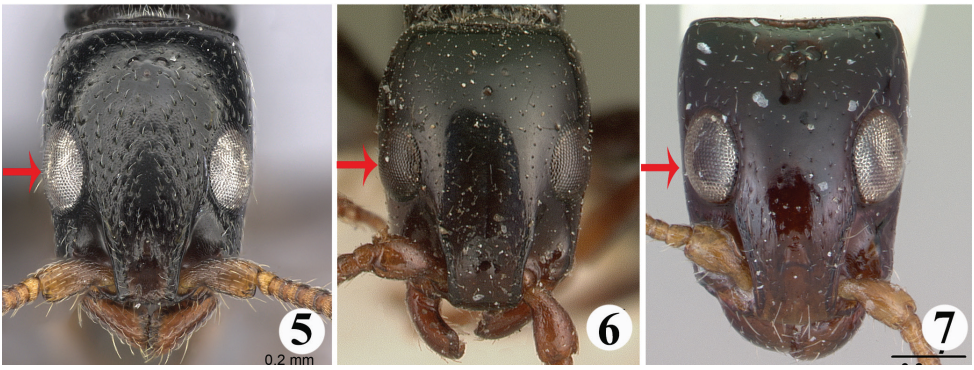
The dorsolateral borders of pronotum in *S. yunnanensis* forms a right angle but never forms longitudinal carina; the original description of *S. yunnanensis* by Chen et al. (2015) needs to be corrected as above.

An illustrated key to species of the *Simopone grandidieri* group based on the worker caste

The following key is built upon the key by Bolton and Fisher (2012).

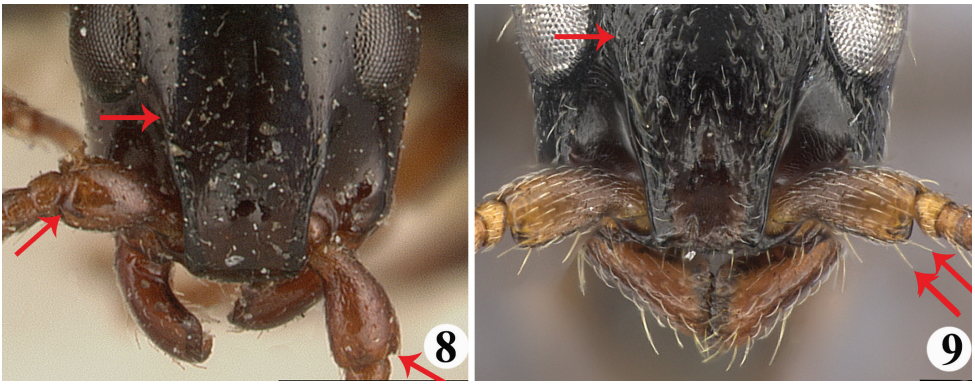
1 In full-face view, outer margins of eye just interrupting lateral margin of head (Figs 5–6)..... **2**

– In full-face view, outer margins of eye not interrupting lateral margin of head (at most toughing the lateral margin as seen in Fig. 7)..... **4**



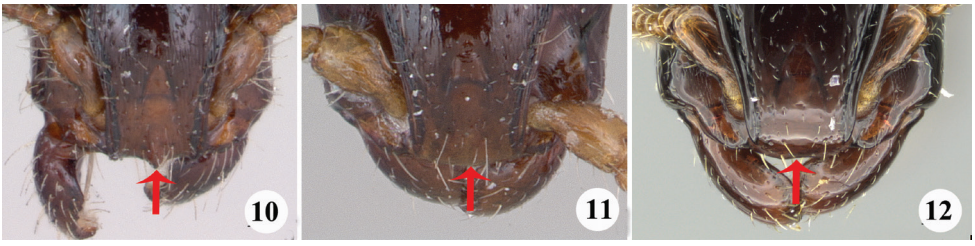
Figures 5–7. Head in full-face view of the worker of *Simopone* spp. **5** *S. elegans*, type (specimen CASENT0492213; photo by Shannon Hartman, available on AntWeb.org) **6** *S. grandidieri*, type (specimen CASENT0101842; photo by April Nobile, available on AntWeb.org) **7** *S. chapmani*, type (specimen CASENT0173044; photo by April Nobile, available on AntWeb.org).

2 Frontal carina relatively short, ending far away from the level of the anterior margins of eyes; leading edge of scape without standing setae (Fig. 8).... ***S. grandidieri***
– Frontal carina relatively long, extending beyond the level of the anterior margins of eye; leading edge of scape with standing setae (Fig. 9) **3**



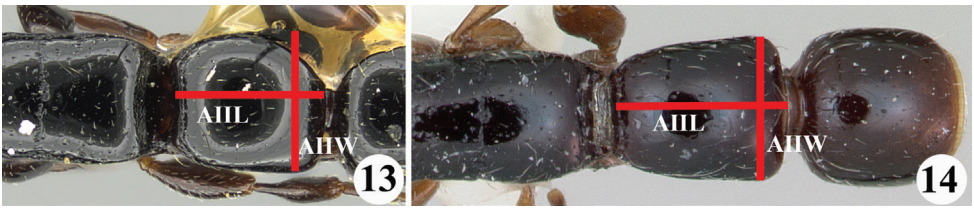
Figures 8–9. Head in full-face view of the worker of *Simopone* spp. **8** *S. grandidieri*, type (specimen CASENT0101842; photo by April Nobile, available on AntWeb.org) **9** *S. elegans* type (specimen CASENT0492213; photo by Shannon Hartman, available on AntWeb.org).

3 Eyes located far back on head (EP 1.90)..... ***S. laevissima***
– Eyes located slightly more anteriorly on head (EP 0.74–0.84)..... ***S. elegans***
4 Anterior margin of clypeus with a prominent tooth at its midpoint (Fig. 10)..... ..... ***S. bakeri***
– Anterior margin of clypeus broadly rounded, and without a tooth at its midpoint (Figs 11, 12)..... **5**



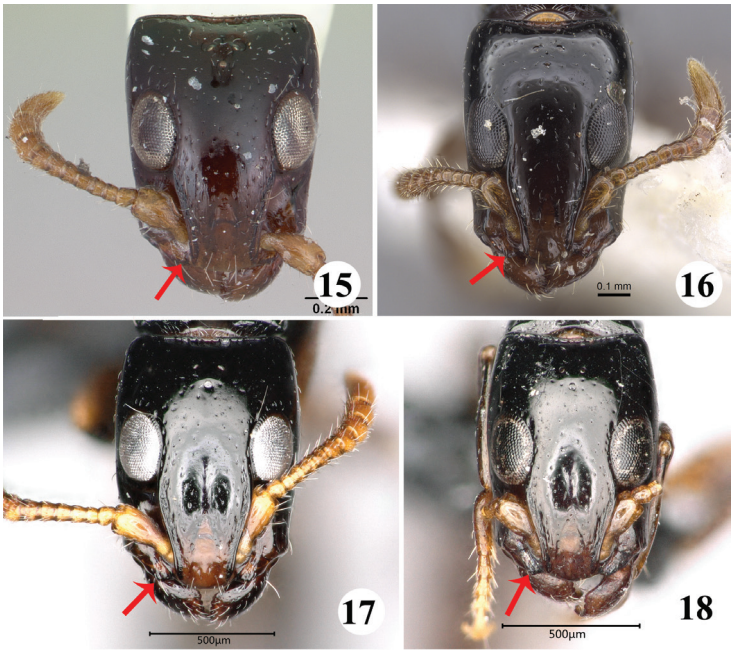
Figures 10–12. Head in full-face view **10** *S. bakeri* gyne (specimen CASENT0173045; photo by April Nobile, available on AntWeb.org) **11** *S. chapmani* type (specimen CASENT0173044; photo by April Nobile, available on AntWeb.org) **12** *S. gressitti* type (specimen CASENT0249114; photo by Ryan Perry, available on AntWeb.org).

5 AII almost as broad as long (AIIW/AIIL 0.96) (Fig. 13) ..... ***S. gressitti***
– AII distinctly longer than broad (AIIW/AIIL ≤ 0.86) (Fig. 14). 7



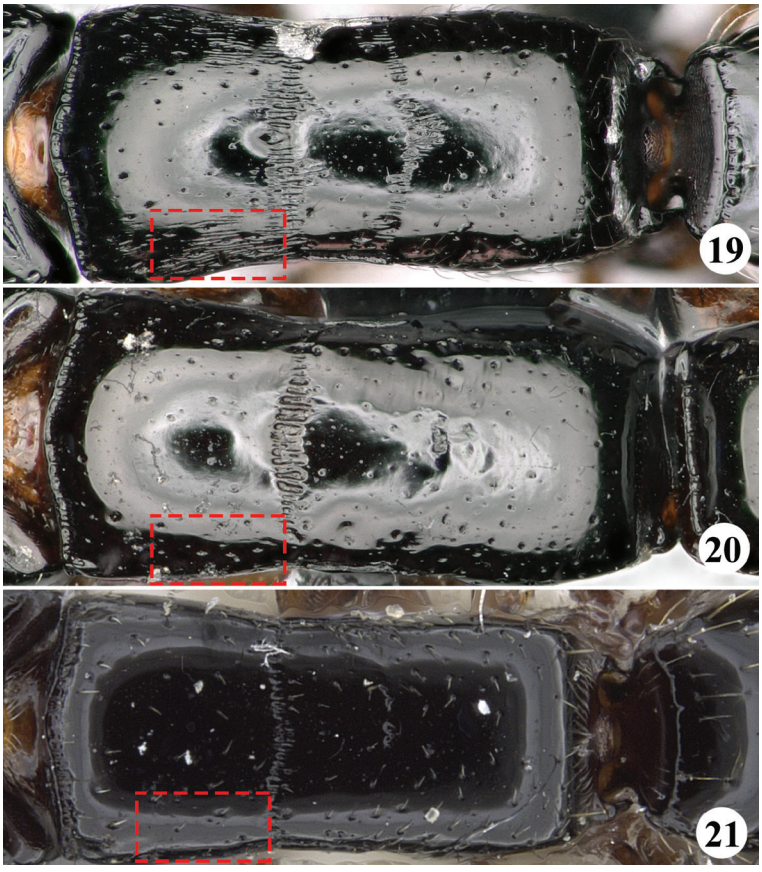
Figures 13, 14. Petiole (AII) in dorsal view of the worker of *Simopone* spp. **13** *S. gressitti*, type (specimen CASENT0249114; photo by Ryan Perry, available on AntWeb.org) **14** *S. chapmani*, type (specimen CASENT0173044; photo by April Nobile, available on AntWeb.org).

6 Head in full-face view distinctly trapezoidal; lateral tooth of clypeus inconspicuous (Fig. 15).....***S. chapmani***
– Head nearly rectangular in full-face view (Figs 18, 19); lateral tooth of clypeus conspicuous (Figs 16–18).....7



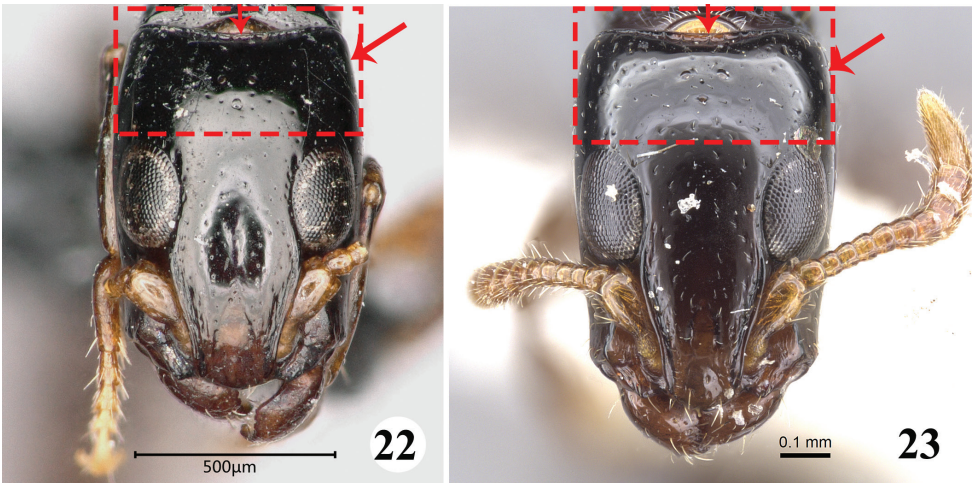
Figures 15–18. Head in full-face view of the worker of *Simopone* spp. **15** *S. chapmani*, type (specimen CASENT0173044; photo by April Nobile, available on AntWeb.org) **16** *S. oculata*, type (specimen CASENT0917355; photo by Kate Martynova, available on AntWeb.org) **17** *S. fisheri*, type (photo by Zhlin Chen) **18** *S. yunnanensis* type (photo by Zhlin Chen).

7 Large species (TL ≥ 8.0 mm); maximum diameter of eye smaller than the minimum distance between eyes; posterolateral portion of dorsal face of pronotum striate longitudinally (Fig. 19) ..... ***S. fisheri* sp. n.**
– Medium-sized or small species (TL ≤ 6.5 mm); the maximum diameter of eye equal to the minimum distance between eyes; posterolateral portion of dorsal face of pronotum smooth and shining (Figs 20, 21). **8**



Figures 19–21. Mesosoma in dorsal view of the worker of *Simopone* spp. **19** *S. fisheri*, type (photo by Zhlin Chen) **20** *S. yunnanensis*, type (photo by Zhlin Chen) **21** *S. oculata*, type (specimen CASENT0917355; photo by Kate Martynova, available on AntWeb.org).

8 Medium-sized species (TL = 6.5 mm); posterior margin of head distinct concave; lateral side of 1/3 posterior head gradually convergent (Fig. 22) ..... ***S. yunnanensis***
– Small species (TL = 5.5 mm); posterior margin of head almost straight; lateral side of 1/3 posterior head gradually divergent (Fig. 23). ..... ***S. oculata***



Figures 22–23. Head in full-face view of the worker of *Simopone* spp. **22** *S. yunnanensis*, type (photo by Zhlin Chen) **23** *S. oculata*, type (specimen CASENT0917355; photo by Kate Martynova, available on AntWeb.org).

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RESEARCH ARTICLE

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An annotated list of the Chamaemyiidae (Diptera, Acalyptrata) of Turkey with new records and additional data

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Abstract

A list of all the species of Chamaemyiidae known from Turkey is compiled from the literature and supplemented by new records. A total of 40 species in five genera is given with updated nomenclature. One undescribed species is illustrated but not named for lack of males. The distribution of each species outside Turkey is summarised.

Keywords

Silver-flies, faunistics, taxonomy, distribution

Introduction

Silver flies of the family Chamaemyiidae have an interesting biology with a potential for the biological control of pest species of aphids and adelgids (Aphidoidea) and scales and mealybugs (Coccoidea) that attack crops, horticultural plants, and forest trees. Silver flies are found in all continents except Antarctica, but much remains to be discovered in nearly all zoogeographical regions.

When compared to the other zoogeographical regions, the Palaearctic is relatively well studied with many species having been associated with their prey and the prey with their host plants, largely summarised by Tanasijtshuk (1986). Nevertheless, the distribution of most species remains inadequately known and there is always more to discover about the biology of a majority of the species. Southern Europe and Turkey through to Central Asia is a region rich in species, reflecting the diversity of habitats and flora. No doubt, more species await discovery and description. Their taxonomy can be difficult owing to the very similar external appearance of species within each genus.

The first record of a species of Chamaemyiidae from Turkey appears to be that of Süreyya and Hovasse (1931). They record larvae of *Leucopis* sp. very successfully attacking the scale insect *Marchalina hellenica* Genn. damaging pine trees on Princes Islands (Turkey). Bodenheimer (1953) reported on an unspecified species of *Leucopis* Meigen, 1830. Three species of *Neoleucopis* McAlpine, 1971 were examined by McAlpine in his revision of the genus (McAlpine 1971). Several more species were added by various authors since then (Eichhorn 1968; McAlpine 1978; Düzgüneş et al. 1982, Tanasijtshuk 1986, Elmali 1997; Kaydan et al. 2006, Raspi and Ebejer 2008, Raspi 2013). In the last two articles, the authors added more data and in each publication a new species of *Parochthiphila* Czerny, 1904 was described. Kaydan et al. (2006) also gave the prey species and their host plants. In a recent paper, Satar et al. (2015) gave a summary of the biological and ecological role of species in this family, added four new records for Turkey and provided biological data based from their own rearing records of several species of *Leucopis*. They gave no new records of species in other genera.

The aim of this article is to briefly review what is known of the Turkish fauna based on the literature, recent field work of one of us (MB) and supplementary material collected by Dr Jindřich Roháček (Opava, Czech Republic). We list all the species recorded in these earlier papers and add new records for the country and further locality and chorological data on some previously known species. Nomenclature is updated.

Materials and methods

Species are listed in alphabetical order under each genus. Previous records are cited below each species name. Additional locality data based on the recently collected material is included and new records for Turkey are indicated. Depositories of specimens are in the M Barták collection, Czech University of Life Sciences, Prague, unless otherwise stated and given in parenthesis at the end of each data entry thus: **MJE** – MJ Ebejer collection, Cowbridge, UK; **MSO** – Museum Silesiae Opava, Czech Republic. Material cited in this paper was collected by water pan traps (PT), Malaise traps (MT), and by hand held sweep net (SW).

The material treated here originates mainly from Muğla province (Muğla, Akyaka, Toparlar, Gökçeova Gölü, and Dalyan), and some from Samsun province (Samsun). The general distribution of species is summarised mainly from Tanasijtshuk (1984, 1986), Beschovski (1995), Beschovski and Merz (1998), Ebejer (2017), and Raspi (2013). Taxonomy follows Tanasijtshuk (1986) and Raspi and Benelli (2016).

List of species

CHAMAEMYIINAE
***Chamaemyia* Meigen, 1803**

***Chamaemyia aridella* (Fallén, 1823)**
Raspi and Ebejer 2008: 61

Distribution: widespread in Europe, from Britain south to the Mediterranean and Turkey.

***Chamaemyia emiliae* Tanasijtshuk, 1970**

Material examined: 2♂♂, Muğla, 700 m, university campus, MT, 37°09'42"N, 28°22'21"E, 17–22.v.2011; 1♀, 12 km SW of Muğla, 660 m, on *Ferula communis*, 37°07'40"N, 28°16'28"E, 23.v.2011; 1♂, Muğla, 720 m, university campus, MT, 37°09'42"N, 28°22'13"E, xi.2015–iv.2016, H Pala leg.

Distribution: Hungary and Russia eastwards to Kazakhstan. New record for Turkey.

***Chamaemyia geniculata* (Zetterstedt, 1838)**

Material examined: 1♀, Antalya, Yarpuz, 4.7 km W nr cross-road, 1240 m, 37°07'26"N, 31°48'01"E, 16.v.2011, J Roháček leg. (MSO); 1♂, Antalya, Ürünlü, 5.8 km SW, Manavgat River, 440 m, 37°04'30"N, 31°39'25"E, 17.v.2011, J Roháček leg. (MSO).

Distribution: A widespread species in Europe through Ukraine to Middle Asian states and Mongolia. New record for Turkey.

***Chamaemyia juncorum* (Fallén, 1823)**

Material examined: 1♂, Gökçeova Gölü, lake shore, 1750 m, 37°03'42.52"N, 28°48'28.42"E, 20.ix.2012; 1♂, Akyaka, 30 m, forest, SW, 37°03'19"N, 28°19'36"E, 30.iv.–9.v.2013.

Distribution: Widespread across the whole Palaearctic including North Africa. New record for Turkey.

***Chamaemyia polystigma* (Meigen, 1830)**
Raspi 2013: 24

Material examined: 1♀, Antalya, Ödaönü, 1 km S, Alara River shores, 11–13 m, 36°40'24"N, 31°40'57"E, 13.v.2011, J Roháček (MSO); 1♂3♀♀, Antalya, Murtiçi, 1 km S, 490–510 m, 36°52'20"N, 31°46'03"E, 31°40'57"E, 14.v.2011, J Roháček (MSO); 1♀, Antalya, Emiraşıklar, 1 km NW, 950 m, 37°02'45"N, 31°43'48"E, 17.v.2011, J Roháček (MSO); 1♀, Antalya, Ibradı, 3.7 km NW, 1200 m, 37°07'15"N,

31°34'10"E, 17.v.2011, J Roháček (MSO); 1♀, Akyaka, river bank, salty meadow, 37°03'16"N, 28°19'57"E, 16–27.v.2011; 1♀, 11 km E of Muğla, wood + meadow, 1310 m, 37°12'45"N, 28°27'42"E, 1.v.2013; 1♂1♀, Samsun, university campus, 41°22'N, 36°11'E, 22.vi–4.vii.2014; 1♀, Akyaka, 40 m, forest, SW, 37°03'16"N, 28°19'35"E, 26.iv.2016; 1♀, Toparlar, lowland forest, 8 m, SW+PT, 36°59'27"N, 28°38'50"E, 28–30.iv.2016.

Distribution: Widespread in Europe and North Africa, Turkey, and reaches Mongolia.

Chamaemyia sylvatica Collin, 1966

Material examined: 1♂1♀, Muğla, 710 m, university campus, MT, 37°09'39"N, 28°22'20"E, xi–iii.2013; 1♂, 11 km E of Muğla, wood + meadow, 1310 m, 37°12'45"N, 28°27'42"E, 1.v.2013; 2♂♂, 13 km NE of Muğla, pinewood + pasture, 1100–1300 m, 37°15'N, 28°30'E, 2–3.v.2016.

Distribution: Britain and Central Europe to Poland and Bulgaria. New record for Turkey.

Parochthiphila Czerny, 1904
Parochthiphila (*Parochthiphila*) *inconstans* (Becker, 1903)

Material examined: 1♂, Muğla Province, Köyceğiz, Toparlar, waterfall, 44 m, 36°49'N, 28°58'E, 26.iv.2006; 1♂, Muğla, 730 m, university campus, MT, 37°09'38"N, 28°22'11"E, 5–19.viii.2015, H Kavak leg.

Distribution: Iberian Peninsula, Mediterranean islands, North Africa and Arabia. New record for Turkey.

Parochthiphila (*Parochthiphila*) *spectabilis* (Loew, 1858)
Raspi and Ebejer 2008: 61

Material examined: 3♂♂4♀♀, Antalya, Manavgat, 4.4 km S, Manavgat rivershore, 1 m, 36°45'01"N, 31°28'03"E, 15.v.2011, J Roháček leg. (MSO); 1♀, Antalya, Manavgat, 3.5 km S, Titreyen lake, 1 m, 36°45'25"N, 31°27'19"E, 15.v.2011, J Roháček leg. (MSO); 16♂♂8♀♀, Akyaka, river bank, salty meadow, 37°03'16"N, 28°19'57"E, 16–27.v.2011; 2♂♂1♀, same data (MJE); 4♂♂, Akyaka, pasture, 4 m, 37°03'09"N, 28°20'17"E, 23–27.ix.2012; 5♂♂1♀, Toparlar, lowland wood, 60 m, 36°58'39"N, 28°39'30"E, 5–7.v.2013; 5♂♂, Akyaka, pasture, 6 m, SW, 37°03'19"N, 28°20'07"E, 28.iv.–8.v.2013; 1♂, same data (MJE); 5♂♂, Akyaka, salty meadow, SW+PT, 37°12'45"N, 28°27'42"E, 28.iv.–9.v.2013.

Distribution: Widespread in Europe, Turkey, through Russia to the Urals and Kazakhstan.

***Parochthiphila* (*Euestelia*) *argentiseta* Ebejer & Raspi, 2008**

Material examined: 1♂, Samsun, university campus, 41°22'N, 36°11'E, 22.vi.–4.vii.2014; 1♂, 13 km NE of Muğla, pine wood, 1200, 37°14'50"N, 28°30'E, 23–27.vi.2015.

Distribution: Described and so far known only from Turkey.

***Parochthiphila* (*Euestelia*) *decipia* Tanasijtshuk, 1986**
Kaydan et al. 2006: 333

Distribution: Italy, Moldova, Turkey, through the Middle Asian states to Afghanistan.

***Parochthiphila* (*Euestelia*) *ephesi* Raspi, 2013**
Raspi, 2013: 14

Distribution: Described and so far known only from Turkey.

***Parochthiphila* (*Euestelia*) *frontella* (Rondani, 1874)**
Raspi and Ebejer 2008: 61

Material examined: 2♂, Dalyan, farm, MT, 1 m, 36°48'54"N, 28°39'04"E, 8–20. viii.2015, Dursun; 1♂, Muğla, 710 m, university campus, MT, 37°09'39"N, 28°22'20"E, xi–iii.2013; 1♂, Dalyan, orchard, 4 m, 36°49'37"N, 28°39'39"E, 11.ix.2014; 2♂♂, Muğla, 720 m, university campus, 37°09'42"N, 28°22'13"E, 26–27.vi.2015; 1♂, Muğla, 730 m, university campus, MT, 37°09'38"N, 28°22'11"E, 5–19.viii.2015, H Kavak leg.

Distribution: Southern France, Iberia, Italy, and Mediterranean islands to Greece and the Aegean part of Turkey.

***Parochthiphila* (*Euestelia*) *kimmerica* Tanasijtshuk, 1968**
Raspi and Ebejer 2008: 61

Material examined: 2♂♂1♀, Muğla, 700 m, university campus, SW+PT, 37°09'42"N, 28°22'21"E, 29.iv.–10.v.2011; 2♂, 12 km SW of Muğla, 660m, on *Ferula communis*, 37°07'40"N, 28°16'28"E, 23.v.2011; 1♂, Akyaka, 30 m, forest, SW, 37°03'16"N, 28°19'35"E, 30.iv.–9.v.2013; 1♂, Akyaka, 40 m, forest, SW, 37°03'19"N, 28°19'36"E, 26.iv.2016 .

Distribution: from western Russia south to Turkey and Israel.

***Parochthiphila* (*Euestelia*) *nigripes* (Strobl, 1900)**
Raspi and Ebejer 2008: 61; Raspi 2013: 20

Material examined: 1♂, Muğla, 700 m, university campus, MT, 37°09'42"N, 28°22'21"E, 17–22.v.2011; 1♂, 11 km E of Muğla, pinewood + meadow, 1310 m,

37°12'45"N, 28°27'42"E, 23.v.2011; 1♂, 12 km SW of Muğla, 660 m, on *Ferula communis*, 37°07'40"N, 28°16'28"E, 23.v.2011; 1♀, Akyaka, 30 m, forest, SW, 37°03'16"N, 28°19'35"E, 30.iv.–9.v.2013; 4♂♂1♀, Muğla, 700 m, university campus, SW+PT, 37°09'42"N, 28°22'21"E, 29.iv.–10.v.2013; 2♂♂, 13 km NE of Muğla, pinewood, 1200, 37°14'50"N, 28°30'E, 23–27.vi.2015; 1♂, Muğla, 720 m, university campus, MT, 37°09'42"N, 28°22'13"E, 26–27.vi.2016.

Distribution: Spain through to southern Russia, Ukraine, Balkan states, Turkey, Iran, and Afghanistan.

LEUCOPINAE

***Leucopis* Meigen, 1830**

***Leucopis afghanica* Tanasijtshuk, 1998**

Material examined: 1♀, Muğla, 730m, university campus, MT, 37°09'38"N, 28°22'11"E, xi.2015–iv.2016; 1♂, Muğla, 720 m, university campus, MT, 37°09'42"N, 28°22'13"E, iv.–v.2016, H Kavak leg.; 1♀, same data, but H Pala leg.

Distribution: Previously known only from Afghanistan. New record for Turkey.

***Leucopis annulipes* Zetterstedt, 1848**

Düzgüneş et al. 1982: 92 (as *Leucopis caucasica* Tanasijtshuk, 1961); Tanasijtshuk 1986: 244; Yoldaş et al. 2011: 63; Satar et al. 2015: 175

Material examined: 1♂, 13km NE of Muğla, pine wood + pasture, 1100–1300 m, 37°15'N, 28°30'E, 2–3.v.2016.

Distribution: all of Europe to western Russia, Turkey, and Iran.

***Leucopis argentata* Heeger, 1848**

Leucopis conciliata McAlpine & Tanasijtshuk, 1972: 1871; Düzgüneş et al. 1982: 93

Material examined: 1♂2♀♀, Antalya, Ödaönü, 1 km S, Alara River shores, 11–13 m, 36°40'24"N, 31°40'57"E, 13.v.2011, J Roháček leg. (MSO); 3♂♂1♀, Akyaka, river bank, salty meadow, 37°03'16"N, 28°19'57"E, 16–27.v.2011; 6♂♂, Akyaka, pasture, 4 m, 37°03'09"N, 28°20'17"E, 23–27.ix.2012; 10♂♂, Akyaka, salty meadow, SW+PT, 37°12'45"N, 28°27'42"E, 28.iv.–9.v.2013; 2♂♂, same data (MJE); 3♂♂, Toparlar, lowland wood, 60 m, 36°58'39"N, 28°39'30"E, 5–7.v.2013; 2♂♂, Akyaka, pasture, 8 m, 37°03'11"N, 28°20'33"E, 27.iv.2016.

Distribution: Central and southern Europe and from the Iberian Peninsula to Turkey and the Middle East including the Arabian Peninsula, and to Mongolia.

***Leucopis artemisiae* Tansijtshuk, 1986**

Raspi and Ebejer 2008: 62

Distribution: Southeastern Russia, Turkey.

***Leucopis compacta* Tanasijtshuk, 1972**
Tanasijtshuk 1986: 269

Distribution: France and Bulgaria through Ukraine, Turkey, and Middle Asian states to Mongolia.

***Leucopis formosana* Hennig, 1938**
Satar et al. 2015: 175

Distribution: one of the most widespread species of the genus occurring from Cape Verde Islands to Cyprus and Middle East including Arabia, and in the Far East from China south through Asian countries to Australia. In tropical Africa found from Côte d'Ivoire to east, South Africa, and on the Mascarene Island of Réunion in the Indian Ocean. A full account of this species is given in Tanasijtshuk (1999).

***Leucopis gallicola* Tanasijtshuk, 1972**
Şahbaz and Uysal 2006: 122

Distribution: Russia, Turkey, Iran, and Middle Asian states.

***Leucopis glyphinivora* Tanasijtshuk, 1958**
Düzgüneş et al. 1982: 93; Tanasijtshuk 1986: 292; Raspi and Ebejer 2008: 63; Satar et al. 2015: 175

Material examined: 1♂, Antalya, Manavgat, 7 km SE, mouth of Manavgat River, 0–1 m, 36°44'17"N, 31°29'44"E, 11.v.2011, J Roháček leg. (MSO); 1♀, Antalya, Güçlüköy, 2 km E, 610 m, 36°49'06"N, 31°46'21"E, 15.v.2011, J Roháček leg. (MSO); 1♂, Muğla, 700 m, university campus, MT, 37°09'42"N, 28°22'21"E, 17–22.v.2011; 5♂♂, Akyaka, river bank, salty meadow, 37°03'16"N, 28°19'57"E, 16–27.v.2011; 1♂, Toparlar, lowland wood, 60 m, 36°58'39"N, 28°39'30"E, 5–7.v.2013; 1♂, 5 km S of Muğla, on flowers, 670 m, 37°08'27"N, 28°22'05"E, 6.v.2013.

Distribution: Iberian Peninsula through Europe and south to the Mediterranean and Turkey, through the Middle East to Mongolia.

***Leucopis grunini* Tanasijtshuk, 1979**

Material examined: 1♀, Muğla, 700m, university campus, MT, 37°09'42"N, 28°22'21"E, 17–22.v.2011; 1♂1♀, Muğla, 700m, university campus, MT, 37°09'42"N, 28°22'21"E, iv.–v.2013, O Dursun leg.

Distribution: Italy, Cyprus, southern Russia, and Middle Asian states. New record for Turkey.

Leucopis hennigrata **McAlpine, 1978**
Eichhorn 1968: 210 (as *Leucopis* n. sp.); McAlpine 1978: 350

Distribution: Germany, France, Switzerland, Austria, former Yugoslavia, Greece, Turkey, and introduced into Canada (found in British Columbia, Alberta, New Brunswick, Newfoundland), and USA (found in Washington, Oregon, Arizona).

Leucopis kerzhneri **Tanasijtshuk, 1970**
Elmali 1997: 174

Distribution: North Africa, Greece, Mongolia.

Leucopis minuscula **Rondani, 1875**
Şahbaz and Uysal 2006: 122 (as *Leucopis auraria* Tanasijtshuk, 1961)

Distribution: Italy, Malta, eastern Russia, Mongolia.

Leucopis monticola **Tanasijtshuk, 1961**
Raspi and Ebejer 2008: 63

Distribution: Iberian Peninsula through Central Europe to Russia, Ukraine, Turkey.

Leucopis ninae **Tanasijtshuk, 1966**
Düzgüneş et al. 1982: 94; Tanasijtshuk 1986: 272

Material examined: 1♂, Antalya, Manavgat, 7 km SE, Titreyen lake, 0–1 m, 36°44'17"N, 31°29'44"E, 11.v.2011, J Roháček leg. (MSO); 1♀, Antalya, Dolbazlar, 1.3 km NW, 21 m, 36°51'01"N, 31°24'24"E, 15.v.2011, J Roháček leg. (MSO); 2♂♂1♀, Akyaka, pasture, 4 m, 37°03'08.9"N, 28°20'17.4"E, 16–22.ix.2012; 1♀, Akyaka, pasture, 8 m, 37°03'11"N, 28°20'33"E, 27.iv.2016.

Distribution: England through Europe to southern Russia, Bulgaria, Ukraine, through the Middle East and north Africa, and to the Middle Asian states through to Mongolia.

Leucopis pallidolineata **Tanasijtshuk, 1961**
Düzgüneş et al. 1982: 94; Tanasijtshuk 1986: 257; Raspi and Ebejer 2008: 63

Material examined: 5♂♂, Muğla, 720 m, university campus, MT, 37°09'42"N, 28°22'13"E, 26–27.vi.2016.

Distribution: Central Europe through southern Russia, Ukraine, through Middle Asian states and Mongolia.

***Leucopis pseudomelanopus* Tanasijtshuk, 1961**
Düzgüneş et al. 1982: 95; Tanasijtshuk 1986: 306

Distribution: Central Europe, southern Russia, Ukraine, Middle Asia.

***Leucopis revisenda* Tanasijtshuk, 1970**
Satar et al. 2015: 175

Distribution: Central Europe, southern Russia, Ukraine, through Middle Asian states and Mongolia.

***Leucopis rufithorax* Tanasijtshuk, 1958**
Satar et al. 2015: 175

Distribution: Central and southern Europe, southern Russia, Ukraine, through Middle Asian states and Mongolia.

***Leucopis spyrothecae* Raspi, 2003**
Satar et al. 2015: 175

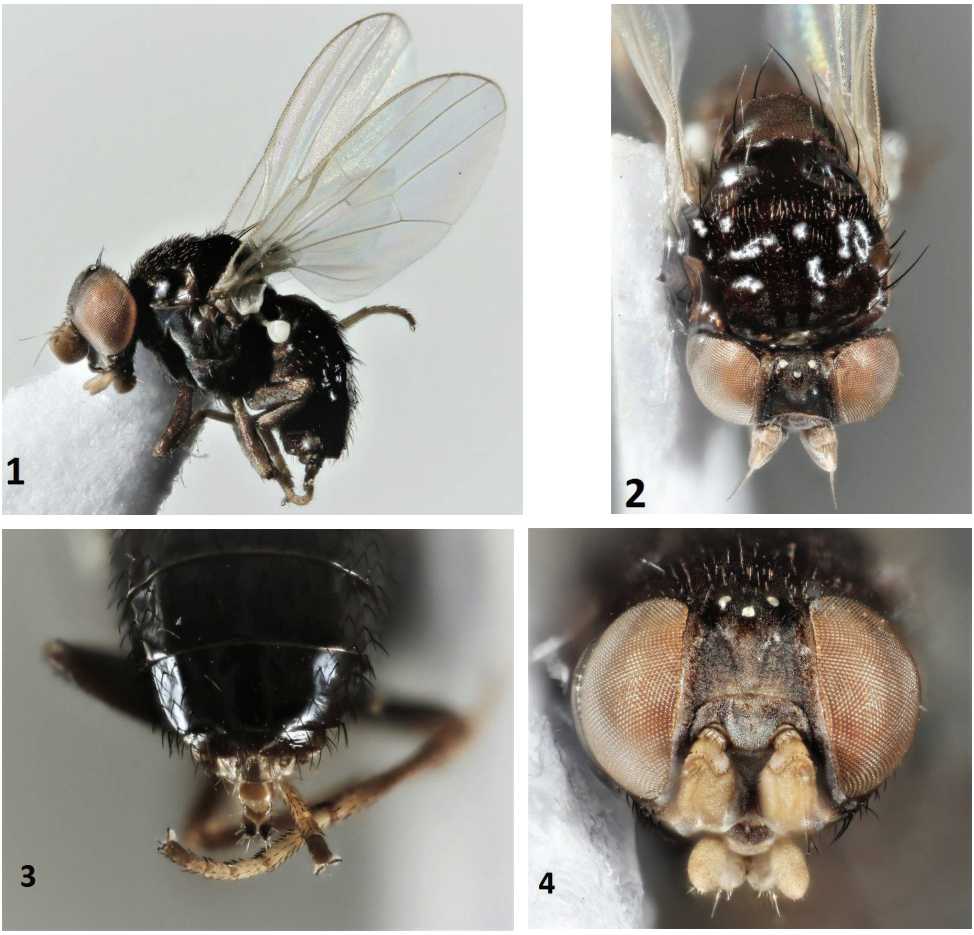
Distribution: Italy, Turkey.

***Leucopis* sp. n.**
Figs 1–4

Material examined: 1♀, Muğla, 730m, university campus, MT, 37°09'38"N, 28°22'11"E, xi.2015–iv.2016; 1♀, Muğla, 720 m, university campus, 37°09'42"N, 28°22'13"E, iv–v.2016, H Pala leg.

Remarks. This distinctive species appears to be undescribed, but for lack of males it cannot be named here. Another dark species of *Leucopis* (*L. albostriata* Czerny, 1936) exhibits distinct sexual dimorphism and so it may eventually prove difficult to correctly associate males with these specimens in the future. The two specimens noted here are dark, shiny, brownish black with a thin coating of pollinosity only on the head and on the pleura. Neither specimen is teneral, but one has the palp and the whole antenna yellow and the other has the palp, pedicel, and post pedicel dark brown. In other respects they are identical. Such small differences can be attributed equally to closely related species or to intraspecific variation. This supports our caution in not naming this species. The safest way to determine if these are one or two species would be to rear males and females simultaneously from a single colony.

Distribution: Turkey.



Figures 1–4. *Leucopis* sp. n., female; **1** habitus, lateral **2** head and thorax, dorsal **3** abdomen dorsal **4** head anterior.

Leucopis sp.

Material examined: 1♂, 13 km NE of Muğla, pinewood + pasture, 1100–1300 m, 37°15'N, 28°30'E, 2–3.v.2016.

Remarks. A single male specimen of *Leucopis* could not be identified. It is probably a variant of one of the commoner species as it shows no differentiating external characters but only small differences in the shape of the aedeagus. Without more material it is not possible to come to any definitive conclusion on the taxonomic status of this specimen.

***Leucopomyia* Malloch, 1921**

***Leucopomyia palliditarsis* (Rondani, 1875)**
Kaydan et al. 2006: 333 (as *Leucopomyia alticeps* (Czerny, 1936))

Distribution: from Iberian Peninsula through Central Europe to Russia and Middle Asian states.

***Leucopomyia silesiaca* (Egger, 1862)**
Ülgentürk 1999: 76, 2001: 371; Kaydan et al. 2006: 333

Distribution: From Britain through Central Europe, Russia, Ukraine, to Middle Asian states.

***Neoleucopis* Malloch, 1921**

***Neoleucopis atratula* (Ratzeburg, 1844)**
Eichhorn 1968: 210; McAlpine 1971: 1868; Tanasijtshuk 1986: 173

Distribution: From Britain through Central Europe to the Balkan states and Turkey. Introduced into North America, Hawaii, New Zealand, and Argentina.

***Neoleucopis kartliana* (Tanasijtshuk, 1986)**
Düzgüneş et al. 1982: 92 (as *Leucopis caucasica* Tanasijtshuk, 1961); Ülgentürk et al. 2013: 533

Material examined: 1♀, Akyaka, 30 m, forest, SW, 37°03'16"N, 28°19'35"E, 30.iv.–9.v.2013; 1♂, 13 km NE of Muğla, pinewood + pasture, 1100–1300 m, 37°15'N, 28°30'E, 2–3.v.2016.

Remarks. Gaimari et al. (2007) provided a detailed redescription with biological notes on this species, studied in Greece, and speculated that it ought to occur in Turkey, evidently unaware that it had been already recorded from there. More information on the biology of this species in Turkey was given by Ülgentürk et al. (2013).

Distribution: Georgia, Italy, Greece, Turkey.

***Neoleucopis obscura* (Haliday, 1833)**
Eichhorn 1968: 210; McAlpine 1971: 1862; Tanasijtshuk 1986: 169

Distribution: North and Central Europe to the Balkan states and Turkey. Introduced into eastern and western North America.

***Neoleucopis tapiae* (Blanchard, 1964)**
McAlpine 1971: 1866

Distribution: Europe, from Britain south to Gibraltar and west to western Russia. Introduced to North and South America and New Zealand.

Conclusions

Many scientists consider Anatolia to have been an important Pleistocene glacial refugium, which together with the heterogeneous topography and geographical position of Anatolia at the junction of three biodiversity hotpots, the Caucasus, Irano-Anatolian, and Mediterranean (Gür 2016), may have contributed to a very high animal diversity. This, alongside an insufficient level of faunistic research, may explain the recent increase in the number of known Chamaemyiidae from Turkey.

Turkey may have one of the most diverse faunas of Chamaemyiidae in the Southern Palaearctic. We list 40 species in five genera including seven new records and one undescribed new species. Notwithstanding this list, we think the fauna still remains poorly known. There are several species present in adjacent countries that have not yet been found in Turkey, a country that offers a very diverse topography and plant life. Sampling in as many diverse habitats as possible, in different seasons, and rearing silver flies from populations of their hosts will yield interesting results, thus adding to the knowledge of the biology and ecology of this family.

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RESEARCH ARTICLE

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Cryptic diversity in *Lithobates warszewitschii* (Amphibia, Anura, Ranidae)

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Abstract

Lithobates warszewitschii is a species of ranid frog distributed from southern Honduras to Panama. This species suffered severe population declines at higher elevations (above 500 m a.s.l.) from the 1980s to early 1990s, but there is more recent evidence of recovery in parts of its range. Here we advocate for the status of *Lithobates warszewitschii* as a candidate cryptic species complex based on sequence data from mitochondrial genes CO1 and 16S. Using concatenated phylogenies, nucleotide diversity (K2P-π), net between group mean distance (NBGMD) (π_{net}) and species delimitation methods, we further elucidate cryptic diversity within this species. All phylogenies display polyphyletic lineages within Costa Rica and Panama. At both loci, observed genetic polymorphism (K2P-π) is also high within and between geographic populations, surpassing proposed species threshold values for amphibians. Additionally, patterns of phylogeographic structure are complicated for this species, and do not appear to be explained by geographic barriers or isolation by distance. These preliminary findings suggest *L. warszewitschii* is a wide-ranging species complex. Therefore, we propose further research within its wider range, and recommend integrative taxonomic assessment is merited to assess species status.

Keywords

Área de Conservación Guanacaste (ACG), barcoding; biodiversity, CO1, phylogenetics; phylogeography, 16S

Introduction

Cryptic species are poorly defined and highly heterogeneous. Identification of potential singular, nominal species may be masked when morphological traits are shared within and between sister taxa (Bickford et al. 2007). Evolutionary mechanisms that produce cryptic species are also diverse and may best be explained by recent divergence, niche conservatism, and morphological convergence (Fišer et al. 2018). Although considered evidence of incomplete species inventories, or potential sources of bias within biodiversity research (Fišer et al. 2018), cryptic species are evidently common (Adams et al. 2014) and extensive among animal phyla (Perez-Ponce de León and Poulin 2016). Species concepts have been a topic of debate since Darwin's Origin of Species (Mallet 2008), yet most contemporary biologists conceptually envisage separately evolving segments of metapopulation-level evolutionary lineages (Mayden 1997, de Queiroz 1998, 1999, Hey et al. 2003, Bock 2004, Hey 2006).

Given that the majority of species remain undescribed, endeavours to explain and catalogue biodiversity are inevitable to both understanding and preventing extinctions (Pimm et al. 2014). For amphibians especially, being the most threatened group of vertebrates (Stuart et al. 2004), identifying cryptic diversity is fundamental to their conservation. Habitat loss, fragmentation, climate change and disease epidemics have produced a global decline in amphibian populations (Baillie et al. 2004, Stuart et al. 2004). Losses reflect patterns of ecological preference, range and taxonomic association, with montane stream dwelling species most affected (Stuart et al. 2004). It is also probable that the number of amphibian species is highly underestimated (Fouquet et al. 2007a, Vieites et al. 2009).

Whereas some species are presumed to be widely distributed, those within a cryptic complex may have smaller ranges or different ecological requirements (Stuart et al. 2006), meaning failure to recognize these taxa can leave them susceptible to mismanagement. However, when genetic differentiation is established, it can unveil previously unknown units of diversity and endemism (Bickford et al. 2007) that may subsequently warrant protection or species status (Whitfield et al. 2016).

High levels of genetic diversity in Costa Rican and Panamanian frog populations are well recognized (Crawford 2003), as are cryptic species (Wang et al. 2008). *Lithobates warszewitschii* (Ranidae) (Schmidt, 1857) is a proposed candidate species – a provisional designation pending further systematic investigation (Vieites et al. 2009). Crawford et al. (2010) (Suppl. material 1) showed that within the amphibian community at El Copé (Omar Torrijos National Park), Panama, *L. warszewitschii* displayed 14.7% pairwise divergence between conspecifics at the CO1 locus. This is an unusually high degree of polymorphism for a single species in sympatry (Crawford 2003, Vences et al. 2005), providing additional evidence this taxon likely contains candidate cryptic lineages (Mallet 2008). Paz et al. (2015) compared El Copé with allopatric populations from Brewster (Chagres National Park), revealing 11% pairwise divergence. Consequently, breeding strategy, dispersal and landscape resistance may help explain this variation between both sites.

Lithobates warszewitschii occurs from Honduras to Panama and has been recorded at elevations up to 1740 meters above sea level (m a.s.l.). They are fairly common, diurnal and generally abundant frogs in forests near streams where they breed (Savage 2002). In Costa Rica, population declines occurred in montane areas such as Tapantí, Monteverde, and Braulio Carrillo (Bolaños 2002, Puschendorf et al. 2006). Post-decline it was found to be rare in San Vito (Santos-Barrera et al. 2007) and vanished but found again at San Ramón (IUCN 2015). *Lithobates warszewitschii* was also found to be abundant at mid-elevation sites in Guayacán (Kubicki 2008), Corcovado, Ciudad Colón, and Tinamastes (IUCN 2015). A population decline also occurred at lowland site La Selva (Whitfield et al. 2007); however, it is not generally abundant at lower elevations (IUCN 2015). Pre-decline it was one of the most abundant tadpoles encountered in streams at El Copé, Panama, (Ranvestel et al. 2004), but was later extirpated following the emergence of a virulent pathogen (Crawford et al. 2010). In Nicaragua, it was found to be abundant in Río San Juan (Sunyer et al. 2009) and numbers were increasing at Quebracho (Barquero et al. 2010) post decline, although Nicaragua's amphibian decline history is much more nebulous than Costa Rica's. No data was found for Honduras, and additional research is needed to ascertain population sizes, distributions, trends and threats throughout its full range (IUCN 2015).

In this study we expand the research on cryptic diversity within *L. warszewitschii,* based on published sequence data from two localities in Panama (Crawford et al. 2010, Paz et al. 2015) and samples collected from the Área de Conservación Guanacaste (ACG) in northwestern Costa Rica. Using phylogenetic data, species delimitation methods and nucleotide diversity within CO1 and 16S loci we make inferences about phylogeographic structure and proposed candidate status across its wider range.

Methods

Field sampling

Lithobates warszewitschii were sampled from five field sites within the Área de Conservación Guanacaste (ACG), Costa Rica: Pitilla, San Gerardo, Maritza, Cacao, and Caribe (Figure 1; for further detail see https://www.acguanacaste.ac.cr) between June 2015 – August 2017 (Table 1). Streams and surrounding forest are preferred habitat for *L. warszewitschii* (Savage 2002), and sampling was conducted within these habitats. Each individual was captured, housed separately in moist bags (Beaupre et al. 2004), identified based on morphology (Savage et al. 2002, Leenders 2016), and toe-clipped (Perry et al. 2011). Individuals were then released back at the point of capture.

A total of 34 samples were collected from ACG and obtained from GenBank, but only 29 had both CO1 and 16S available and therefore used in this analysis. All data for *L. warszewitschii* samples collected in Panamanian sites El Copé and Brewster were obtained from other studies (Crawford et al. 2010, Paz et al. 2015).

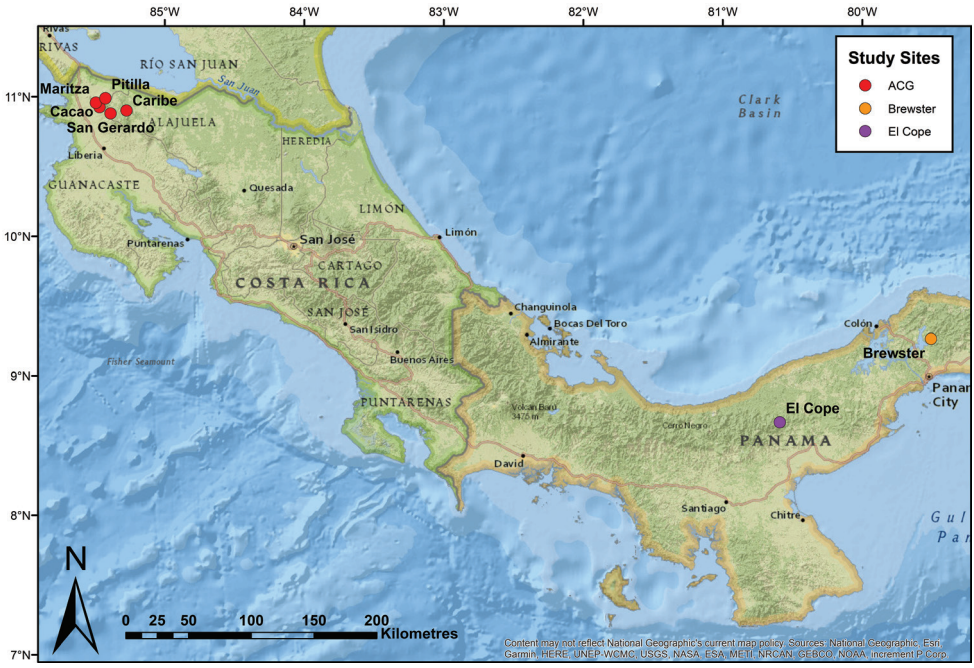

Figure 1. Study sites included in phylogenetic analysis of *L. warszewitschii*. Sites: Cacao, Caribe, Maritza and San Gerardo are within the Área de Conservación Guanacaste (ACG), Costa Rica. Sites El Copé and Brewster are within Panama.

Table 1. Information on study sites.

Sites	Collection dates	No. tissue samples	Habitat	Longitude	Latitude	Elevation(m)	Reference
Pitilla	August, 2016	1	Rainforest	10.989	-85.426	650–750	Field data – this study
	June, 2017	1					
San Gerardo	August, 2017	2	Rainforest/ pastureland	10.881	-85.389	470–640	Field data – this study
Maritza	June, 2015	7	Dry/wetforest	10.956	-85.495	570–610	Field data – this study
	August, 2015	7					
	November, 2016	6					
	July, 2017	3					
	August, 2017	5					
Cacao	November, 2016	4	Rain/cloud forest	10.923	-85.468	980–1130	Field data – this study
	August, 2017	3					
Caribe	June, 2015	4	Rainforest	10.902	-85.275	370	Field data – this study
El Copé	July, 2010	NA	Rainforest	8.667	-80.592	700–750	(KRL0823) Paz et al. 2015
Brewster	June, 2015	NA	Rainforest	9.265	-79.508	130–810	(CH6868) Paz et al. 2015

Description of sites where populations of *Lithobates warszewitschii* were sampled. Habitat type, georeferences, and information sources (field data GPS coordinates, or external sources, e.g., other researchers, ACG website, or literature) are included.

Lab work

In order to extract DNA from tissue samples a standard ammonium acetate protocol was used (Nicholls et al. 2000). The Cytochrome c oxidase subunit I (CO1) and 16S ribosomal RNA (16S) mitochondrial genes were targeted for amplification by PCR. 16S primers (16Sar-L +16Sbr-H) and reaction protocols were adapted from Kessing et al. (2004). Multiple primers were used in the CO1 reactions to maximize the number of successful PCR products. CO1 primers (dgLCO-1490 + dgHCO-2198) and reaction protocols were adapted from Meyer et al. (2005) and CO1 primers (Chmf4 + Chmr4; Che et al. 2012) followed reaction protocols by Ivanova et al. (2008).

Extracted DNA from a subset of samples was sent to the Canadian Centre of DNA barcoding for PCR amplification and sequencing. These samples used CO1 primers (C_VF1LFt1 + C_VF1LRt1) in PCR reactions (Ivanova et al. 2007). The remaining samples were amplified in-house. Thermocycler (*Techne Prime Gradient*) programmes differed depending on the primer and reaction used. CO1 (dgLCO-1490 + dgHCO-2198) and 16S (16Sar-L + 16Sbr-H) reactions were run using the protocol outlined by Crawford et al. (2010). Primer set (CO1, Chmf4 + Chmr4) followed thermocycler profiles by (Ivanova et al. 2008). Two percent agar gels were used for electrophoresis with 1% TAE (Smith et al. 2008). Gels were visualized using an *ImageQuant LAS4000* and *Nanodrop 2000* quantification was performed on each successful PCR product visualized at the correct length, prior to dilution.

Bioinformatics

Concatenated gene alignments were used in the phylogenetic analyses. GENEIOUS v11.0.5 (Kearse et al. 2012) bioinformatics software was used to assemble forward and reverse sequences from returned CO1 and 16S chromatographs. Forward and reverse (compliment) sequences were aligned using Geneious' alignment (Global alignment with free end Gaps; Cost matrix = 65% similarity (5.0/-4.0); Gap open penalty = 12; Extension penalty = 3). Sequences were trimmed at the 3' and 5' ends where low quality base calls were present. Consensus sequences were produced for each sample, ranging from 609–658 base pairs (bp) in length for CO1 and 578–601bp for 16S. For both CO1 and 16S, a BLAST search (Altschul et al. 1990) was conducted using a consensus sequence derived from all Costa Rican sequences. Additional *Lithobates* species sequence data were downloaded to represent an ingroup for *L. warszewitschii* based on previous phylogenetic studies (e.g., Hillis and Wilcox 2005, Frost et al. 2006, Che et al. 2007, Huang et al. 2016): *Lithobates clamitans* (Latreille, 1801), *Lithobates catesbeiana* (Shaw, 1802), *Lithobates maculata* (Brocchi, 1877), *Lithobates palmipes* (Spix, 1824), *Lithobates septentrionalis* (Baird, 1854), *Lithobates sylvaticus* (LeConte, 1825), *Lithobates vaillanti* (Brocchi, 1877), *Rana maoershanensis* (Lu et al., 2007) was used as an outgroup (Zhou et al. 2017). All sequences were archived in Genbank (Benson et al. 2012; Table 2). All relevant sequences for each gene were then Geneious aligned (Maddison 1997). Only individuals which had sequence data for both genes were included

Table 2. Genbank (NCBI) Voucher ID & Accession numbers.

Species	Study site	Voucher ID	CO1 Genbank Accession #	16S Genbank Accession #
L. warszewitschii	Maritza	RP 388	MH559513	MH603380
	Maritza	RP 389	MH559517	MH603379
	Pitilla	RP 435	NA	MH603378
	San Gerardo	RP 466	MH559519	MH603377
	San Gerardo	RP 475	MH559514	MH603376
	Maritza	RP 496	MH559518	MH603375
	Maritza	RP 500	MH559515	MH724925
	Cacao	RP 878	NA	MH724926
	Cacao	RP 885	MH559516	MH724927
	Cacao	RP 887	NA	MH724928
	Caribe	RP Fw142	MH559500	MH603393
	Caribe	RP Fw144	MH559501	MH603392
	Caribe	RP Fw147	MH559502	NA
	Maritza	RP Fw455	MH559503	MH603391
	Maritza	RP Fw457	MH559504	MH603390
	Pitilla	RP Fw570	MH559505	MH603389
	Cacao	RP Fw591	MH559506	MH603388
	Cacao	RP Fw597	MH559507	MH603387
	Cacao	RP Fw601	MH559508	MH603386
	Cacao	RP Fw616	NA	MH603385
	Maritza	RP Fw618	MH559509	MH603384
	Maritza	RP Fw619	MH559510	MH603383
	Maritza	RP Fw620	MH559511	MH603382
	Maritza	RP Fw635	MH559512	MH603381
	Brewster	CH6868	KR863019	KR863275
	Brewster	AJC1794	KR863021	KR863277
	Brewster	AJC1798	KR863026	KR863282
	Brewster	CH6658	KR863027	KR863283
	Brewster	CH6659	KR863028	KR863284
	El Copé	KRL 0823	FJ766749	FJ84384
	El Copé	KRL 1540	FJ766751	FJ84552
	El Copé	KRL 1508	KR911913	KR911916
	El Copé	KRL 1496	KR911914	KR911917
	El Copé	KRL 1567	KR911915	KR911918
L. catesbeiana	*NA*	–	KX686108*	KX686108*
L. clamitans	*NA*	–	EF525879	KY677813
L. maculata	*NA*	–	*NA*	AY779207
L. palmipes	*NA*	CFBHT12435	KU494586	KU495379
L. septentrionalis	*NA*	–	EF525896	AY779200
L. sylvaticus	*NA*	–	KP222281*	KP222281*
L. vaillanti	*NA*	–	KY587190	AY779214
R. maoershanensis	*NA*	SYNU08030061	KX1397728	KX1397722

Voucher ID and GenBank accession numbers for all individuals and sequences of *Lithobates warszewitschii* used in this study. (*) indicates that gene sequences derived from a whole mitochondrial genome sequence.

in the concatenated alignment for the phylogenetic analyses. *Lithobates clamitans*, *L. maculata*, *L. septentrionalis* and *L. vaillanti* were represented by different individuals on 16S and CO1 phylogenetic analyses.

Separate Bayesian consensus trees for the CO1 and 16S alignments were estimated independently using MR BAYES v3.2.6 (Ronquist et al. 2013) to ensure they did not conflict with each other. After establishing that there were no conflicts, columns with gaps were removed from the two individual alignments, which were then concatenated end to end with PhyUtility v.2.7.1 (Smith et al. 2008). This concatenated alignment was then used to construct trees using a Bayesian framework (Mr. Bayes with default settings used for Markov chain Monte Carlo (MCMC) analysis—1,000,000 generations, 4 chains, 2 runs, a sample frequency of 500, and a 25% burn-in) and a maximum likelihood framework (RAxML; Stamatakis 2014); 20 maximum-likelihood trees generated on distinct starting trees, 1000 bootstrap replicates calculated and annotated on the best maximum-likelihood tree). The alignment was partitioned by gene, meaning model parameters were unlinked across the partition, to account for the different evolutionary histories of the CO1 and 16S genes. The General Time Reversible (GTR) model of substitution (Tavaré 1986) was used for all trees in order to be consistent between the Bayesian and maximum likelihood approaches since GTR is the model implemented in RAxML. Rate variation among sites was modelled as a discrete gamma distribution with four rate categories. Trees were rooted on the outgroup (*R. maoershanensis)* and visualised in FigTree v1. 4. 2 (Rambaut 2014).

Species boundaries were assessed in two ways. The first using the GENEIOUS plugin SPECIES DELIMITATION (Masters et al. 2011), which calculates the probability of reciprocal monophylly against the null model of random coalescence (Rosenberg 2007) for single panmictic populations (Rodrigo et al. 2008) and presents the probability for correct identification for putative species, given the data (Ross et al. 2008). Groups with P (Randomly Distinct) values of 0.05 – 1, represent branching events that would be expected under a coalescent model in a Wright-Fisher population and a strict molecular clock (Rodrigo et al. 2008, Masters et al. 2011). The second method used the Automatic Barcode Gap Discovery for primary species delimitation (ABGD; Puillandre et al. 2012) via a web interface (http://wwwabi.snv.jussieu.fr/public/abgd/). A maximum of ten, and minimum of two samples per geographic locality of the focal species were used as required for the minimum estimation of genetic divergence (Hickerson et al. 2007), a minimum of one sample was considered adequate for interspecific analysis (Aliabadian et al. 2009). Where possible, the same individuals were used in the analyses of both genes. Intraspecific and interspecific genetic distances were also calculated and analysed. Average, K2P-corrected (Kimura 1980) pairwise distance (K2P-π) and net between group mean distance (NBGMD) (π_{net}) (Nei and Li 1979) were calculated in MEGA v6 (Tamura et al. 2013) to assess nucleotide diversity ($\boldsymbol{\pi}$) and cryptic speciation within and between sites.

Results

Phylogenetic comparison

Concatenated phylogenetic trees reconstructed using Bayesian inference and Maximum likelihood (Figure 2) methods, show similar topology of three major clades within the focal species. Geographic samples from ACG and Brewster formed well-supported independent monophyletic groups. However, samples from El Copé presented a polyphyletic structure. Four out of five individuals (KRL 1496, KRL 1508, KRL 1540, KRL 1567) formed an independent clade, sister to the ACG clade, whereas sample KRL 0823 formed a clade with samples from Brewster – revealing the presence of two taxa at El Copé. Subsequently, three clades are recognized: ACG and El Copé, containing samples exclusively from these areas, and Brewster (including sample KRL 0823 from El Copé). Single gene trees showed a similar topology to the concatenated ones (Suppl. material 1: Figures S1, S2).

CO1 operational taxonomic units (OTUs) delimitation results

CO1 species delimitation in GENEIOUS yielded three OTUs (Table 3). Focal clades ACG, Brewster (+KRL 0823), and El Copé (KRL 1496, KRL 1508, KRL 1540, KRL 1567) had P values <0.05, indicating they are not conforming to the expected Wright-Fisher criteria. According to this assumption and the data present, all clades were taxonomically distinct. ABGD analysis identified four OTUs within *L. warszewitschii*, with KRL 0823 forming its own OTU (p= 0.0359). ABGD also supported the three distinct OTUs outlined by species delimitation in GENEIOUS (p= 0.0599, Suppl. material 1: Table S1 and Suppl. material 1: Figure S3).

CO1 and 16S nucleotide diversity

K2P-π at the CO1 and 16S loci showed a mean value of 7.2% and 3.4%, respectively, within all *L. warszewitschii* samples (Table 4). Samples from El Copé had the highest intra-group mean distance at 6.3% and 3.2%, respectively, whereas samples from ACG had 0.4% and 0.3% and within Brewster 0.1% and 0.2%, respectively. Mean intraspecific distances between ACG and Brewster samples (CO1/16S) were the highest at 15.7%/7.2% (Suppl. material 1: Tables S2, S3). Samples from ACG and El Copé shared the lowest distance at 10.7%/6.2%, and the intermediate distance was 13.8%/6.7% between Brewster and El Copé samples. Interspecific comparisons within the genus resulted in lower interspecific distances among recognized species (COI/16S), such as: *L. clamitans* and *L. catesbeiana* (5.7%/2%), *L. septentrionalis* and *L. clamitans* (8.3%/3.1%), *L. septentrionalis* and *L. catesbeiana* (8.6%/2.2%).

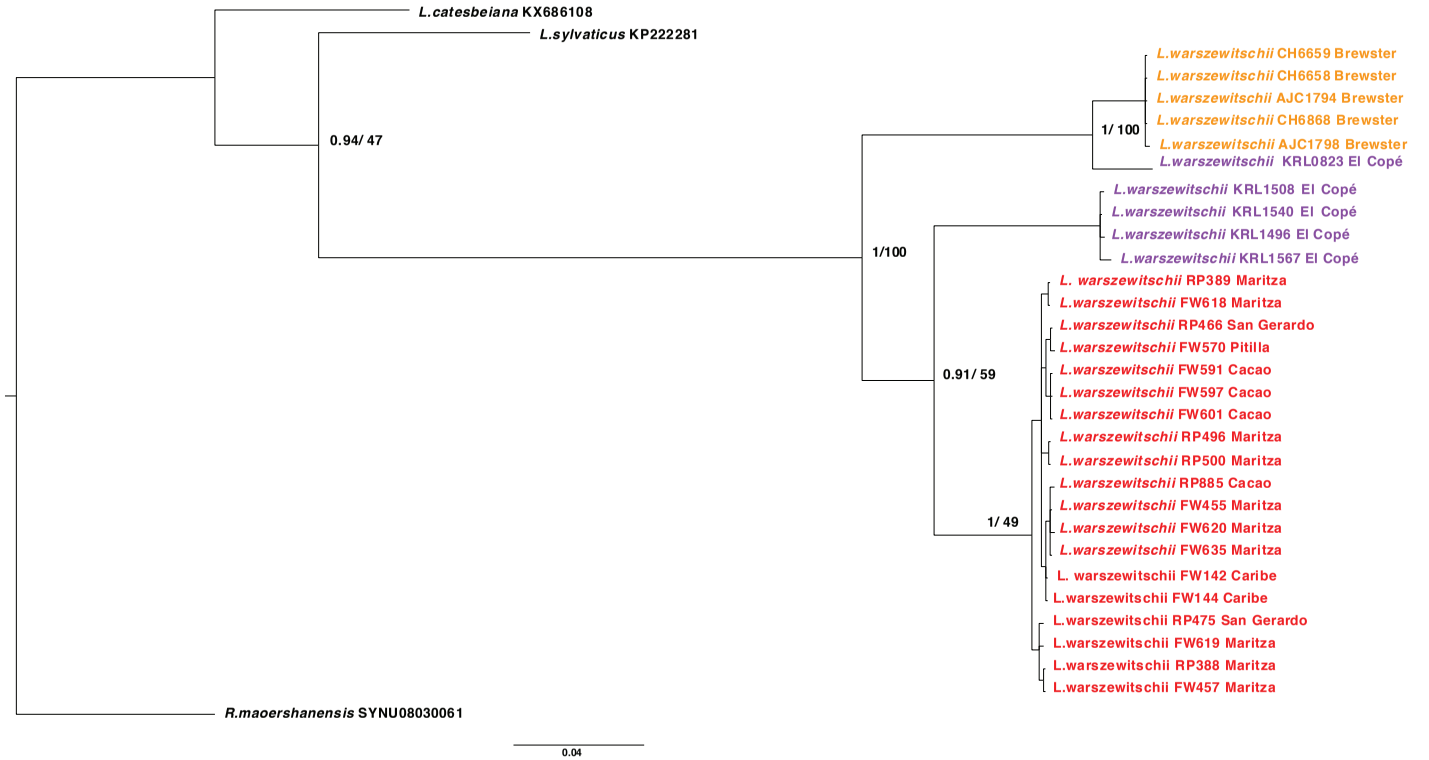

Figure 2. Phylogenetic reconstruction of *Lithobates warszewitschii* relationships between Costa Rican and Panamanian populations using concatenated alignments of CO1 and 16S. Node support values (posterior probabilities) and percentages calculated from 1000 bootstrap replicates are annotated at nodes. Samples collected in different localities are represented by different colours: individuals from Área de Conservación Guanacaste (ACG; Cacao, Caribe, Maritza, Pitilla, and San Gerardo) highlighted in red, individuals from Brewster highlighted in purple, and individuals from El Copé highlighted in orange. Sample information can be found in Table 2. Separate trees were constructed in Mr. Bayes and RAxML using a GTR model of molecular evolution, both with similar topologies, therefore node supports were included in a single tree. Scale of branch lengths is in nucleotide substitutions per site.

Table 3. CO1 Species delimitation results.

OTU	Closest OTU	Monophyletic?	Intra Dist	Inter Dist – Closest	Intra/Inter	PID(Strict)	PID(Liberal)	Av(MRCA-tips)	P(Randomly Distinct)	Rosenberg's P(AB)
1: ACG	2: El Copé	yes	0.01	0.109	0.08	0.97 (0.91,1.0)	0.99 (0.96,1.0)	0.0076	0.05	8.10E-06
2: El Copé	1: ACG	yes	0.01	0.109	0.06	0.83 (0.69,0.97)	0.97 (0.86,1.0)	0.0047	0.05	8.10E-06
3: Brewster & KRL 0823	2: El Copé	yes	0.02	0.197	0.08	0.88 (0.75,1.0)	0.97 (0.87,1.0)	0.0211	0.05	1.10E-07
4: *L. palmipes*	5: *L. vaillanti*	yes	0	0.114	0	0	0.96 (0.83,1.0)	0	NA	1
5: *L. vaillanti*	4: *L. palmipes*	yes	0	0.114	0	0	0.96 (0.83,1.0)	0	NA	1
6: *L. catesbeiana*	7: *L. clamitans*	yes	0	0.057	0	0	0.96 (0.83,1.0)	0	NA	1
7: *L. clamitans*	*L. catesbeiana*	yes	0	0.057	0	0	0.96 (0.83,1.0)	0	NA	1
8: *L. septentrionalis*	7: *L. clamitans*	yes	0	0.092	0	0	0.96 (0.83,1.0)	0	NA	0.33
9: *L. sylvaticus*	8: *L. septentrionalis*	yes	0	0.238	0	0	0.96 (0.83,1.0)	0	NA	0.17

Species delimitation results of *Lithobates warszewitschii* in Costa Rica and Panama using partial sequences of the CO1 gene. Analysis conducted in Geneious using the Species Delimitation plugin (Masters et al. 2011). Clades defined in phylogenetic analysis: ACG, Brewster (+ sample KRL 0823) and El Copé are all represented as putative species. The table also includes ingroup and outgroup species.

Table 4. Intraspecific nucleotide diversity (π) within geographic groups of *L. warszewitschii*.

Population	Mean(π)	Range(π)
	CO1	
ACG	0.004	0-0.008
El Copé	0.063	0.002-0.154
Brewster	0.001	0-0.002
L.warszewitschii	0.072	0-0.166
	16S	
ACG	0.003	0-0.009
El Copé	0.032	0-0.076
Brewster	0.002	0-0.006
L.warszewitschii	0.034	0-0.079

Nucleotide diversity (π) within *Lithobates warszewitschii* for the geographic groups ACG, Brewster and El Copé based on pairwise values for CO1 and 16S sequences. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter = 4).

CO1 and 16S Net between group mean distance (NBGMD) (π_{net})

At the CO1 and 16S loci the largest NBGMD (π_{net}) was 15.4% and 6.9%, respectively, between ACG and Brewster samples (Suppl. material 1: Tables S2, S3). Samples from ACG and El Copé shared the lowest distance at 7.3% and 4.5%, respectively, and the intermediate distance was 10.6% and 5%, respectively, between El Copé and Brewster samples. Most intraspecific distances between the geographic groups within *L. warszewitschii*, surpassed the interspecific values between recog-

nized species within the genus (CO1/16S), such as: *L. catesbeiana* and *L. clamitans* (5.7%/2%), *L. clamitans* and *L. septentrionalis* (8.3%/3.1%), *L. catesbeiana* and *L. septentrionalis* (8.6%/2.2%).

Discussion

The concatenated phylogenetic trees consistently outlined three distinct clades within *Lithobates warszewitschii* supported by high posterior probabilities, bootstrap values, and taxonomic distinctness at the CO1 locus. No field sites within the ACG exhibited any well-defined cladistic structure, indicating it is a larger panmictic population. The individuals from El Copé were polyphyletic, revealing the presence of two OTUs at this site. Geographic groups within *L. warszewitschii* also exhibited greater genetic distances than many other recognized species pairs within the genus, suggesting cryptic species may be present.

In the analyses of nucleotide diversity and NBGMD, isolation by distance (IBD) (Wright 1943) does not explain all patterns of genetic variation, as samples from ACG and El Copé are most closely related in all scenarios. Additionally, the range of 16S (K2P- π) distance values within El Copé reached the highest for any geographic group at both loci. Thus, there is evidence that IBD contributes towards greater polymorphism in the most isolated allopatric populations, but other intrinsic (dispersal capability) and extrinsic (environmental and ecological) factors may explain large variation within and between finer geographic scales.

Isolation by distance may be the main driver of divergence or speciation among conspecific populations (Slatkin 1993) in allopatry (Vences and Wake 2007), other drivers include, low vagility due to limitations of physiology (Balinsky 1981, Navas and Otani 2007) and dispersal (Blaustein et al. 1994). However, recurrent hybridization, secondary contact, or overlap with sister species can decrease this genetic distance correlation (Fouquet et al. 2007b). If populations follow a simple pattern of IBD, they may be considered with some probability, conspecific (Fouquet et al. 2007a). Conversely, where large variations in genetic distance cannot be explained by this concept, it is likely that cryptic speciation is present.

Lithobates warszewitschii is widely distributed throughout Central America, and the possibility of vicariance may explain mechanisms for genetic divergence. The Talamanca mountain range divides the Pacific and Atlantic versants at ~2000m altitude (Savage 1982). Many of the Isthmian fauna disperse through the Caribbean lowlands but have disjunct distribution along Costa Rica's Pacific southwest (McDiarmid and Savage 2005) that historically contained more dry forest. Crawford et al. (2007) hypothesized that the presence of a filter barrier (Remington 1968), caused by extreme topography and narrowing of the rainforest corridor in Panama's Bocas del Toro province induced the deepest phylogeographical split between northern and southern populations of *Craugastor* rainforest species. For *Craugastor fitzingeri* (Schmidt, 1857), a generalist species, these effects were much less accentuated and

its phylogenetic structure may be attributed to a more recent range expansion. For *L. warszewitschii*, gene flow is still possible, even if regional dry forests were transformed into savannah during the Pleistocene glacial maxima (Piperno and Pearsall 1998), patches of gallery forest that allowed reproduction in freshwater could permit dispersal westward into Costa Rica.

Although vicariance does divide sister species (Avise et al. 1987), it fails to form a general explanation for divergence in the tropics (Antonelli et al. 2010). Barriers such as mountains do not impede gene flow directly, but promote ecological gradients (Janzen 1967). An alternative explanation for the phylogeographic structure within *L. warszewitschii* could be peripatric (Mayr 1954) or dichopatric (Bush 1994) speciation – a common mode of evolution in amphibians (Vences and Wake 2007).

Paz et al. (2015) used a trait-based phylogeographic approach to model environmental and ecological variables in Panamanian frog populations. Indirect development encouraged greater dispersal and species with large ranges had lower genetic divergence – a characteristic associated with generalists (Duminil et al. 2007). Despite being oviparous and wide-ranging, *L. warszewitschii* scored highest when modelling landscape resistance (resistance to dispersal caused by environmental conditions) and was highly divergent between Brewster and El Copé, with large genetic distances in proportion to their geographical distance. A possible explanation for this pattern could be a secondary contact during the post-glacial maxima (Schneider 1993) or selection for different ecological roles, such as within habitat or resource use (Alizon et al. 2008). It is true that *L. warszewitschii*'s colouration, habitat use, elevation range, and distribution vary (Savage 2002, Leenders 2016). Thus, high intraspecific diversity may be attributed to ecological specialization (Schluter 2000) in allopatry or coexistence of sister species in sympatry, such as in El Copé. For example, even if broad colouration of this species is genuine, frogs use non-morphological signals such as advertisement calls, cuticular hydrocarbons and other pheromones in mating systems and species recognition (Bickford et al. 2007), meaning they often remain inconspicuous. Divergent or cryptic species should therefore be considered a hypothesis of separately evolving entities (Hey et al. 2003, de Quieroz 2007, Fiser et al. 2018) and species status further scrutinized through integrative taxonomic methods (Padial et al. 2010).

Polyphyly can be used as indication of undescribed species in a lineage (Fouquet et al. 2007a). However, its presence complicates the classification of species in phylogenies as it may represent transitional stages in the evolution of taxa (Hörandl and Stuessy 2010, Xiang et al. 2012). Cryptic species often show morphological, ecological or genetic differentiation and usually a degree of reproductive isolation, which may occur through phenotypic plasticity or single locus polymorphisms. Hybridization may persist, leaving traces of introgression, speciation or hybrid vigour. Alternatively, fusion may be resisted by disruptive/divergent selection or postzygotic isolation (Sasa et al. 1998). This continuum is evident across large geographic ranges to highly localized areas, providing explanations for the evolutionary transitions of ecological races

to species (Mallet 2008). Consequently, in *L. warszewitschii*, patterns of polyphyly, relatedness between ACG and El Copé samples, or large pairwise ranges in sympatry may reflect occasional or historical gene flow from migrants, hybridization, introgression, retention of ancestral polymorphisms or incomplete lineage sorting when using mitochondrial genes (Moritz and Cicero 2004). Alternatively, the presence of two sympatric OTUs at El Copé, may reflect human-induced introduction. Because of these scenarios, nuclear DNA is also recommended in subsequent evolutionary and taxonomic studies (Vences et al. 2005).

At both CO1 and 16S loci, K2P-π mean (Meyer and Paulay 2005) intraspecific ingroup values overlapped with interspecific species values, surpassing proposed general thresholds: 8% at CO1 and 2% 16S (Crawford et al. 2010), 10% CO1, 5% 16S (Vences et al. 2005) and for neotropical amphibians at 16S (>3%) (Fouquet et al. 2007a). This indicates a wider ranging cryptic complex is present, and advocates for the use of both genes in comparative amphibian phylogenetics (Vences et al. 2005). Ultimately, concatenated genes may yield the best phylogenies (Gadagkar et al. 2005), however, interspecific comparisons are limited in this study due to having one individual representing each congeneric species, and an incomplete taxonomy that can hamper results (Meyer and Paulay 2005).

Conclusions

The type specimen of *Lithobates warszewitschii* originated from Volcán Chiriqui, western Panama (Schmidt 1857, Savage 1970), a locality near the Costa Rican border at almost equal distance between ACG and Brewster. Whilst the topotype locality was not sampled, all clades in this study may represent cryptic species. We have extended the research on cryptic diversity within *L. warszewitschii* by revealing an additional clade from ACG, and propose this clade is a candidate cryptic species that warrants further taxonomic investigation. Determination of evolutionary mechanisms are beyond the scope of this study, but an additional paraphyletic lineage from Costa Rica suggests it is probably a wide-ranging species complex, a likely scenario for many neotropical amphibians. Population trends in Costa Rica and Panama reflect both historical factors and recent habitat destruction, declines and introduced disease. Further sampling within Costa Rica, Nicaragua, and Honduras is likely to yield more cryptic diversity, and extirpation of a candidate lineage within El Copé (Crawford et al. 2010) highlights the importance of DNA barcoding in rapid, preliminary species identification. Such assessments are necessary to inform biodiversity estimates, taxonomic progress, and conservation of amphibian species. Phylogeographic structure in *L. warszewitschii* highlights the difficulty in explaining mechanisms of speciation in Mesoamerican amphibian fauna. Evolutionary theory, supported by morphological, ecological, physiological and multiple genetic methods are necessary to evaluate divergent processes in this group, and in achieving species status of sister taxa in this complex.

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Supplementary material 1

Supplementary tables and figures

Authors: James Cryer, Felicity Wynne, Stephen J. Price, Robert Puschendorf

Data type: molecular data

Explanation note:

Table S1. ABGD analysis from CO1 using all species presented in Table S2.

Table S2. Estimates of evolutionary divergence (π), and net evolutionary divergence (πnet) over CO1 sequence pairs between groups.

Table S3. Estimates of evolutionary divergence (π), and net evolutionary divergence (πnet) over 16S sequence pairs between groups.

Figure S1. CO1 phylogenetic tree. Geographic populations ACG (red), Brewster (orange), El Copé (purple) of *L. warszewitschii* are represented.

Figure S2. 16S phylogenetic tree. Geographic populations ACG (red), Brewster (orange), El Copé (purple) of *L. warszewitschii* are represented.

Figure S3. Prior intraspecific genetic divergence and number of OTUs using the ABGD algorithm.

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RESEARCH ARTICLE

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A new species of *Pista* Malmgren, 1866 (Polychaeta, Terebellidae) from the north-western Mediterranean Sea

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Abstract

A new species of Terebellidae, *Pista colini* **sp. n.**, has been identified from the harbour of Banyuls-sur-Mer, north-western Mediterranean Sea. This new species was found in very high densities, exclusively in gravelly sand deposited manually, and was not found in the original source habitat of the gravel. This species is characterized by the colour of the ventral shields with pinkish anterior part and a blood red posterior part in live specimens, a pair of unequal-sized plumose branchiae inserted on segment II and anterior thoracic neuropodia with long-handled uncini. The presence of long-handled uncini even in the smallest specimens constitutes the major difference between *Pista colini* **sp. n.** and other *Pista* species with a single pair of branchiae such as *P. lornensis* and *P. bansei*.

Keywords

Annelida, gravel deposits, harbour, *Pista colini* sp. n., taxonomy, Terebellida

Introduction

Terebellids belong to a very species-rich group of sedentary polychaetes, widely distributed in most marine benthic substrates, from shallow waters to deep-sea environments (Hutchings 2000, Rouse and Pleijel 2001). A recent review of Terebellidae Johnston, 1846 has been undertaken by Hutchings et al. (2017). The genus *Pista* Malmgren, 1866 currently includes 74 valid species (Hutchings et al. 2017). Difficulties in observing morphological characters and lack of geographically relevant literature have led to misidentifications of specimens belonging to this group. For example, *Pista cristata* (Müller, 1776) has been considered as a cosmopolitan species, but represents a complex of species (Gil 2011, Hutchings and Kupriyanova 2018). Recently, many changes have occurred in the *Pista sensu lato* group (Hutchings et al. 2017, Jirkov and Leontovich 2017) which currently includes seven genera: *Axionice* Malmgren, 1866, *Eupistella* Chamberlin, 1919, *Lanicides* Hessle, 1917, *Paraxionice*, Fauchald, 1972, *Pista* Malmgren, 1866, *Pistella* Hartmann-Schröder, 1996, and *Scionella* Moore 1903. Among these genera, only four have some species with a single pair of branchiae: *Pista*, *Lanicides*, *Pistella,* and *Scionella*. *Pista* and *Lanicides* can be differentiated from each other by the shape of avicular uncini and particularly by the presence of long-handled uncini (Hutchings et al. 2017). Nogueira et al. (2010, 2015) highlighted the presence of distally serrated notochaetae in *Lanicides,* which are absent in *Pista*. Furthermore, according to Nogueira et al. (2010), species of *Scionella* have a single pair of branchiae on segment IV while those of *Pistella* have a single pair of branchiae on segment II. Mikac and Hutchings (2017) provide a generic diagnosis of *Pistella* versus *Pista.* According to them, the main difference between these two genera is that *Pistella*'s neurochaetae are all short-handled avicular uncini while *Pista*'s neurochaetae are long-handled avicular uncini, at least on some anterior neuropodia.

Currently, morphological-based studies on *Pista*-like genera (Saphronova and Jirkov 2001; Gil 2011) consider the number of pairs of branchiae and the presence of long-handled anterior thoracic uncini as size-related characters, and therefore synonymized several genera and suggested some species have very wide distributions. However, it is clear that a detailed revision of all these genera is required using both morphological and molecular techniques. Ontogenetic studies could also clarify if the development of the long-handled uncini present in anterior thoracic neuropodia is a size-related character or is fixed for a species within a genus. Hutchings et al. (2017) and the present study accept them as stable generic characters and therefore reject these synonymies of Saphronova and Jirkov (2001). Currently, only seven species in the genus *Pista* are characterised by possessing a single pair of branchiae: *P. dibranchis* Gibbs, 1971 known from the Solomon Islands, *P. godfroyi* (Gravier, 1911) and *P. spinifera* (Ehlers, 1908) from Antarctica; *P. mirabilis* McIntosh, 1885 from deep water off Argentina; *P. bansei* Saphronova, 1988 described from Northern Pacific Ocean (although no specific type locality is given in the original description, but recently confirmed by Hutchings unpublished data.); *P. lornensis* (Pearson, 1969) from a Scottish loch, and finally *P. adriatica* Mikac & Hutchings, 2017 recently described from the Adriatic Sea.

The present study provides the description of a new species of *Pista* from the north-western Mediterranean Sea, based on morphological characters. Molecular data (COI gene) are provided for further investigations.

Materials and methods

Sampling and morphological analyses

The first specimens of the new *Pista* species were sampled in 2012 in the harbour of Banyuls-sur-Mer (French Mediterranean Sea; WGS84: 42°28.87'N, 3°08.15'E; 3 m depth; Fig. 1). Specimens examined in this study were collected in 2012 and 2017 using a van Veen grab. Live specimens (anaesthetised with menthol) were examined under a Zeiss stereomicroscope (V20 discovery-Plan S objective 1.0×) equipped with a camera (Axiocam 105) and preserved specimens with a Nikon SMZ25 stereomicroscope (Nikon DS-Ri 2) camera, a Nikon Eclipse E400 microscope, and a Zeiss Axio Lab.A1 microscope. Slides for uncini were prepared with lactic acid and observed under 100× oil immersion lens. A posterior parapodium of paratype MNHN-IA-TYPE 1853 was removed and fixed in 100% ethanol for molecular studies. All other material was fixed in 4% formaldehyde seawater solution, then transferred to 70% ethanol for morphological analyses. Several specimens were dehydrated in ethanol, critical point dried and covered with gold, and examined under a scanning electron microscope (SEM) at Macquarie University (JEOL JSM 6480LA) and at Arcachon Marine Station (Hitachi TM3030).

Holotype and most paratypes were deposited at the Museum national d'Histoire naturelle, Paris (**MNHN**), other paratypes were deposited in the Australian Museum, Sydney (**AM**). Non-type additional material was lodged in collections of Banyuls-sur-Mer and Arcachon Marine Stations in France.

DNA isolation, amplification, and sequencing

Samples for DNA analysis were removed from a live specimen (paratype MNHN-IA-TYPE 1853) placed in ethanol 96% and frozen at -20 °C. Extraction of DNA was done with QIAamp DNA Micro Kit (QIAGEN) following protocol supplied by the manufacturers. Approximately 650 bp of COI (cytochrome c oxidase subunit I) genes were amplified using primers polyLCO and polyHCO (Carr et al. 2011). The PCR (Polymerase Chain Reaction) was carried out with Gotaq G2 Flexi DNA Polymerase (PROMEGA), with 50 µL mixtures contained: 10µL of 5X Colorless GoTaq Reaction Buffer (final concentration of 1X), 1.5 µL of $MgCl_2$ solution (final concentration of 1.5mM), 1 µL of PCR nucleotide mix (final concentration of 0.2 mM each dNTP), 0.5 µl of each primer (final concentration of 1µM), 0.2 µl of GoTaq G2 Flexi DNA Polymerase (5U/µl), 1 µl template DNA and 33.8 µL of nuclease-free water. The tem-

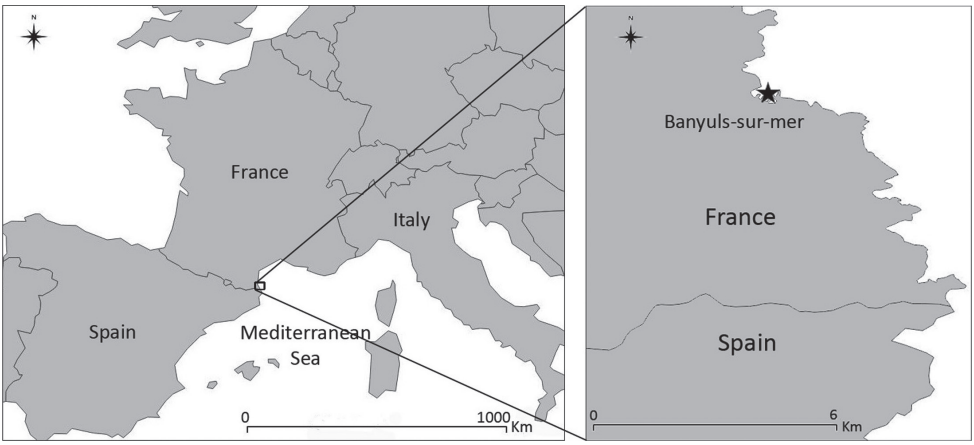

Figure 1. Location of Banyuls-sur-Mer harbour, France, where *P. colini* sp. n. was collected.

perature profile was as follows: 94 °C/600s – (94 °C/40s-44 °C/40s-72 °C/60s) *5 cycles -(94 °C/40s-51 °C/40s-72 °C/60s) *35 cycles -72 °C/300s -4 °C. Amplified PCR products were analysed by electrophoresis in a 1 % p/v agarose gel stained with ethidium bromide and were sent to GATC Biotech Company to complete double strain sequencing, using same set of primers as used for PCR. Overlapping sequence (forward and reverse) fragments were merged into consensus sequences and aligned using Clustal Omega. Sequences were translated into amino acid alignment and checked for stop codons to avoid pseudogenes. The minimum length coverage was around 660 bp. Sequence obtained in this study has been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The accession number is given in the section Genetic data.

Taxonomic account

Family Terebellidae Johnston, 1846

Genus *Pista* Malmgren, 1866

Type species. *Amphitrite cristata* Müller, 1776, by original designation.

Diagnosis. Transverse prostomium attached to dorsal surface of upper lip; basal part as thick crest, eye spots sometimes present; distal part shelf-like. Buccal tentacles all uniformly cylindrical. Peristomium restricted to lips; relatively short upper lip, hood-like; swollen, cushion-like and mid-ventral lower lip. Segment I reduced dorsally, with pair of lobes of variable size and position; segments II–IV also with pairs of lobes of variable size and position, sometimes extending for a few more segments. Anterior segments highly glandular ventrally, with discrete, smooth to slightly corrugated, rectangular to trapezoidal mid-ventral shields. Paired arborescent, pectinate or plumose

branchiae present from segment II, typically two pairs, on segments II and III, rarely a single pair or three pairs. Conical to rectangular notopodia beginning on segment IV, all aligned, typically extending for 17 segments, until segment XX; notochaetae all distally winged, frequently broadly winged. Neuropodia beginning on segment V, as low ridges in conjunction with notopodia and short pinnules posteriorly; neurochaetae as long-handled avicular uncini, at least on anterior neuropodia, frequently until segment X or termination of notopodia, then short-handled; uncini in partial to completely intercalated double rows on segments XI–XX. Nephridial papillae present on segment III, genital papillae on variable number of segments, usually on segments VI–VII, posterior and dorsal to notopodia. Pygidium smooth to slightly crenulated (after Hutchings et al. 2017).

***Pista colini* sp. n.**
http://zoobank.org/0532761D-4534-4C83-8D56-D7683468160B
Figs 2–4

Material examined. ***Type material.*** Banyuls-sur-Mer harbour, Gulf of Lion, Mediterranean Sea, France (42°28.867'N, 3°08.154'E, 3 m depth), subtidal in gravely sands, all collected 16 July 2012 except MNHN-IA-TYPE 1853 collected 12 July 2017. Holotype: MNHN-IA-TYPE 1850, complete, 70 segments, total length 17.6 mm, thoracic length 4.8 mm, anterior width 0.6 mm, Paratypes: AM W.50625, 1 specimen, posteriorly incomplete, total length 11 mm, thoracic length 7 mm, anterior width 1.0 mm; AM W.50626, 3 specimens plus 1 posterior fragment 5 mm with pygidium, 1 complete, total length 11 mm, thoracic length 5 mm, anterior width 0.5 mm, 1 complete, total length 12 mm, thoracic length 5 mm, anterior width 0.5 mm, 1 posteriorly incomplete, length 16 mm, thoracic length 8 mm, anterior width 0.8 mm, 2 specimens mounted for SEM. MNHN-IA-TYPE 1851, 1 specimen, posteriorly incomplete, total length 14.3 mm, thoracic length 3.7 mm, anterior width 0.7 mm; MNHN-IA-TYPE 1852, complete specimen, total length 9.70 mm, thoracic length 4.6 mm, anterior width 0.9 mm; MNHN-IA-TYPE 1853, complete collected 12 July 2017, thoracic length 4.4 mm, anterior width 1.1 mm, posterior part cut for molecular analysis; MNHN-IA-TYPE 1854, complete length 18.2 mm, anterior width 0.7 mm, mounted for SEM; MNHN-IA-TYPE 1855, complete, 1 specimen, total length 9.0 mm, thoracic length 3.1 mm, anterior width 0.7 mm.

Additional material. Banyuls-sur-Mer harbour, Gulf of Lion, Mediterranean Sea, France (42°28.867'N, 3°08.154'E, 3 m depth), subtidal in gravely sands, all collected 16 July 2012. BAN.Pista.08, 1 specimen, complete, total length 10.0 mm, thoracic length 3.7 mm, anterior width 0.8 mm; BAN.Pista.09, 1 specimen gravid, posteriorly incomplete, thoracic length 5.1 mm, anterior width 0.7 mm; BAN.Pista.10, complete, 1 specimen, total length 22.7 mm, thoracic length 7.8 mm, anterior width 0.9 mm; BAN.Pista.12, complete, 1 specimen, total length 12.4 mm, thoracic length 4.6 mm, anterior width 0.6 mm.

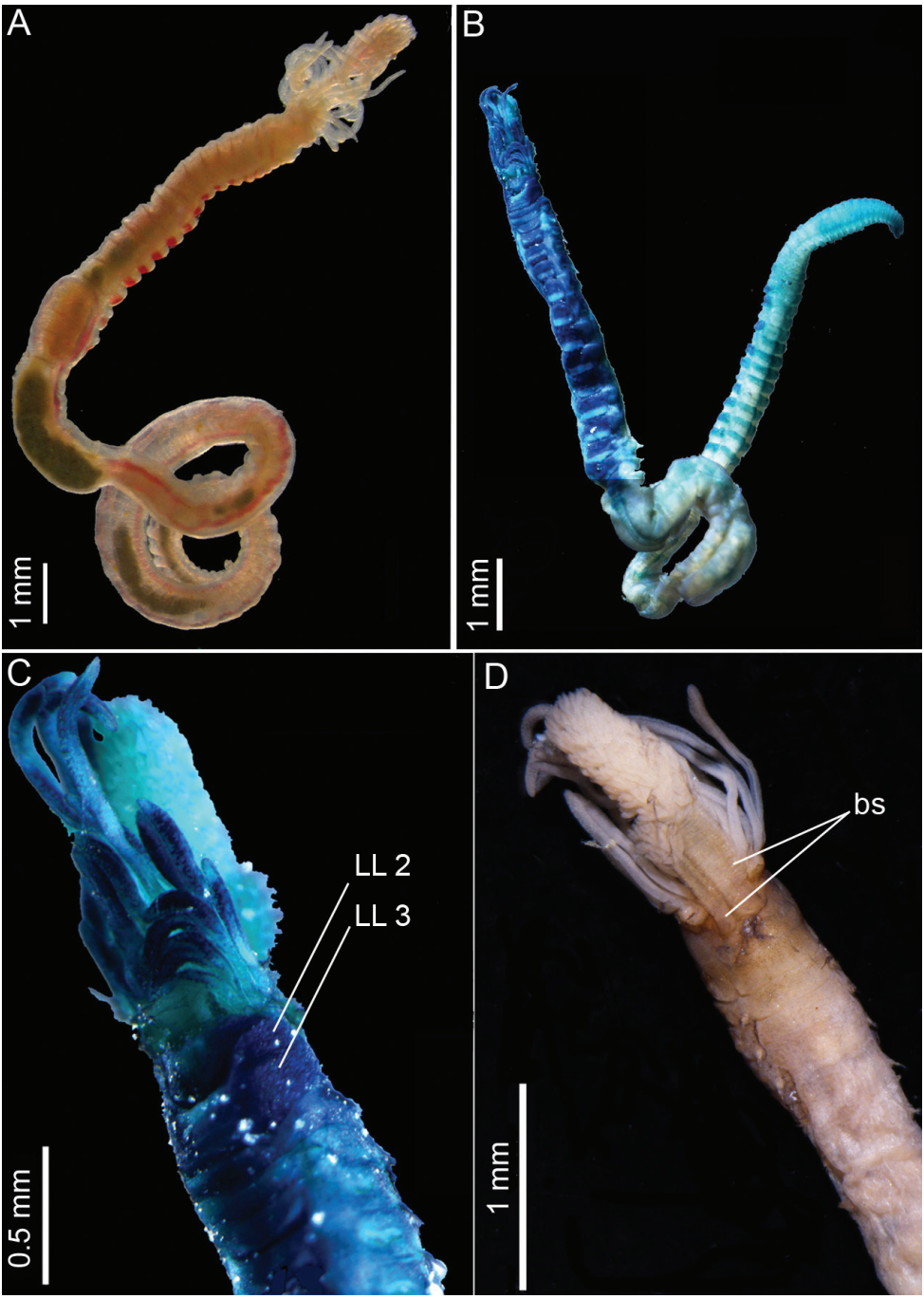

Figure 2. *Pista colini* sp. n.: **A** Live specimen, dorsal view **B** Entire specimen, ventral view, methyl green staining **C** Anterior part, ventral view **D** Anterior part, dorsal view. **B–D** from holotype MNHN-IA-TYPE 1850. Key: LL: lateral lobes, bs: branchial stalks.

Comparative material. *Pista bansei* Saphronova, 1988 Holotype reg. # 47667, 47°41'N, 139°34.1'E, Sea of Japan, Tartary Strait, off Nelma, 105 m; 4 paratypes reg. # 47668 according to Saphronova 1988 (but reg. # 32423 according to label in the museum vial) from same station, deposited in Zoological Museum of Russian Academy of Sciences in St Petersburg.

Additional material from R/V "Vityaz" stations 59, 119, 1587a, 3350, 3569, 1086. (For locality details see Saphronova (1988: table 1, no museum registration numbers allocated) deposited in the Zoological Museum of Moscow State University.

Description (based on holotype). Holotype is a complete specimen, 17.5 mm in length, 0.6 mm in width at segment X and with 70 segments (Fig. 2A, B).

Transverse prostomium attached to dorsal surface of upper lip. Buccal tentacles all of similar width inserted ventrally on prostomium, shorter than smallest branchia; long tentacles situated centrally in dorsal region, longer than largest branchia (Fig. 2C). Peristomium consisting of large rounded upper lip, forming a swollen cushion with one small fold on each side. Lower lip short, irregularly swollen (Fig. 3C). Segment I reduced, V-shaped, situated medio-ventrally (Fig. 3C), without lateral lobes. Segment II with well-developed lateral lobes, with anterior margins rounded merging with ventral pad to form a continuous minutely crenulated ventral collar. One pair of unequal-sized plumose branchiae inserted one just next to the other on segment II; all filaments strongly ciliated (Figs 2D, 3D), arranged in spiral around central axis with dichotomous filaments. Both stalks markedly wrinkled (Fig. 3A, B). Segment III with lateral lobes half width of segment, asymmetrical and slightly displaced dorsally, connected across ventrum (Fig. 3A–C). Segment IV lacking lateral lobes (Fig. 3A, B).

Notochaetae, broad-winged capillaries, with fine tips (Fig. 4A). Neuropodia from segment V (chaetiger 2), initially arranged in single rows, from segments XI to XX arranged in completely intercalated double rows face-to-face and then reverting to single rows on abdomen. Neurochaetae as long-handled avicular uncini on segments V and VI (Fig. 4B) then short-handled. Neuropodia with ca. 14 uncini (arranged in single row), thoracic uncini with dental formula MF: 3–4:5–6:α (Fig. 4D–E). Abdominal neuropodia becoming more erect posteriorly with ca. 12 uncini each, elongate extending from torus, dental formula MF: 6–7:6–7: α: α (Fig. 4C, F). Nephridial papillae on segments VI and VII (chaetigers 3 and 4), inserted posteriorly/laterally to notopodia, small spherical.

Pygidium with slightly crenulated margins (hardly visible even under stereomicroscope but clearly visible under SEM).

Methyl green staining pattern. Branchiae, lips and base of tentacles not stained. Extremity of tentacles staining and retained as blue/brown even after being washed in ethanol for some days (Fig. 2B, C). Thorax until segment XX, strongly staining ventrally, moderately laterally and poorly dorsally. Ventral stain on all shields, anterior half of each shield staining deeply, posterior part not staining (Fig. 2B, C). Anterior abdomen not coloured and posterior abdomen staining ventrally and dorsally with anterior half of each segment staining deeply, posterior part not staining; increasing colouration towards pygidium (Fig. 2B).

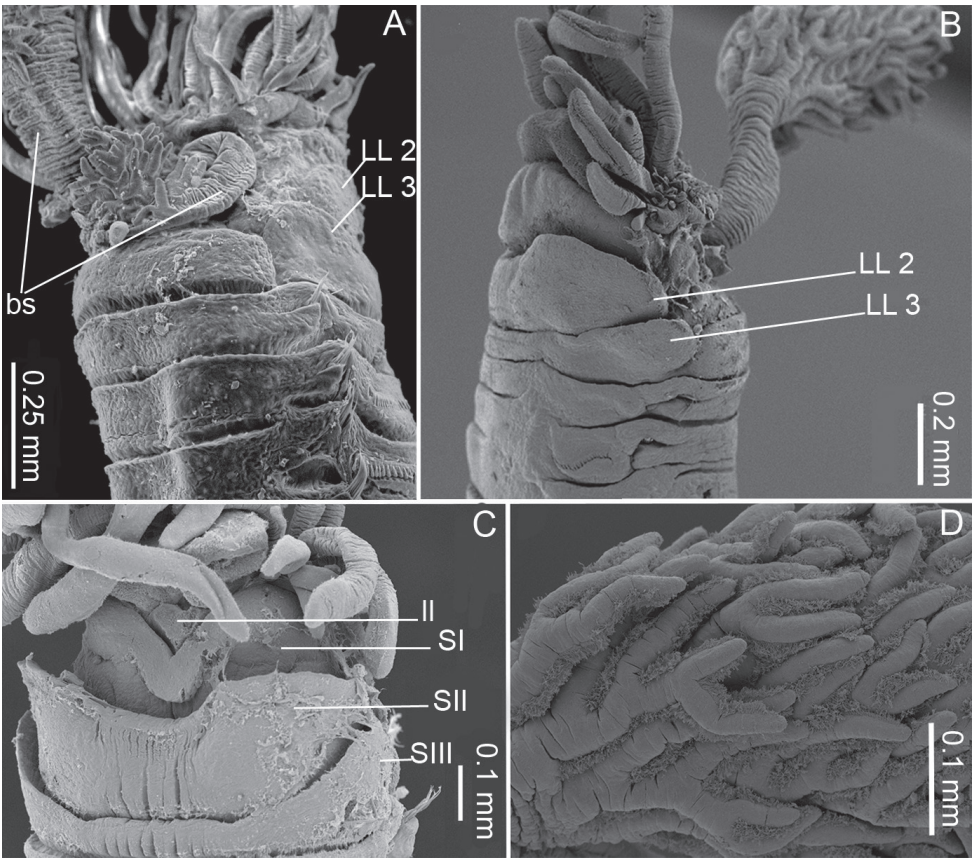

Figure 3. *Pista colini* sp. n., SEM images: **A** Anterior part, dorso-lateral view **B** Anterior part, lateral view **C** Anterior part, ventral view **D** Branchial filaments **A** from paratype MNHN-IA-TYPE 1854 **B–D** from paratype AM W.50626. Key: LL: lateral lobes, bs: branchial stalks, ll: lower lip, SI, SII, and SIII: Segments I, II, and III.

Morphological variation. Complete individuals ranging from 9.0 to 22.7 mm in length, 0.5 to 1.1 mm in width at segment X and between 59 to 72 segments. Thoracic lengths vary between 3.1 and 7.8 mm. One gravid specimen was found (BAN. Pista.09). It was incomplete, but thoracic length was 5.1 mm and anterior width 0.7 mm. These measurements correspond to a small size species. Live specimens pinkish with translucent buccal tentacles; ventral shields divided in two parts, anterior part pinkish, posterior part blood red (Fig. 2A). Preserved specimens pinkish with ventral shields divided transversally in two parts. Crenulation of ventral collar of segment II is difficult to see under the binocular and not always visible under SEM. It probably depends on the contraction of the animal. All specimens, regardless of size, with a single pair of branchiae, one up to twice as long as the other (Fig. 2D). Eleven of the twenty observed specimens had the long branchiae on the right side. Number of anterior

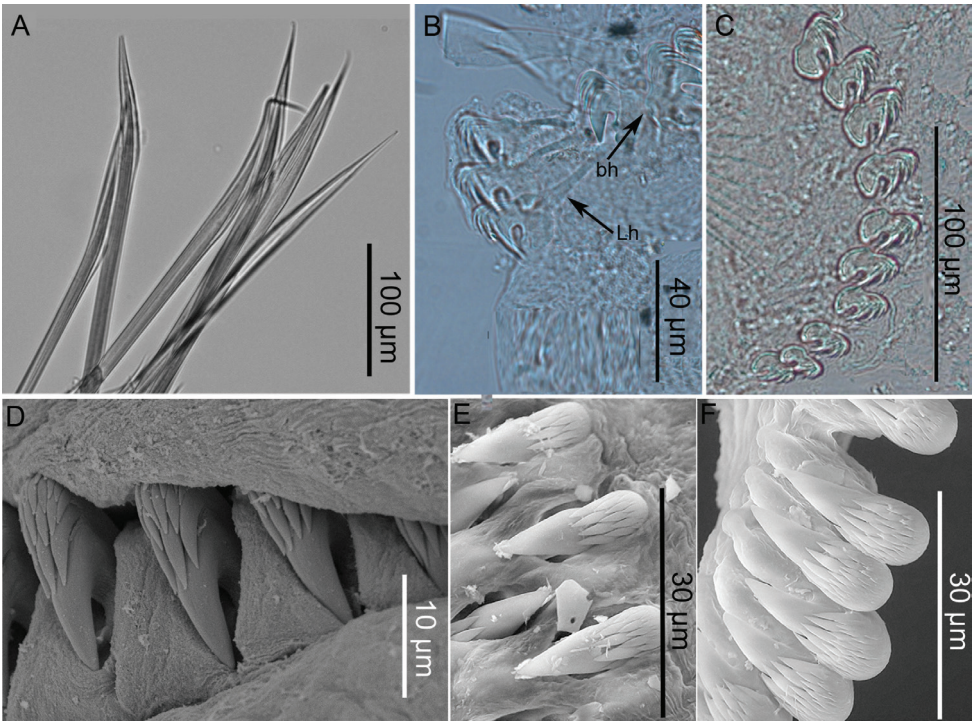

Figure 4. *Pista colini* sp. n.: **A** Thoracic notochaeta of segment VI **B** Thoracic uncini of segment V **C** Abdominal uncini **D** Thoracic uncini in single row **E** Thoracic uncini in double row **F** Abdominal uncini **A** from paratype MNHN-IA-TYPE 1853 **B, C** from additional material BAN.Pista.12 **D** from paratype AM W.50626 (SEM image) **E, F** from paratype MNHN-IA-TYPE 1854 (SEM images). Key: Lh: long-handled uncinus, bh: broken-handled uncinus.

thoracic uncinigers with long-handled uncini is variable (from 2 to 9). This difference seems to not be dependent upon size. Nephridial papillae not always visible.

Etymology. The name of species is dedicated to the nephew of the first author Colin Labrune who is already a little budding naturalist.

Type locality. Only known from Banyuls-sur-Mer harbour, France (Mediterranean Sea).

Ecological notes. *Pista colini* sp. n. was sampled at 3 m depth on gravelly sand recently deposited manually in Banyuls-sur-Mer harbour. It was found in very high densities (446 ind. m^{-2} in April 2012 and 1176 ind. m^{-2} in July 2012) a few weeks after the sediments had been deposited. We sampled again in November 2012 but there was no more gravel and *Pista colini* sp. n. was absent. The species is not found in the harbour if no gravel deposits are present. In the undisturbed part of the harbour, median granulometry was ca. 50 µm while the median granulometry of the gravelly sand in which this species is found was ca. 800 µm. In July 2017, we sampled a week after another fresh load of sediment with gravel had been deposited and we found high

densities of *Pista colini* sp. n. living in tubes made from heterogeneous sediment agglomerated with mucus.

Genetic data. The COI gene was successfully sequenced and published at NCBI GenBank for paratype MNHN-IA-TYPE 1853 with accession number MK584933.

Remarks and discussion. The presence of a single pair of branchiae is a stable character in *Pista colini* sp. n. More than 100 specimens were observed, of different sizes and all of them had a single pair of branchiae, some were also observed alive. Our observations support Hutchings et al. (2017) but are not in agreement with Saphronova and Jirkov (2001), who hypothesised that this character is size-related. A detailed morphological and molecular study needs to be performed in order to investigate this hypothesis across a range of species with varying number of pairs of branchiae.

Although Gil (2011) mentioned *Pista cristata* as having only one pair of branchiae, based on Saphronova (1988), we considered that this species has two pairs of branchiae. The absence of consensus on this species does not have any consequence for this new species which always had some long-handled thoracic uncini, whereas Gil (2011) records *P. cristata* as lacking such long-handled even on large specimens. Among the seven species with a single pair of branchiae, there is no possible confusion of *P. colini* sp. n. with *Pista mirabilis* and with *P. spinifera* as both lack plumose branchiae. *Pista colini* sp. n. is close to *P. adriatica* sharing the following characters: one pair of unequal sized plumose branchiae on segment II and presence of lateral lobes on segments II and III, lacking on segment IV. However, segment II of *P. adriatica* presents narrow lateral lobes while in *P. colini* sp. n. these lateral lobes are well developed. Lateral lobes of segment III are rectangular in *P. adriatica* rather than being asymmetrical and slightly displaced dorsally as in *P. colini* sp. n. Furthermore *P. colini* sp. n. can be differentiated by the absence of glandular ridges on segments II and III, which are present in *P. adriatica*. According to Mikac and Hutchings (2017), *P. godfroyi* and *P. dibranchis* which also have a single pair of branchiae, should be transferred to *Pistella* Hartmann-Schröder 1996 because they lack long-handled uncini. Therefore, they cannot be confused with *P. colini* sp. n. The lack of long-handled uncini is also the case for *Pista lornensis.* Furthermore, when first describing *Pista lornensis*, Pearson (1969) reported two obvious ligaments, one attached below the rostrum and the largest to the posterior basal corner of the uncini. These filaments are not present in *P. colini* sp. n.

According to Gil (2011), *Pista bansei* is the only *Pista* species in Europe to present one pair of "pompom like" branchiae and anterior long-handled uncini. The original description by Saphronova (1988) is based on an incomplete holotype with 16 segments, 3.2 mm wide collected at 105 m in Strait of Tartar, the Sea of Japan, north-western Pacific Ocean, and four damaged paratypes from the same locality. She also designated another eight paratypes (R/V "Vityaz" St 1576, 60°03'N, 168°46'E, 230 m, Olutorsky Bay, off Kamchatka Peninsula, Bering Sea, north-western Pacific Ocean) and 1 paratype (R/V "Sevastopol" St 1086, 495 m, 62°56'N, 9°19'W, between Iceland and Faroe Islands, North Atlantic Ocean), the material is deposited in Zoological Museum of Moscow State University. She also lists additional specimens not designated as type material from localities such as Davis Strait, Norwegian, Kara Sea (off Novaya Zemlya), White Sea in the North Atlantic, and Arctic Oceans, as well as Sea of Ja-

pan, Sea of Okhotsk, and Bering Sea in the north-western Pacific Ocean in depths of 120–606 m. Such a wide distribution is highly unlikely and we suggest that *P. bansei* sensu stricto is restricted to the north-western Pacific Ocean, while the rest of the material, including the one paratype from the North Atlantic Ocean represents another species, most likely part of the same species complex. Although, much of the material in Zoological Museum of Moscow State University is in poor condition, it most certainly belongs to multiple species. Therefore, Saphronova's (1988) hypothesis that only adults have anterior thoracic uncini with well-developed handles, while such handles are absent in juveniles, cannot be accepted. Furthermore, her diagrammatic illustrations indicate neither the sizes of individuals nor where the specimens were collected.

All the specimens of *P. colini* sp. n. examined here, even the smallest (59 chaetigers, thoracic width at segment X: 0.5 mm), which are comparable in size with the individuals that Saphronova (1988) identified as juveniles (width between 0.4 and 1.15mm), had well-developed long-handled uncini, at least on chaetigers 1 and 2. Furthermore, in the original description, Saphronova (1988) described *P. bansei* with (1) an upper lip high and narrow while upper lip of *P. colini* sp. n. is large and rounded (2) large lateral lobes, positioned vertically and connected mid ventrally by a wide fold, although the connection between the two lateral lobes in *P. colini* sp. n. does not form a fold and does not look like the illustration in Saphronova (1988, fig. 8g–i). Moreover, Hilbig (2000) reports *P. bansei* with (1) moderate numbers of tentacles, usually broken off, although all specimens of *P. colini* sp. n. had some short and some long tentacles, rarely broken and (2) glandular ridges on segments II and III, which were not observed in *P. colini* sp. n. Furthermore, based on the holotype 1/47667, paratypes, and several additional specimens, Jirkov and Leontovich (2017) reported the presence of small lateral lobes on segment I in *P. bansei* which are not observed in *P. colini* sp. n. Therefore, *P. colini* sp. n., while similar to *P. bansei* in a number of characters, differs by the presence of long-handled uncini, even in the smallest specimens, and the fact that no glandular ridge was observed on segments II and III. Furthermore, the type locality of *P. bansei* is from the northern Pacific in cold deeper water (105 m).

Based on examination of the type material of Saphronova (1988) in Moscow and St Petersburg museums by Hutchings in 2018 (see Comparative material), *Pista bansei* is a North Pacific species currently only known with certainty from Tartar Strait and therefore, its range cannot overlap with that of any Mediterranean species. For these reasons, we describe *P. colini* as a new species from the Mediterranean Sea. Finally, this paper reinforces the need for a complete revision of the group of terebellids with long-handled uncini using both molecular and morphological data, especially those species with only a single pair of branchiae.

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RESEARCH ARTICLE

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Four new species of *Hexanchorus* Sharp from Ecuador (Coleoptera, Elmidae) with DNA barcoding and notes on the distribution of the genus

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Abstract

The riffle beetle genus *Hexanchorus* Sharp, 1882 is distributed from Mexico to Argentina, forming an important component of the freshwater invertebrate fauna of Latin America. With 21 described species, *Hexanchorus* represents one of the most speciose Larainae genera, but its real diversity is likely much higher. We analysed material from a relatively small area in Ecuador, resulting in the first record of *H. cordillierae* for Ecuador and discovery of four new species and one subspecies: *Hexanchorus virilis* **sp. n.**, *Hexanchorus rostratus* **sp. n.**, *Hexanchorus shepardi* **sp. n.**, *Hexanchorus onorei* **sp. n.** and *Hexanchorus onorei sagittatus* **ssp. n.** For delimiting and characterizing species, both morphological and molecular (mtCOI DNA barcodes) data were used. A distribution map of *Hexanchorus* species is provided based on published records.

Keywords

Andes, diversity, Larainae, Latin America, mtDNA, riffle beetles, new record

Introduction

The Neotropics represent one of the most life-rich regions in the world. With its enormously diverse ecosystems from large lowlands, through Amazonian rainforests up to the snow-covered peaks of the Andes, it provides manifold living conditions

suitable for an inordinate number of various organisms. However, many taxonomic groups inhabiting the Neotropics are still very poorly known including the riffle beetles, despite numerous recently published taxonomic papers describing their diversity (e.g. Maier 2013, Čiampor Jr et al. 2017, Martinéz Román et al. 2017, Polizei and Barclay 2018).

Ecuador is a relatively small country, but due to its great altitudinal variation and the presence of rainforests, it belongs to the top ten most biodiverse countries. The Elmidae of this region were studied mostly by Delève in the 1960s, who recorded 23 species in nine genera (Delève 1968). After forty-five years, these numbers increased to 59 species in 19 genera (Monte and Mascagni 2012).

The genus *Hexanchorus* Sharp, 1882, with 21 known species, is the largest and most likely the most wide-spread genus of Larainae in the Neotropics. The area of its distribution reaches from Mexico through Central America and the West Indies up to northern Argentina (Jäch et al. 2016). In contrast to its great distribution, 1/3 of all known species (seven) can be found in one country. This is almost certainly biased by uneven distribution of the research, pointing to our insufficient knowledge of the *Hexanchorus* fauna from the other countries and probably also to the large diversity of the genus. Here we processed the *Hexanchorus* material from Ecuador, collected at several of the 50 sites surveyed in 2013, including fresh material used for DNA barcode analyses to characterise species.

Material and methods

The studied material was collected by net sampling in small streams flowing in primary or degraded forest or at light. Specimens were fixed in pure alcohol directly in the field. The majority of material was collected in Ecuador. Additional specimens come from two localities in Venezuela and one site in Brazil. For the morphological study, specimens were cleaned and examined under a Leica M205C stereomicroscope at magnifications up to 160×. Male genitalia were studied as temporary glycerine slides at magnifications up to 600×, using a Leica DM1000 light microscope. Drawings were made with a drawing tube, subsequently scanned and finalized in Adobe Photoshop CS5. Habitus photographs were made using a Leica M205C with a Nikon D3s digital camera attached. Morphological terms generally follow Kodada et al. (2016).

Morphometric characters were measured with an ocular grid to the nearest 0.05 mm. Abbreviations used in the text: **CL** – body length (measured from the anterior margin of the pronotum to the elytral apices), **EL** – elytral length, **EW** – maximum elytral width, **PL** – pronotal length, **PW** – maximum pronotal width, **NMW** – Natural History Museum (Vienna, Austria), **CCB** – collection of Fedor Čiampor Jr (Bratislava, Slovakia), **PUCE** – collection of the Pontifical Catholic University of Ecuador (Quito, Ecuador), **MNHN** – National Museum of Natural History (Paris, France),

RBINS – Royal Belgian Institute of Natural Sciences (Brussels, Belgium). All type specimens belong to PUCE, but are presently on long-term loan at the CCB.

For the DNA analyses, 26 adults of *Hexanchorus* and 3 adults of related Larainae species were used. The dataset is available on https://doi.org/10.5883/DS-ELMHEXAN. DNA was isolated from the whole specimens using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocol or by phenol-chlorophorm extraction method. A fragment of the 5' end of the mitochondrial gene for cytochrome c oxidase subunit I (COI) was amplified with primers LCO1490, HCO2198 (Folmer et al. 1994). Amplification products were purified by alkaline phosphatase (FastAP) and exonuclease and sequenced from both sides in Macrogen Europe Inc. (Amsterdam, Netherlands). Raw sequences were assembled and edited in Sequencher v5.1. The genetic distances were measured using K2P model, maximum likelihood tree and bootstrap support was performed in MEGA software v7 (Kumar et al. 2016). The best-fitted substitution model (GTR+I+G) was selected by jModelTest 2 (Darriba et al. 2012). Species delimitation (bPTP, mPTP, ABGD) was run on servers (http://species.h-its.org, http://mptp.h-its.org/#/tree, https://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default settings. For outgroup rooting, sequences of *Potamophilops bostrychophallus* Maier, 2013, *Pseudodisersus goudotii* (Guérin-Méneville, 1843) and *Disersus inca* Spangler & Santiago, 1987 were used. The final tree was edited in FigTree v1.4.2 and Adobe Illustrator CS5. Vouchers are deposited in the CCB, and sequences were sent to GenBank and BOLD (accession numbers and BINs are in Table 1).

Results

Molecular data analysis

Sequences of the barcoding fragment from 26 specimens were used in the analysis, representing six putative *Hexanchorus* species. Four of the five new species described herein are also included, amplification of COI failed for *H. rostratus* sp. n., likely due to degraded DNA. The final fragment was 625bp long with no ambiguous sites or indels. The maximum likelihood (ML) analysis revealed five distinct clades, separated by the genetic distance of 1.1–12.5% (Suppl. material 1: Table S1). Among *H. cordillierae*, *H. onorei* sp. n. and *H. shepardi* sp. n., smaller genetic distances were recorded, ranging from 1.1 to 2.3%. However, all clusters representing these species have robust support and the taxa proposed are further supported by delimitation analyses and distinct morphological characters. The lower genetic distance could be thus attributed to recent speciation. In *H. onorei* sp. n., two clearly distinguishable morphological forms were recovered, and easily recognized by the size and structure of the male genitalia. These forms described here as subspecies are represented by

Table 1. Samples used in the molecular analyses: location of samples, GenBank and BOLD Data Systems BIN accession numbers. (FZ numbers refer to the vouchers used for DNA extraction)

Sample	Location	GenBank no.	BOLD BIN no.
Hexanchorus cordillierae FZ0602	Ecuador, Napo	MK155275	BOLD:ADO9755
Hexanchorus cordillierae FZ0956	Ecuador, Napo	MK155252	BOLD:ADO9755
Hexanchorus cordillierae FZ0966	Ecuador, Pastaza	MK155257	BOLD:ADO9755
Hexanchorus cordillierae FZ0972	Ecuador, Napo	MK155265	BOLD:ADO9755
Hexanchorus cordillierae FZ0987	Ecuador, Pastaza	MK155279	BOLD:ADO9755
Hexanchorus cordillierae FZ1242	Ecuador, Pastaza	MK155280	BOLD:ADO9755
Hexanchorus cordillierae FZ1243	Ecuador, Pastaza	MK155271	BOLD:ADO9755
Hexanchorus cordillierae FZ1244	Ecuador, Napo	MK155282	BOLD:ADO9755
Hexanchorus cordillierae FZ1245	Ecuador, Napo	MK155270	BOLD:ADO9755
Hexanchorus cordillierae FZ1248	Ecuador, Napo	MK155277	BOLD:ADO9755
Hexanchorus onorei sagittatus FZ0773	Ecuador, Morona-Santiago	MK155259	BOLD:ADB7879
Hexanchorus onorei sagittatus FZ0970	Ecuador, Morona-Santiago	MK155262	BOLD:ADB7879
Hexanchorus onorei sagittatus FZ1252	Ecuador, Morona-Santiago	MK155261	BOLD:ADB7879
Hexanchorus onorei onorei FZ0621	Ecuador, Morona-Santiago	MK155268	BOLD:ADB7879
Hexanchorus onorei onorei FZ0969	Ecuador, Morona-Santiago	MK155263	BOLD:ADB7879
Hexanchorus onorei onorei FZ0971	Ecuador, Morona-Santiago	MK155258	BOLD:ADB7879
Hexanchorus onorei onorei FZ1246	Ecuador, Morona-Santiago	MK155256	BOLD:ADB7879
Hexanchorus shepardi FZ0967	Ecuador, Napo	MK155264	BOLD:ADO9756
Hexanchorus shepardi FZ0968	Ecuador, Napo	MK155276	BOLD:ADO9756
Hexanchorus shepardi FZ1247	Ecuador, Napo	MK155267	BOLD:ADO9756
Hexanchorus virilis FZ0623	Ecuador, Pastaza	MK155281	BOLD:ADB7877
Hexanchorus virilis FZ0960	Ecuador, Pastaza	MK155251	BOLD:ADB7877
Hexanchorus virilis FZ1241	Ecuador, Pastaza	MK155253	BOLD:ADB7877
Hexanchorus virilis FZ1250	Ecuador, Pastaza	MK155266	BOLD:ADB7877
Hexanchorus tarsalis FZ1249	Brazil, Rio Grande do Sul	MK155255	BOLD:ADO9167
Hexanchorus tarsalis FZ1253	Brazil, Rio Grande do Sul	MK155278	BOLD:ADO9167
Potamophilops bostrychophallus FZ0383	Venezuela, El Caura	MK155274	BOLD:ADB8887
Pseudodisersus goudotii FZ0855	Ecuador, Pastaza	MK155254	BOLD:ADB9256
Disersus inca FZ0788	Ecuador, Morona-Santiago	MK155273	BOLD:ADB8448

well-separated clades (molecular data) with robust support in the ML tree, but with very small genetic distance (0.3%).

Taxonomy

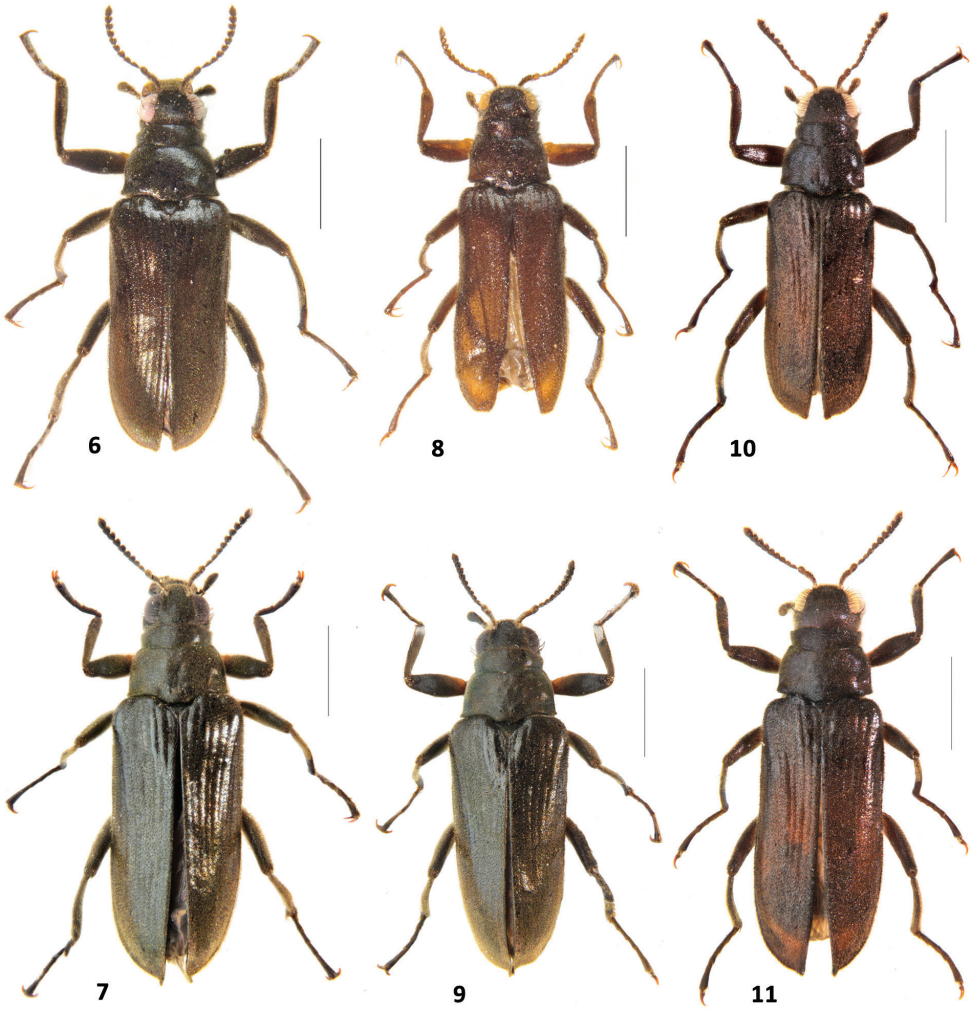
***Hexanchorus cordillierae* (Guérin Méneville, 1843)**
Figs 1, 2, 12, 13, 23, 24, 36

Material examined. (PUCE, NMW, CCB): 21 ♂♂, 10 ♀♀: "Ecuador, Napo prov., river near Don Napo ranch, Río Anzu, 01°14'17.2"S, 77°52'56.0"W 542m a.s.l., 13.8.2013, at light, Čiampor & Čiamporová-Zaťovičová lgt."; 1 ♂ "Ecuador, Pastaza prov., 10 de Agosto env., 01°21'37.1"S, 77°51'55.7"W 900m a.s.l., 16.8.2013, stream ca 1m wide, above confluence with larger stream, fast flowing with boulders, Čiampor

Figures 1–5. *Hexanchorus* habitus: **1** *H. cordillierae* male **2** *H. cordillierae* female **3** *H. virilis* sp. n. male **4** *H.* cf *virilis* sp. n. female **5** *H. rostratus* sp. n. male. Scale: 1 mm.

& Čiamporová-Zaťovičová lgt."; 5 ♂♂, 5 ♀♀ "Ecuador, Napo prov., road to Coca, Sumaco env., 00°43'29.0"S, 77°46'01.4"W 1109m a.s.l., 17.8.2013, confluence of two larger streams, with gravel, stones, boulders, Čiampor & Čiamporová-Zaťovičová lgt."; 5 ♂♂, 5 ♀♀ "Ecuador, Pastaza prov., Río Uklan, 01°17'13.8"S, 77°38'52.5"W 468m a.s.l., 18.8.2013, bigger river with lowland character, stream ca 15m wide, slow flowing with small riffles, with boulders, rock tables and sand, Čiampor & Čiamporová-Zaťovičová lgt.".

Diagnosis. *Hexanchorus cordillierae* can be distinguished from all species of the genus by a combination of the following male characters: 1) smaller size (CL: 2.96 – 3.16 mm); 2) mesotibiae with medial pubescent area long, reaching short of apex and lateral pubescent area long, extending to 2/3 of tibia; 3) mesotibiae with short thorn-like carina on inner apex; 4) metatibiae with feeble thorn-like carina on inner apex; 5) elytra with rounded apices; 6) fifth ventrite deeply and



Figures 6–11. *Hexanchorus habitus*: **6** *H. onorei onorei* sp. n. male **7** *H. onorei onorei* sp. n. female **8** *H. onorei sagittatus* ssp. n. male **9** *H. onorei sagittatus* ssp. n. female **10** *H. shepardi* sp. n. male **11** *H. shepardi* sp. n. female. Scale: 1 mm.

broadly emarginate; 7) aedeagus with right margin slightly dilated in middle in ventral view.

Redescription. Male. Body elongate, subparallel, dorsum moderately convex (Fig. 1). Length (CL) 2.96 – 3.16 mm; greatest width (EW) 1.12 – 1.15 mm; dorsal side dark brown with greenish iridescence; venter brown to almost black, tarsal claws reddish-brown. Dorsal surface densely covered with short recumbent setae and sparser, longer, dark, semi-erect setae; ventral surface densely covered with longer, golden, recumbent setae, especially on trochanters.

Head partly retractable into prothorax. Clypeus with anterior margin straight, about three times wider than long, shorter and narrower than labrum. Labrum feebly emarginate anteromedially, expanded laterally with sides broadly rounded, densely setose. Frontoclypeal suture visible, almost straight. Eyes suboval in lateral view, protruding from head outline, bordered by long black curved setae ("eyelashes") that arise near dorsal and ventral sides of eyes and extend toward middle of eye. Antenna moniliform, 11-segmented, pubescent; first two segments with dense long, dark brown setae, rest of antenna with only few such setae on sides; scape curved, about twice as long as pedicel, remaining segments about three times longer than first and second combined; segments 3–10 short, subtriangular; terminal segment subglobular with slightly pointed apex.

Pronotum (PL) 0.69 – 0.77 mm long, widest (PW: 0.88 – 0.92 mm) at base; with complete transversal depression at apical third and small basolateral impressions, with two prescutellar foveae; sublateral carinae absent; lateral margins convex before and after depression, basal angles slightly projected outwards; disc raised with concave sides near base; two tiny depressed dots medially near base; middle portion of base produced posteriorly; basal margin straight on sides, broadly rounded before scutellum. Scutellum subtriangular. Hypomeron narrow, straight. Prosternum extremely short in front of procoxae; prosternal process parallel-sided, apical portion subtriangular. Mesoventrite short with a deep, broad, V-shaped depression for reception of prosternal process. Metaventrite long and wide, slightly depressed along midline; discrimen thin and long, reaching abdomen. Legs slender, long. Procoxae and mesocoxae rounded, metacoxae transverse. Forelegs shortest, with all segments slightly wider than remaining pairs. Mesotibiae with medial pubescent area long, reaching before apex and lateral pubescent area long, extending to 2/3 of tibia. Mesotibiae with short thorn-like carina on inner apex, metatibiae with feeble thorn-like carina on inner apex. Tarsi simple, fourth tarsal segment with fine, nearly erect setae ventrally, fifth segment longest. Tarsal claws long and stout.

Elytra (EL) 2.28 – 2.42 mm long, widest (EW: 1.12 – 1.15 mm) across humeri; subparallel in anterior 4/5, with ten rows of small punctures forming striae; punctures separated by a distance three to four times the puncture diameter; humeral area slightly swollen. First four or five striae distinct, in nearly straight lines, remaining ones feebly visible, obscured apically. Epipleuron thin, widest in anterior third. Apical margin of elytra narrowly rounded.

Abdomen with five clearly visible ventrites (Fig. 12). Intercoxal process subtriangular with rounded apex. First three ventrites depressed medially; fifth ventrite deeply and broadly emarginate. Cuticle densely covered with short, golden, recumbent setae. Aedeagus (Figs 23, 24) elongate. Penis in ventral view narrowing from short basal apophyses towards rounded apex with right margin slightly dilated in middle, in lateral view slender, sinuate, with widened basal fourth; with corona membranous, fibula not visible, curved oblong sclerotized structure present in middle. Parameres slightly longer than half of penis, in lateral view widest at base, moderately tapering towards rounded

apex, in ventral view jointed in middle, with rounded apex. Phallobase parallel-sided, curved in lateral view. Penis and parameres with sparse fine spines.

Female. Externally similar to male (Figs 2, 13) except bigger; elytra broader with slightly produced apex; meso – and metatibiae without carina on inner apex; first three ventrites medially convex and fifth ventrite very broadly but shallowly emarginate. Females vary in size (CL: 3.25 – 3.36 mm, PL: 0.70 – 0.71 mm, PW: 0.86 – 0.95 mm, EL: 2.54 – 2.66 mm, EW: 1.14 – 1.26 mm).

Variation. We observed variation in color from dark brown to brown, size and pubescence, especially on abdominal sterna. Scale of green iridescence differed substantially.

Distribution. Until now, the species was known only from Colombia. We recorded *H. cordillierae* at two localities in the Napo Province and three localities in Pastaza Province (Fig. 36). This is the first record of *H. cordillierae* for Ecuador.

Note. We had habitus and aedaeagus photographs of the type available in this study, and were kindly provided with a redescription by Cinzia Monte, which was made based on the study of the type specimen. Based on the comparison of our specimens with the redescription of *H. cordillierae*, we have assigned the studied specimens to *H. cordillierae*.

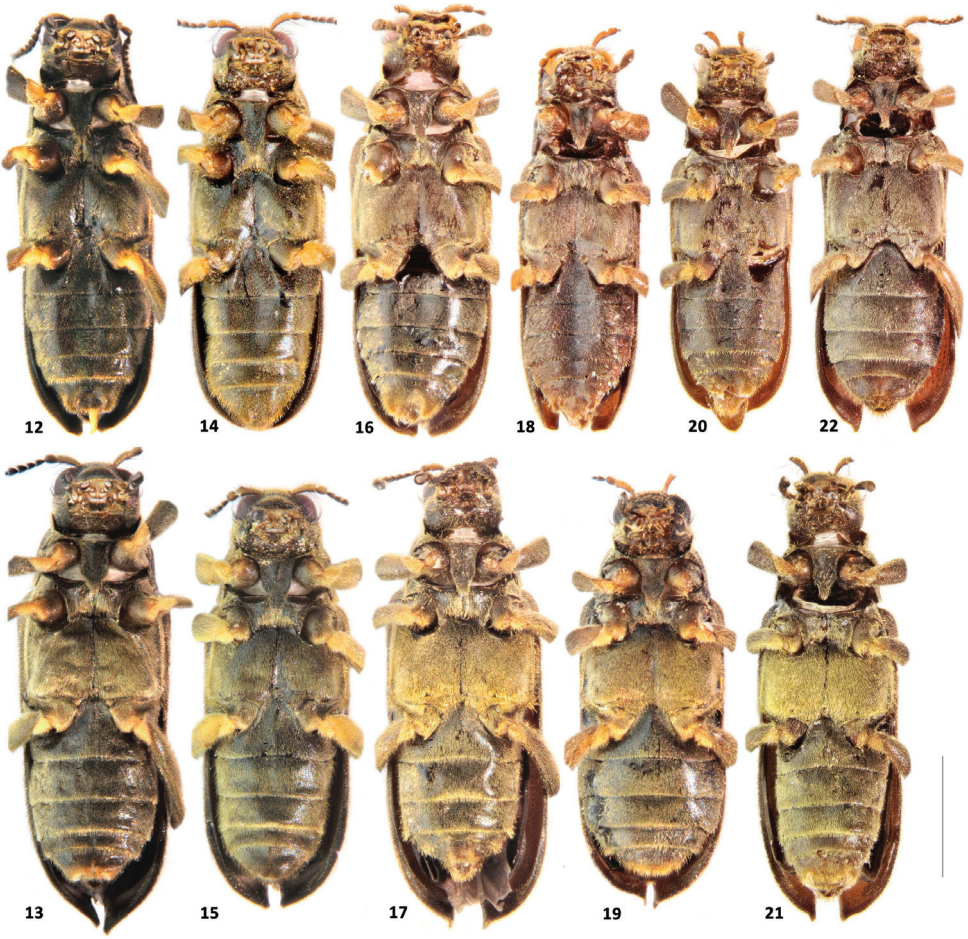
***Hexanchorus virilis* sp. n.**
http://zoobank.org/E4223A38-3093-4EB0-B4EF-C07705D555A0
Figs 3, 4, 14, 15, 25, 26, 36

Material examined. Holotype (PUCE) ♂: "Ecuador, Pastaza prov., Río Uklan, 01°17'13.8"S, 77°38'52.5"W 468m a.s.l., 18.8.2013, bigger river with lowland character, stream ca 15m wide, slow flowing with small riffles, with boulders, rock tables and sand, Čiampor & Čiamporová-Zaťovičová lgt.". **Paratypes** (PUCE, NMW, CCB): 10 ♂♂ with the same locality as holotype.

Diagnosis. *Hexanchorus virilis* sp. n. can be distinguished from all species of the genus by combination of the following male characters: 1) smaller size (CL: 2.78 – 2.97 mm); 2) protibiae apically dilated; 3) mesotibiae with medial pubescent area long, reaching to 2/4 of tibia and lateral pubescent area short, only in first fourth; 4) mesotibiae with small tubercle on inner apex; 5) metatibiae with indistinct tubercle on inner apex; 6) elytra with rounded apices; 7) fifth ventrite moderately deeply but narrowly emarginate; 8) aedeagus with slightly zagged apical portion in ventral view.

Description. Male. Body elongate, subparallel, dorsum moderately convex (Fig. 3). Length (CL) 2.78 – 2.97 mm; greatest width (EW) 1.02 – 1.07 mm, dorsal side dark brown with greenish iridescence; venter brown to almost black, tarsal claws reddish-brown. Dorsal surface densely covered with short recumbent setae and sparser, longer, dark, semi-erect setae; ventral surface densely covered with longer, golden, recumbent setae, especially on trochanters.

Head partly retractable into prothorax. Clypeus with anterior margin straight, about three times wider than long, shorter and narrower than labrum. Labrum feebly



Figures 12–22. *Hexanchorus* ventral view: **12** *H. cordillierae* male **13** *H. cordillierae* female **14**) *H. virilis* sp. n. male **15** *H.* cf *virilis* sp. n. female **16** *H. onorei onorei* sp. n. male **17** *H. onorei onorei* sp. n. female **18** *H. onorei sagittatus* ssp. n. male **19** *H. onorei sagittatus* ssp. n. female **20** *H. shepardi* sp. n. male **21** *H. shepardi* sp. n. female **22** *H. rostratus* sp. n. male. Scale: 1 mm.

emarginate anteromedially, expanded laterally with sides broadly rounded, densely setose. Frontoclypeal suture visible, almost straight. Eyes suboval in lateral view, protruding from head outline, bordered by long black curved setae ("eyelashes") that arise near dorsal and ventral sides of eyes and extend toward middle of eye. Antenna moniliform, 11-segmented, pubescent; first two segments with dense long, dark brown setae, rest of antenna with only few such setae on sides; scape curved, about twice as long as pedicel, remaining segments about three times longer than first and second combined; segments 3–10 short, subtriangular; terminal segment subglobular with slightly pointed apex. Pronotum (PL) 0.65 – 0.69 mm long, widest (PW: 0.81 – 0.83 mm) at base; with complete transversal depression at apical third and small basolateral impressions, with two prescutelar foveae; sublateral carinae absent; lateral margins convex before and

after depression, basal angles slightly projected outwards; disc raised with concave sides near base; two tiny depressed dots medially near base; middle portion of base produced posteriorly; basal margin straight on sides, broadly rounded before scutellum. Scutellum subtriangular. Hypomeron narrow, straight. Prosternum extremely short in front of procoxae; prosternal process parallel-sided, apical portion subtriangular. Mesoventrite short with a deep, broad, V-shaped depression for reception of prosternal process. Metaventrite long and wide, slightly depressed along midline; discrimen thin and long, reaching abdomen. Legs slender, long. Procoxae and mesocoxae rounded, metacoxae transverse. Forelegs shortest, with all segments slightly wider than remaining pairs. Protibiae apically widened, emarginated before apex. Mesotibiae with medial pubescent area long, reaching to 2/4 of tibia and lateral pubescent area short, only in first fourth. Mesotibiae with small tubercle on inner apex, metatibiae with small tubercle on inner apex. Tarsi simple, fourth tarsal segment with fine, nearly erect setae ventrally, fifth segment longest. Tarsal claws long and stout.

Elytra (EL) 1.91 – 2.16 mm long, widest (EW: 1.02 – 1.07 mm) across humeri; subparallel in anterior 4/5, with ten rows of small punctures forming striae; punctures separated by a distance three to four times the puncture diameter; humeral area slightly swollen. First four or five striae distinct, in nearly straight lines, remaining ones feebly visible, obscured apically. Epipleuron thin, widest in anterior third. Apical margin of elytra narrowly rounded.

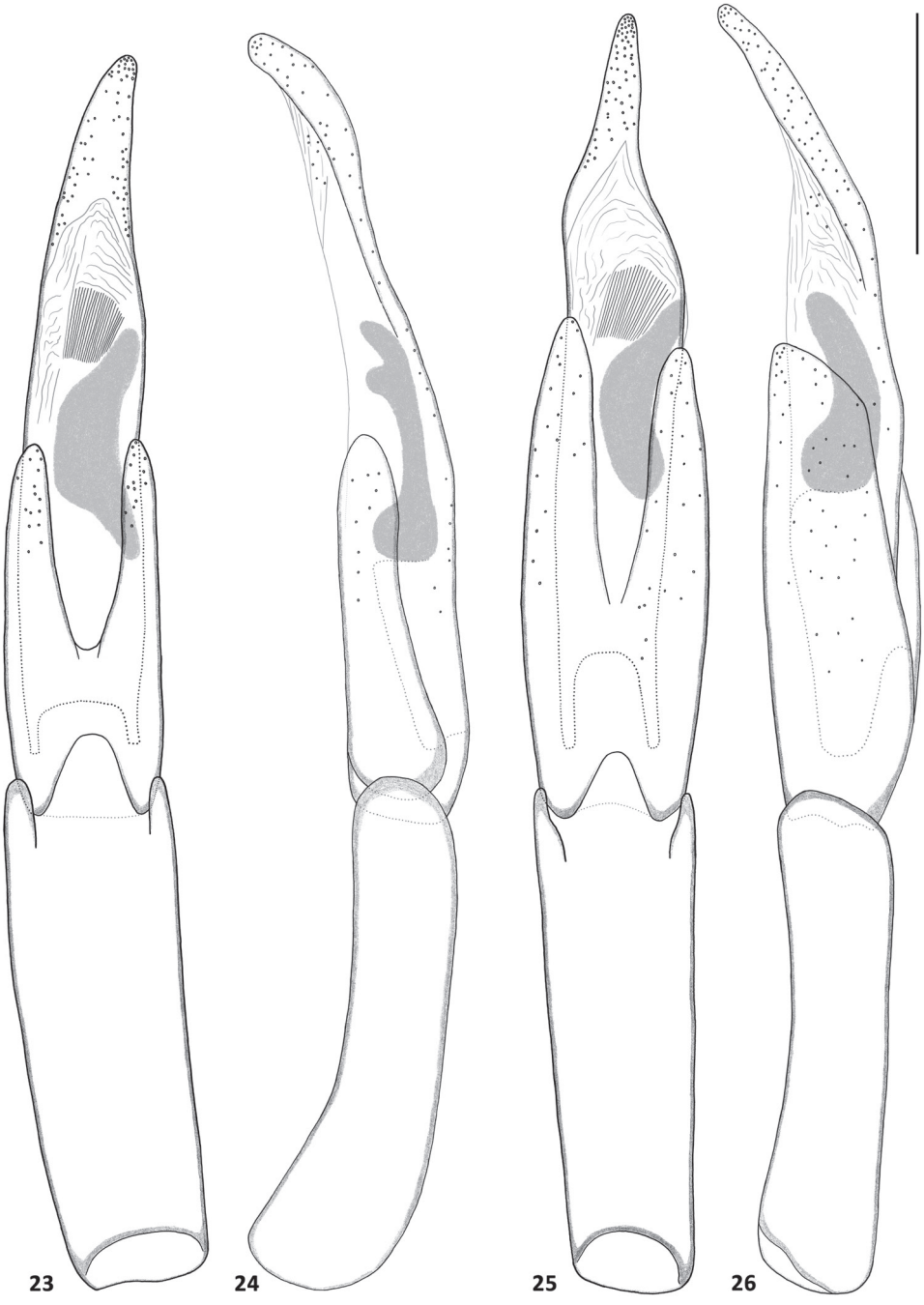
Abdomen with five clearly visible ventrites (Fig. 14). Intercoxal process subtriangular with rounded apex. First three ventrites depressed medially; fifth ventrite moderately deeply but narrowly emarginate. Cuticle densely covered with short, golden, recumbent setae. Aedeagus (Figs 25, 26) elongate. Penis in ventral view subparallel with long apophyses, apical part slightly zagged, firstly wide then strongly narrowing into long apical portion with rounded apex, in lateral view slender, sinuate, with widened basal third; with corona membranous, fibula not visible, curved oblong sclerotized structure present in middle. Parameres asymmetrical, about 1.5x shorter than penis, in lateral view subparallel, widest in middle, feebly tapering towards rounded apex, skewed on one side, in ventral view jointed in middle, with rounded apex. Phallobase parallel-sided, feebly curved in lateral view. Penis and parameres with sparse fine spines.

Female. Even females were collected at the same locality as males, we failed to get molecular data from them to confirm their conspecificity. Due to that we refrained from formal description of females and including them in the type series, but we provide their habitus photographs (Figs 4, 15).

Variation. We observed variation in size, color from dark brown to brown and pubescence, especially on abdominal sterna. Scale of green iridescence differed substantially.

Etymology. Latin, *virilis* (manly, masculine, virile), in reference to male sexual dimorphism.

Distribution. Known only from the one locality in Pastaza Province (Fig. 36).



Figures 23–26. Aedeagi of *Hexanchorus*: **23** *H. cordillierae* ventral view **24** *H. cordillierae* lateral view **25** *H. virilis* sp. n. ventral view **26** *H. virilis* sp. n. lateral view. Scale: 0.1 mm.

***Hexanchorus rostratus* sp. n.**
http://zoobank.org/FCBFC399-3D18-45D2-B0A8-5CEA797CD5DB
Figs 5, 22, 27, 28, 36

Material examined. Holotype (PUCE) ♂: "Ecuador, MoronaSantiago prov., Limón env., Río Yungantza, 02°59'49.3"S, 78°29'18.9"W 1522m a.s.l., 27.8.2013, stream ca 3m wide, fast flowing, partly shaded, with boulders, stones, gravel, Čiampor & Čiamporová-Zaťovičová lgt.". **Paratypes** (PUCE): 2 ♂♂ with the same data as holotype.

Diagnosis. *Hexanchorus rostratus* sp. n. can be distinguished from all species of the genus by combination of the following male characters: 1) bigger size (CL: 3.46 – 3.58 mm); 2) mesotibiae with medial pubescent area extremely short, only at base and lateral pubescent area short, reaching to 1/4 of tibia 3) mesotibiae with indistinct tubercle on inner apex; 4) metatibiae with indistinct tubercle on inner apex; 5) elytra with slightly acute, almost rounded apices; 6) fifth ventrite moderately deeply but narrowly emarginate; 7) aedeagus with beak-like apical portion in lateral view.

Description. Male. Body elongate, subparallel, dorsum moderately convex (Fig. 5). Length (CL) 3.46 – 3.58 mm; greatest width (EW) 1.25 – 1.32 mm, dorsal side brown with greenish iridescence; venter brown to almost black, tarsal claws reddish-brown. Dorsal surface densely covered with short recumbent setae and sparser, longer, dark, semi-erect setae; ventral surface densely covered with longer, golden, recumbent setae, especially on trochanters.

Head partly retractable into prothorax. Clypeus with anterior margin straight, about three times wider than long, shorter and narrower than labrum. Labrum feebly emarginate anteromedially, expanded laterally with sides broadly rounded, densely setose. Frontoclypeal suture visible, almost straight. Eyes suboval in lateral view, protruding from head outline, bordered by long black curved setae ("eyelashes") that arise near dorsal and ventral sides of eyes and extend toward middle of eye. Antenna moniliform, 11-segmented, pubescent; first two segments with dense long, dark brown setae, rest of antenna with only few such setae on sides; scape curved, about twice as long as pedicel, remaining segments about three times longer than first and second combined; segments 3–10 short, subtriangular; terminal segment subglobular with slightly pointed apex.

Pronotum (PL) 0.77 – 0.85 mm long, widest (PW: 0.96 – 1.03 mm) at base; with complete transversal depression at apical third and small basolateral impressions, with two prescutelar foveae; sublateral carinae absent; lateral margins convex before and after depression, basal angles slightly projected outwards; disc raised with concave sides near base; two tiny depressed dots medially near base; middle portion of base produced posteriorly; basal margin straight on sides, broadly rounded before scutellum. Scutellum subtriangular. Hypomeron narrow, straight. Prosternum extremely short in front of procoxae; prosternal process parallel-sided, apical portion subtriangular. Mesoventrite short with a deep, broad, V-shaped depression for reception of prosternal process. Metaventrite long and wide, slightly depressed along midline; discrimen thin and long,

reaching abdomen. Legs slender,long. Procoxae and mesocoxae rounded, metacoxae transverse. Forelegs shortest, with all segments slightly wider than remaining pairs. Mesotibiae with medial pubescent area extremely short, only at base and lateral pubescent area short, reaching to 1/4 of tibia. Mesotibiae and metatibiae with indistinct tubercle on inner apex. Tarsi simple, fourth tarsal segment with fine, nearly erect setae ventrally, fifth segment longest. Tarsal claws long and stout.

Elytra (EL) 2.69 – 2.73 mm long, widest (EW: 1.25 – 1.32 mm) across humeri; with ten rows of small punctures forming striae; punctures separated by a distance three to four times the puncture diameter; humeral area slightly swollen. First four or five striae distinct, in nearly straight lines, remaining ones feebly visible, obscured apically. Epipleuron thin, widest in anterior third. Apical margin of elytra acutely produced.

Abdomen with five clearly visible ventrites (Fig. 22). Intercoxal process subtriangular with rounded apex. First three ventrites depressed medially; fifth ventrite moderately deeply but narrowly emarginate. Cuticle densely covered with short, golden, recumbent setae. Aedeagus (Figs 27, 28). elongate. Penis in ventral subparallel with distinct apophyses, narrowest in middle, with rounded apex, in lateral with subglobular apex skewed from below, strongly constricted then widened in basal half; with corona membranous, fibula not visible, straight oblong sclerotized structure present in apical half. Parameres about half as long as penis, in lateral view widest in basal half, tapering towards rounded apex, in ventral view with thin rounded apex, distinctly widening in apical half; Phallobase long, parallel-sided, curved in lateral view. Penis and parameres with sparse fine spines.

Female. Unknown.

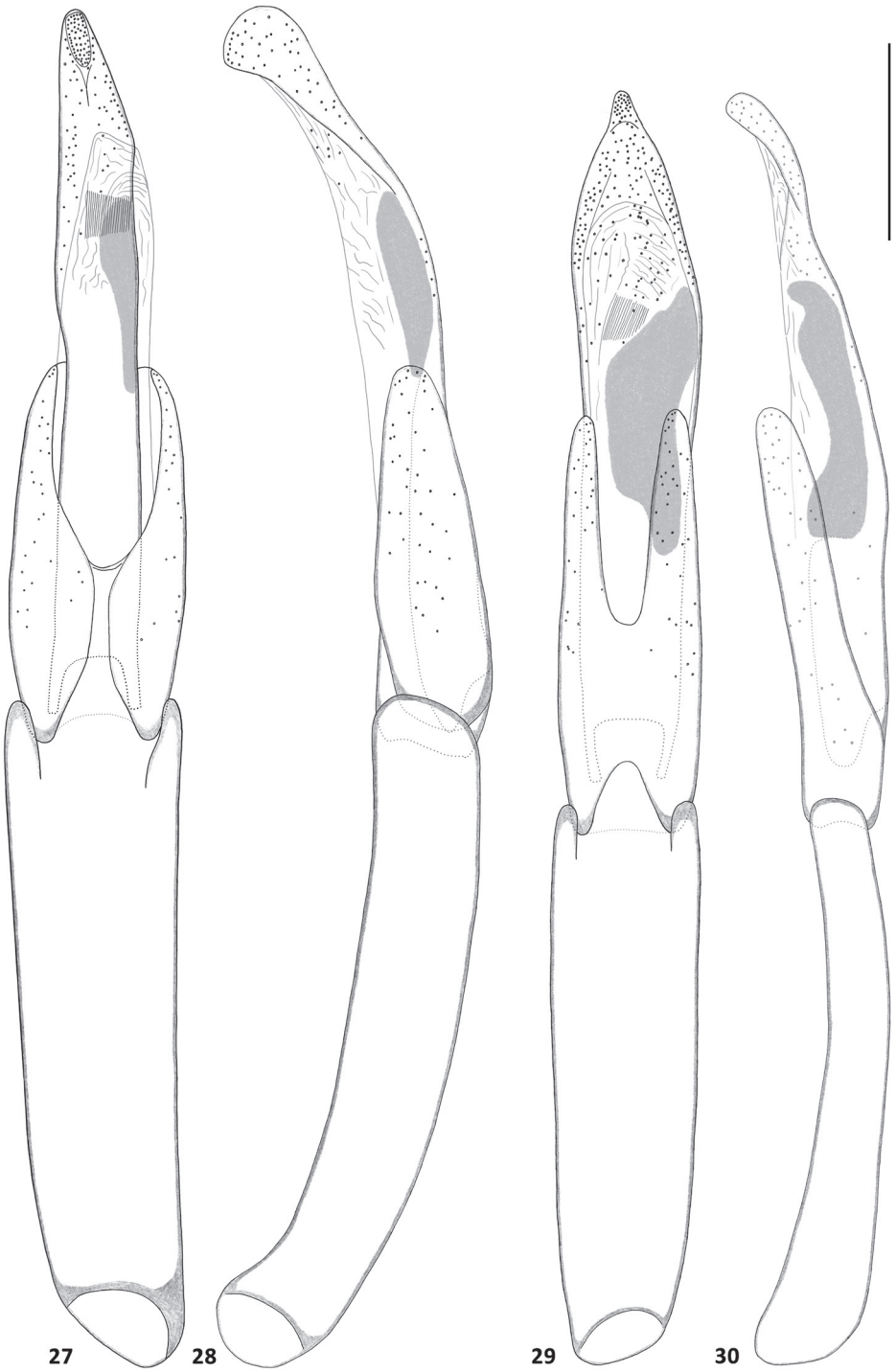
Variation. We observed variation in size and pubescence, especially on abdominal sterna. Scale of green iridescence differed substantially.

Etymology. Latin, *rostrātus* (beak-shaped), in reference to the apical part of penis in lateral view that resembles an upper beak of some birds.

Distribution. Known only from the one locality in Morona-Santiago Province (Fig. 36).

***Hexanchorus onorei onorei* ssp. n.**
http://zoobank.org/BF0BB8DA-D2DB-4C16-ABEE-F46AD8648913
Figs 6, 7, 16, 17, 31, 32, 36

Material examined. Holotype (PUCE) ♂: "Ecuador, Morona-Santiago prov., Indanza env., Río Crusado, 03°02'55.0"S, 78°30'03.5"W 972m a.s.l., 24.8.2013, stream ca 5m wide, fast flowing with rapids, in forest, with gravel, boulders, Čiampor & Čiamporová-Zaťovičová lgt." **Paratypes** (PUCE, NMW, CCB): 1 ♂, 2 ♀♀ with the same data as holotype; 2 ♂♂ "Ecuador, Morona-Santiago prov., Río Indanza, Indanza env., 03°04'09.3"S, 78°28'07.9"W 772m a.s.l., 28.8.2013, at light, Čiampor & Čiamporová-Zaťovičová lgt.".



Figures 27–30. Aedeagi of *Hexanchorus*: **27** *H. rostratus* sp. n. ventral view **28** *H. rostratus* sp. n. lateral view **29** *H. shepardi* sp. n. ventral view **30**) *H. shepardi* sp. n. lateral view. Scale: 0.1 mm.

Diagnosis. *Hexanchorus onorei onorei* sp. n. can be distinguished from all species of the genus by combination of the following male characters: 1) bigger size (CL: 3.44 – 3.57 mm); 2) mesotibiae with medial pubescent area through entire tibia and lateral pubescent area reaching half of tibia; 3) mesotibiae with small tubercle on inner apex; 4) metatibiae with indistinct tubercle on inner apex; 5) elytra with slightly acute, almost rounded apices; 6) fifth ventrite deeply and broadly emarginate; 7) aedeagus broad with protruded apex in ventral view.

Description. Male. Body elongate, subparallel, dorsum moderately convex (Fig. 6). Length (CL) 3.44 – 3.57 mm; greatest width (EW) 1.28 – 1.36 mm, dorsal side dark brown with greenish iridescence; venter brown to almost black, tarsal claws reddish-brown. Dorsal surface densely covered with short recumbent setae and sparser, longer, dark, semi-erect setae; ventral surface densely covered with longer, golden, recumbent setae, especially on trochanters.

Head partly retractable into prothorax. Clypeus with anterior margin straight, about three times wider than long, shorter and narrower than labrum. Labrum feebly emarginated anteromedially, expanded laterally with sides broadly rounded, densely setose. Frontoclypeal suture visible, almost straight. Eyes suboval in lateral view, protruding from head outline, bordered by long black curved setae ("eyelashes") that arise near dorsal and ventral sides of eyes and extend toward middle of eye. Antenna moniliform, 11-segmented, pubescent; first two segments with dense long, dark brown setae, rest of antenna with only few such setae on sides; scape curved, about twice as long as pedicel, remaining segments about three times longer than first and second combined; segments 3–10 short, subtriangular; terminal segment subglobular with slightly pointed apex.

Pronotum (PL) 0.82 – 0.85 mm long, widest (PW: 1.04 – 1.09 mm) at base; with complete transversal depression at apical third and small basolateral impressions, with two prescutelar foveae; sublateral carinae absent; lateral margins convex before and after depression, basal angles slightly projected outwards; disc raised with concave sides near base; two tiny depressed dots medially near base; middle portion of base produced posteriorly; basal margin straight on sides, broadly rounded before scutellum. Scutellum subtriangular. Hypomeron narrow, straight. Prosternum extremely short in front of procoxae; prosternal process parallel-sided, apical portion subtriangular. Mesoventrite short with a deep, broad, V-shaped depression for reception of prosternal process. Metaventrite long and wideslightly depressed along midline; discrimen thin and long, reaching abdomen. Legs slender, long. Procoxae and mesocoxae rounded, metacoxae transverse. Forelegs shortest, with all segments slightly wider than remaining pairs. Mesotibiae with medial pubescent area through entire tibia and lateral pubescent area reaching to half. Mesotibiae with small tubercle on inner apex, metatibiae with indistinct tubercle on inner apex. Tarsi simple, fourth tarsal segment with fine, nearly erect setae ventrally, fifth segment longest. Tarsal claws long and stout.

Elytra (EL) 2.63 – 2.72 mm long, widest (EW: 1.28 – 1.36 mm) across humeri; subparallel in anterior 4/5, with ten rows of small punctures forming striae; punctures separated by a distance three to four times the puncture diameter; humeral area slightly

swollen. First four or five striae distinct, in nearly straight lines, remaining ones feebly visible, obscured apically. Epipleuron thin, widest in anterior third. Apical margin of elytra narrowly rounded.

Abdomen with five clearly visible ventrites (Fig. 16). Intercoxal process subtriangular with rounded apex. First three ventrites depressed medially; fifth ventrite deeply and broadly emarginate. Cuticle densely covered with short, golden, recumbent setae. Aedeagus (Figs 31, 32) elongate. Penis in ventral view subparallel with short apophyses, apical part narrowing towards protruded rounded apex, in lateral view slender, sinuate, with widened basal third, with corona membranous, fibula not visible, curved oblong sclerotized structure present in middle. Parameres about 1.5 times shorter than penis, in lateral view widest at base, tapering towards rounded apex, in ventral view jointed in middle, with rounded apex. Phallobase parallel-sided, curved in lateral view. Penis and parameres with fine, sparse spines.

Female. Externally similar to male (Figs 7, 17) except bigger (CL: 3.83 – 3.88 mm); elytra with pointed and vertically curved apices; meso – and metatibiae without carina on inner apex; first three ventrites medially convex and fifth ventrite only feebly emarginate. Females vary in size (PL: 0.75 – 0.77 mm, PW: 1.10 – 1.12 mm, EL: 3.07 – 3.11 mm, EW: 1.40 – 1.43 mm).

Variation. We observed variation in color from dark brown to brown, size and pubescence, especially on abdominal sterna. Scale of green iridescence differed substantially.

Etymology. This species is named after our friend, Prof. Giovanni Onore, President of the Otonga Foundation, to express our gratitude for his altruistic help and support for research of Elmidae fauna of Ecuador.

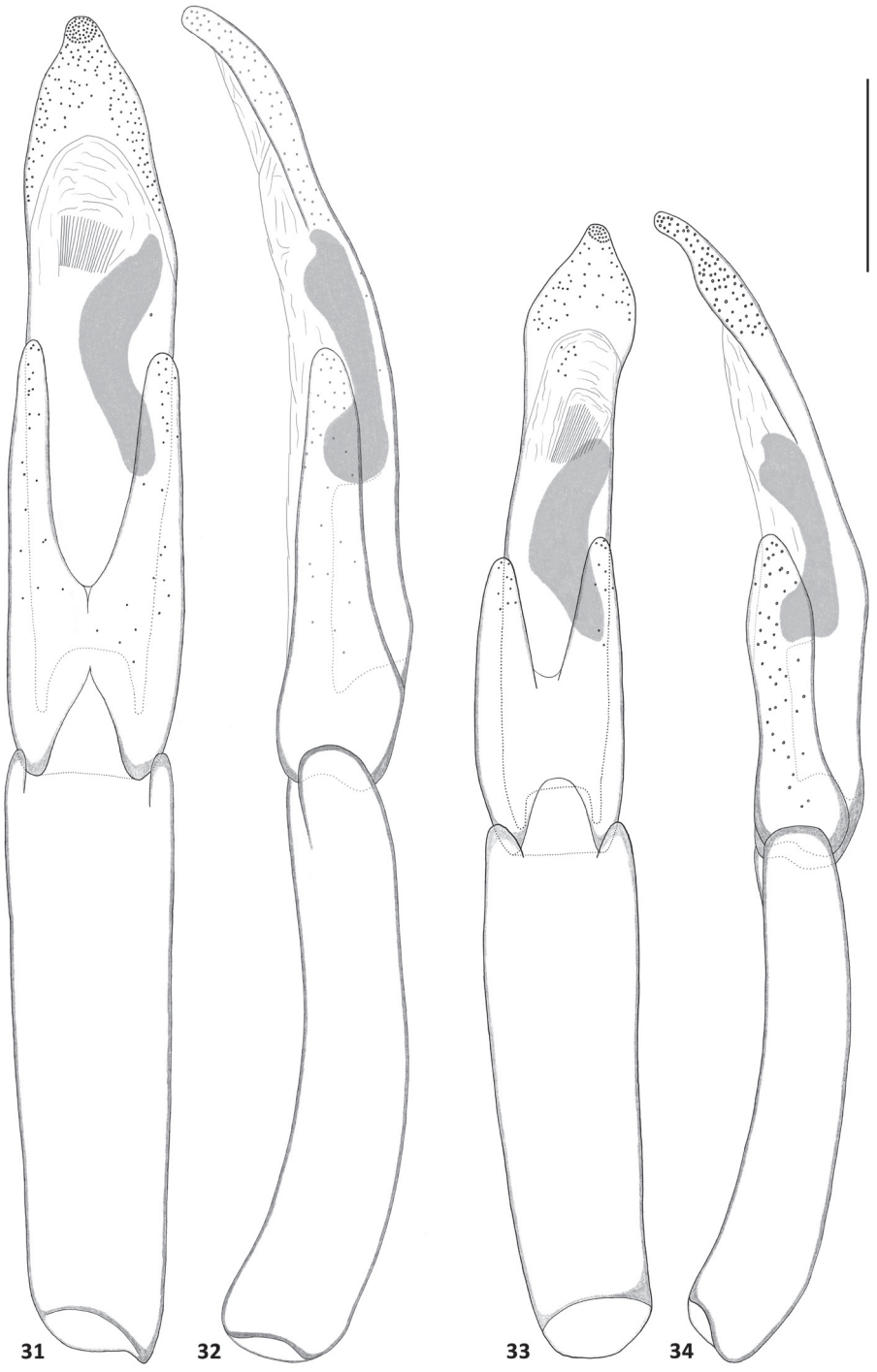
Distribution. Known from the two localities in Morona-Santiago Province (Fig. 36).

***Hexanchorus onorei sagittatus* ssp. n.**
http://zoobank.org/EEDA9052-48D5-49C7-909A-854C020F9765
Figs 8, 9, 18, 19, 33, 34, 36

Material examined. Holotype (PUCE) ♂: "Ecuador, Morona-Santiago prov., Río Indanza, Indanza env., 03°04'09.3"S, 78°28'07.9"W 772m a.s.l., 28.8.2013, at light, Čiampor & Čiamporová-Zaťovičová lgt.". **Paratypes** (PUCE, NMW, CCB): 1 ♂, 5 ♀♀ with the same data as holotype.

Diagnosis. *Hexanchorus onorei sagittatus* ssp. n. (Figs 8, 18) is externally similar to *Hexanchorus onorei onorei* sp. n. but can be distinguished by combination of the following male characters: 1) smaller size (CL: 3.22 – 3.25 mm vs 3.44 – 3.57 mm); 2) fifth ventrite distinctly wider emarginate; 3) aedeagus arrow-like in ventral view.

Description. Male. Aedeagus (Figs 33, 34) elongate. Penis in ventral view arrow-like, mostly subparallel with short apophyse, tapering towards apex, then widened into subglobular portion with protruded, rounded apex, in lateral view with apex emarginate, then slender, sinuate, with widened basal third, with corona membranous, fibula not visible, curved oblong sclerotized structure present in middle. Parameres slightly asymmetrical,



Figures 31–34. Aedeagi of *Hexanchorus*: **31** *H. onorei onorei* sp. n. ventral view **32** *H. onorei onorei* sp. n. lateral view **33** *H. onorei sagittatus* ssp. n. ventral view **34** *H. onorei sagittatus* ssp. n. lateral view. Scale: 0.1 mm.

about half as long as penis, in lateral view widest at base, narrowest in middle, apical part with rounded apex, in ventral view jointed in middle, with rounded apex. Phallobase parallel-sided, curved in lateral view. Penis and parameres with sparse fine spines.

Female. Externally similar to male (Figs 9, 19) except bigger (CL: 3.65 – 3.68 mm); elytra with pointed and vertically curved apices; meso – and metatibiae without carina on inner apex; first three ventrites medially convex and fifth ventrite only feebly emarginated. Females vary in size (PL: PL: 0.76 – 0.78 mm, PW: 1.07 – 1.09 mm, EL: 2.88 – 2.90 mm, EW: 1.35 – 1.37 mm).

Variation. We observed variation in size and pubescence, especially on abdominal sterna. Scale of green iridescence differed substantially.

Etymology. Latin, *sagittātus* (formed like arrow), in reference to its arrow-like shape of penis.

Distribution. Known only from the one locality in Morona-Santiago Province (Fig. 36).

***Hexanchorus shepardi* sp. n.**
http://zoobank.org/F8C4E2E8-15F5-4588-B249-F68DF2FD2A0D
Figs 10, 11, 20, 21, 29, 30, 36

Material examined. Holotype (PUCE) ♂: "Ecuador, Napo prov., road to Coca, Sumaco env., 00°42'25.7"S, 77°43'10.0"W 1138m a.s.l., 17.8.2013, stream ca 2–3 m wide, fast flowing, with boulders, stones, gravel, submerged wood, Čiampor & Čiamporová-Zaťovičová lgt." **Paratypes** (PUCE, NMW, CCB): 1 ♂, 5 ♀♀ with the same data as holotype.

Diagnosis. *Hexanchorus shepardi* sp. n. can be distinguished from all species of the genus by combination of the following male characters: 1) moderate size (CL: 3.22 – 3.36 mm); 2) mesotibiae with medial pubescent area long, extending before apex and lateral pubescent area shorter reaching behind first third; 3) mesotibiae with small tubercle on inner apex; 4) metatibiae with indistinct tubercle on inner apex; 5) elytra with slightly acute, almost rounded apices; 6) fifth ventrite deeply and broadly emarginate; 7) aedeagus with ovate apical portion in ventral view.

Description. Male. Body elongate, subparallel, dorsum moderately convex (Fig. 10). Length (CL) 3.22 – 3.36 mm; greatest width (EW) 1.31 – 1.37 mm, dorsal side dark brown with greenish iridescence; venter brown to almost black, tarsal claws reddish-brown. Dorsal surface densely covered with short recumbent setae and sparser, longer, dark, semi-erect setae; ventral surface densely covered with longer, golden, recumbent setae, especially on trochanters.

Head partly retractable into prothorax. Clypeus with anterior margin straight, about three times wider than long, shorter and narrower than labrum. Labrum feebly emarginated anteromedially, expanded laterally with sides broadly rounded, densely setose. Frontoclypeal suture visible, almost straight. Eyes suboval in lateral view, protruding from head outline, bordered by long black curved setae ("eyelashes") that arise near

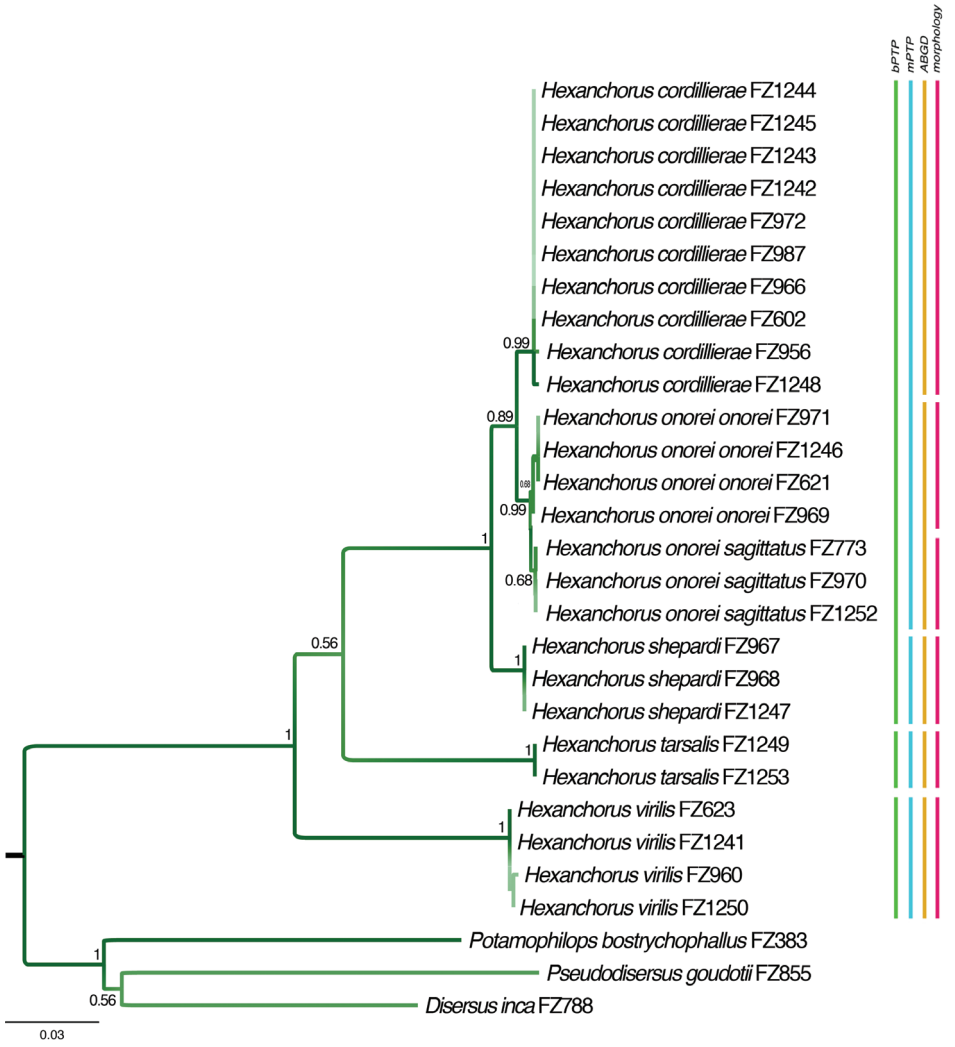


Figure 35. Maximum likelihood analysis tree based on 625 bp barcoding fragment of COI with species delimitation according to different approaches used. Numbers next to branches represent Bootstrap support (ML).

dorsal and ventral sides of eyes and extend toward middle of eye. Antenna moniliform, 11-segmented, pubescent; first two segments with dense long, dark brown setae, rest of antenna with only few such setae on sides; scape curved, about twice as long as pedicel, remaining segments about three times longer than first and second combined; segments 3–10 short, subtriangular; terminal segment subglobular with slightly pointed apex.

Pronotum (PL) 0.83 – 0.87 mm long, widest (PW: 1.07 – 1.08 mm) at base; with complete transversal depression at apical third and small basolateral impressions, with two prescutelar foveae; sublateral carinae absent; lateral margins convex before and after depression, basal angles slightly projected outwards; disc raised with concave

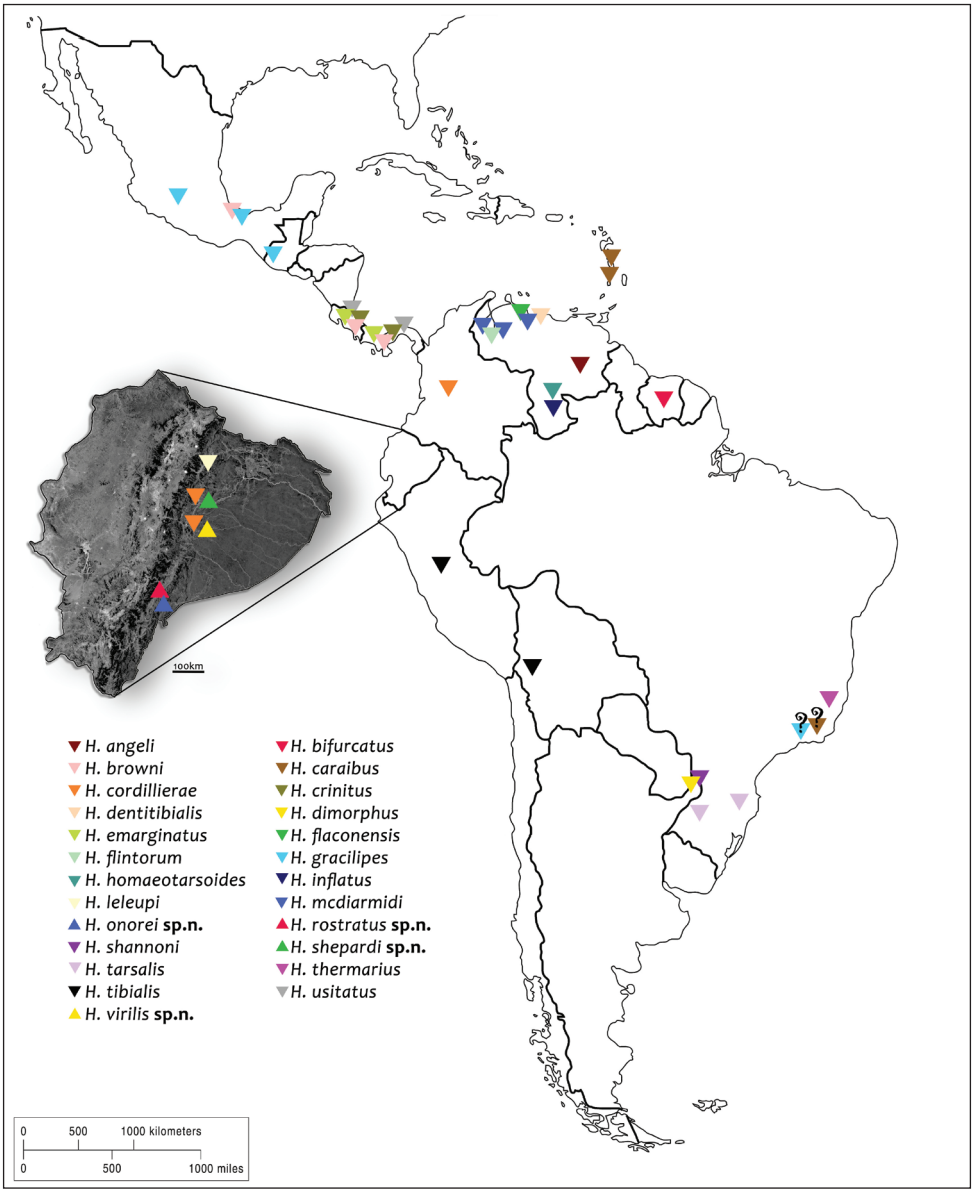


Figure 36. Distribution map of all known *Hexanchorus* species. (top down triangle – known species, top up triangle – new species, question mark – doubtful distribution).

sides near base; two tiny depressed dots medially near base; middle portion of base produced posteriorly; basal margin straight on sides, broadly rounded before scutellum. Scutellum subtriangular. Hypomeron narrow, straight. Prosternum extremely short in front of procoxae; prosternal process parallel-sided, apical portion subtriangular. Mesoventrite short with a deep, broad, V-shaped depression for reception of prosternal process. Metaventrite long and wide, slightly depressed along midline;

discrimen thin and long, reaching abdomen. Legs slender, long. Procoxae and mesocoxae rounded, metacoxae transverse. Forelegs shortest, with all segments slightly wider than remaining pairs. Mesotibiae with medial pubescent area long, extending before apex and lateral pubescent area shorter reaching behind first third. Mesotibiae with small tubercle on inner apex, metatibiae with indistinct tubercle on inner apex. Tarsi simple, fourth tarsal segment with fine, nearly erect setae ventrally, fifth segment longest. Tarsal claws long and stout.

Elytra (EL) 2.42 – 2.63 mm long, widest (EW: 1.31 – 1.37 mm) across humeri; subparallel in anterior 4/5, with ten rows of small punctures forming striae; punctures separated by a distance three to four times the puncture diameter; humeral area slightly swollen. First four or five striae distinct, in nearly straight lines, remaining ones feebly visible, obscured apically. Epipleuron thin, widest in anterior third. Apical margin of elytra acutely produced.

Abdomen with five clearly visible ventrites (Fig. 20). Intercoxal process subtriangular with rounded apex. First three ventrites depressed medially; fifth ventrite deeply and broadly emarginate. Cuticle densely covered with short, golden, recumbent setae. Aedeagus (Figs 29, 30) elongate. Penis in ventral view subparallel with short apophyses, apical part ovate with rounded apex, in lateral view slender, sinuate, with widened basal third, with corona membranous, fibula not visible, curved oblong sclerotized structure present in middle. Parameres about 1.7x shorter than penis, in lateral view widest at base, moderately tapering towards rounded apex, in ventral view jointed in middle, with rounded apex. Phallobase parallel-sided, in later view curved and slender. Penis and parameres with sparse fine spines.

Female. Externally similar to male (Figs 11, 21) except bigger (CL: 3.58 – 3.62 mm); meso – and metatibiae without carina on inner apex; first three ventrites medially convex and fifth ventrite very broadly but shallowly emarginate. Females vary only slightly in size (PL: 0.77 – 0.78 mm, PW: 1.00 – 1.01 mm, EL: 2.81 – 2.84 mm, EW: 1.24 – 1.27 mm).

Variation. We observed variation in size and in pubescence, especially on abdominal sterna, was observed. Scale of green iridescence differed substantially.

Etymology. The species is named after Prof. William D. Shepard, great American coleopterologist and expert on dryopoid beetles.

Distribution. Known only from the one locality in Napo Province (Fig. 36).

Discussion

Ecuador is one of the richest countries in the world, in regard to its biodiversity. Here we focused on the riffle beetle genus *Hexanchorus*. Although we analysed the material from a relatively small area, the results clearly demonstrate that the diversity of the genus is almost certainly much higher than it would appear based on the previous knowledge. With its 25 species, *Hexanchorus* is the most diverse Larainae genus in Latin America, forming an important part of the Elmidae fauna in the region.

Most of the *Hexanchorus* species are very similar concerning their external morphology and usually it is very hard to identify species without examining male genitalia. Moreover, different species sometimes inhabit the same stream or are collected together at light, which makes assigning females to the species difficult. Hence we also employed molecular data (DNA barcoding), which has proved very useful for Elmidae taxonomy in previous studies (e.g. Čiampor Jr et al. 2013, 2016, 2017) and allowed for the inclusion of females in the type series in most of the described species.

The description of subspecies in Elmidae genera is usually based on subtle morphological differences between geographically isolated populations (e.g. Jeng and Yang 1993; Jäch 1994). The two subspecies of *H. onorei* sp. n. were collected at the same locality. They clearly differ in the morphology of the male genitalia, but due to the small genetic distance they most probably represent separate lineages of the same species occuring sympatrically after a short period of isolation. However, subspecies designation could be useful not only for allopatric populations, but also in situations where secondary contact between distinct populations has occurred (Monroe 1982). The latter could be the case of the subspecies of *H. onorei*.

Regarding Ecuador, only *H. leleupi* Delève, 1968 was known from this country until now (Monte and Mascagni 2012). This species was collected at high altitudes (3300 m a.s.l.), which contrasts with other species occurring mostly up to 1500 m a.s.l. (Spangler and Santiago-Fragoso 1992 and our own observation).

Based on the limited literature sources (Guérin-Méneville 1843, Coquerel 1851, Hinton 1935, Hinton 1937, Delève 1968, Spangler and Santiago-Fragoso 1992, Spangler and Staines 2004, Maier 2013, Maier and Short 2014, Passos et al. 2010, Shepard and Chaboo 2015,) and our data, we illustrated the current distribution of all *Hexanchorus* species (Fig. 36). Specimens of the genus can be found also in El Salvador (Gutiérrez-Fonseca 2010), possibly in Chile (Vera Solís 2012) and certainly in several other countries, but we used only published species distribution data. Records of two species (*H. caraibus*, *H. gracilipes*) from Rio de Janeiro State, Brazil (Passos et al. 2010) are clearly too distant from their known distribution area including the type locality, and due to potentially erroneous determination these data were not considered. The distributional data showed that *Hexanchorus* is widely distributed from as far north as Mexico to southern Brazil. Most species are concentrated in Central America, while southern regions, including Peru, Bolivia, Paraguay or Argentina are covered by a single species records only. This indicates that for a comprehensive survey of the *Hexanchorus* diversity and distribution, intensive exploration of mainly southern areas would be required.

The revision of *Hexanchorus* material from a few localities in Ecuador and summary of published information clearly show that we still know very little about this genus. The differences in molecular distances among species and its incongruence with morphological differences in some cases highlight the importance of using DNA barcodes, because if combined, the morphological and molecular data improve significantly the robustness of the proposed taxonomy of Elmidae genera. Further research is greatly needed, employing conventional and modern techniques to better understand the true diversity of the Neotropic riffle beetles.

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Supplementary material 1

Table S1. Genetic distance among specimens and species (Kimura 2-parameter distance) using 625bp fragment of COI gene

Authors: Marek Linský, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: Genetic distances

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RESEARCH ARTICLE

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A new soil centipede from South-East Asia with a unique arrangement of ventral glands, and a revised synopsis of Gonibregmatidae (Chilopoda, Geophilomorpha)

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Abstract

A new gonibregmatid centipede, *Vinaphilus unicus* **gen. n.**, **sp. n.**, is described based on two females from a single location in northern Vietnam. The new genus and species are distinguished mainly by the arrangement of the ventral pore-fields, which is unique among all Chilopoda. A critically revised synopsis of the Gonibregmatidae is also given. In particular, three species are provisionally recognized in *Himatosoma* Pocock, 1891: *H. bidivisum* Silvestri, 1919, *H. porosum* Pocock, 1891 (= *H. typicum tridivisum* Silvestri, 1919, **syn. n.**), and *H. typicum* Pocock, 1891. The genera *Dschangelophilus* Verhoeff, 1937 and *Tweediphilus* Verhoeff, 1937, with their species *D. coloratus* Verhoeff, 1937 and *T. malaccanus* Verhoeff, 1937, are moved to the Gonibregmatidae, whereas *Geoporophilus aporus* Attems, 1930 is moved to the Oryidae as *Orphnaeus aporus* (Attems, 1930), **comb. n.**

Keywords

Chilopoda, Gonibregmatidae, new genus, new species, ventral pore-fields, Vietnam

Introduction

Gonibregmatids are frequent members of the centipede communities inhabiting the soils throughout southern Asia (Lewis 1981, Bonato and Zapparoli 2011). They are among the largest and most elongated geophilomorphs, often more than 10 cm long when adults (Bonato 2011). Because of their conspicuous body size, gonibregmatids were among the first centipedes reported from tropical regions (since Newport 1843a, 1843b). Nevertheless, they are still one of the least known centipede groups, even with regards to morphology and species diversity. Moreover, they have been one of the first lineages of geophilomorphs to be distinguished at the family level (Cook 1896), yet researchers are still far from reaching a consensus on the circumscription and diagnosis of Gonibregmatidae (compare e.g. Attems 1929; Bonato 2011; Bonato et al. 2014).

Until now, less than 20 species have been referred to Gonibregmatidae, almost all of them from southern Asia (see Appendix). Most species belong to three morphologically well characterised genera: *Eucratonyx* Pocock, 1899, the best-known genus, with two species; *Gonibregmatus* Newport, 1843, comprising six poorly described and rarely recorded species, of which four are from southern Asia; *Himantosoma* Pocock, 1891, for which up to four nominal taxa have been distinguished and illustrated. A few other incompletely described species of gonibregmatids have been separated in other nominal genera: *Disargus* Cook, 1896, *Geoporophilus* Silvestri, 1919, and *Sogophagus* Chamberlin, 1912.

Recent field sampling in northern Vietnam allowed us to discover a new species that adds significantly to the known morphological diversity of Gonibregmatidae and of Chilopoda as a whole, especially for the unprecedented arrangement of the pores of the sternal glands. The peculiar morphological features of the new species are here described, and its taxonomic position is discussed in the framework of an updated synopsis of the Gonibregmatidae.

Methods

Specimens were collected by manual search and fixed in 70% ethanol. Morphological examination was performed with a stereoscopic microscope (Leica MZ 125) with magnifications 8–100× and with a biological microscope (Leica DMLB) with magnifications 100–400×, by means of temporary mounts in ethylene glycol and also after dissection of the head and the mouth parts (Pereira 2000). Light photographs were taken with digital cameras applied to the two microscopes (Leica EC3 and DFC420, respectively). Line drawings were drawn from the digital photos. For the morphological description, we followed the standard terminology for centipedes as defined in Bonato et al. (2010).

In order to evaluate the taxonomic position of the new species, we browsed the primary taxonomic and faunistic literature to collate all available morphological information and geographical records for all species either referred or possibly related to Gonibregmatidae. Morphological data were critically revised, and published records were re-interpreted providing modern geographical names.

Results

Two specimens were found as representatives of a new species of Gonibregmatidae, which deserves to be distinguished in a new genus. It is described below as *Vinaphilus unicus* gen. n. sp. n.

Vinaphilus gen. n.

http://zoobank.org/6BD441E1-3AB8-47FB-BD63-B6DA3717C02E

Diagnosis. Geophilomorpha differing from all other known genera for the combination of the following characters: both anterior and posterior ends relatively stout; head and forcipular segment quite short; all appendages relatively short; club-like sensilla on multiple distal antennal articles; labrum with broad mid-part separating the lateral pieces; labral margin slightly concave and fringed with elongate, pointed projections; mandible with a single row of many short teeth; telopodite of second maxillae composed of three articles and bearing a claw provided with two marginal rows of thin projections; forcipular tergite wider than long, and as wide as the head; forcipules without denticles; no paratergites; all walking legs with similar claws; sternites with pore-fields, including two paired anterior fields and a single medial posterior field; ultimate leg-bearing segment with entire pleuropretergite; coxal organs opening separately through many pores, most of which aggregated on the meso-ventral side; legs of the ultimate pair much longer than those of the penultimate pair, with two tarsal articles and without claw; female gonopods basally touching, short and non-articulate.

The main diagnostic differences between *Vinaphilus* gen. n. and the other genera of Gonibregmatidae from Asia are summarized in Table 1 and discussed below in Discussion.

Type species. *Vinaphilus unicus* sp. n.

Table 1. Main differences between *Vinaphilus* gen. n. and all genera of Gonibregmatidae, or possibly belonging to Gonibregmatidae, reported from Asia (see Appendix).

Nominal genus	Type species	Labrum	Second maxillae	Anterior part of trunk			Ultimate pair of legs		
		Narrow, convex mid-part	Filaments on pretarsus	Pincer-like claws	Paratergites	Posterior circular pore-field	Aggregated coxal pores	Distinctly elongate leg	Claw
Vinaphilus gen. n.	*Vinaphilus unicus* sp. n.	–	+	–	–	+	+	+	–
Disargus Cook, 1896	*Himantarium striatum*, by original designation	?	?	?	?	–	–/+	–	+
Dschangelophilus Verhoeff, 1937	*Dschangelophilus coloratus*, by monotypy	–	+	?	–	–	–	–	+
Eucratonyx Pocock, 1899	*Himantarium meinerti*, by original designation	–	+	+	–	–	–	+	–
Geoporophilus Silvestri, 1919	*Geoporophilus angustus*, by original designation	–	+	?	–	–	–/+	+	–?
Gonibregmatus Newport, 1843	*Gonibregmatus cumingii*, by monotypy	+	–	–	+	–	–	+/–	–
Himantosoma Pocock, 1891	*Himantosoma typicum*, by original designation	–	+	–	–	–	–/+	–	+
Luangana Attems, 1953	*Luangana varians,* by monotypy	–	+	?	?	–	–	?	–
Sogophagus Chamberlin, 1912	*Geophagus serangodes*, by direct substitution	–	+	+	?	–	–	+	–
Tweediphilus Verhoeff, 1937	*Tweediphilus malaccanus*, by monotypy	–	+	?	–	–	–	?	–

Name derivation. From "Vina", an alternative name of Vietnam, and the suffix *-philus*, which is frequently used in names of genera of Geophilomorpha. Gender: masculine.

***Vinaphilus unicus* sp. n.**
http://zoobank.org/E9E2B7EC-DB0F-4F51-ACA8-27C1F78F1144
Figs 1–3

Material examined. *Holotype*: IEBR-Chi 001, ♀, 65 mm long, with 109 leg-bearing segments, with developed gonopods; in ethanol 70%; collected by Anh D. Nguyen, 11–19 September 2016, originally labelled ML01a; originally entire, subsequently divided into four pieces (cephalic capsule with right mandible and part of maxillae; left mandible; left half of the second maxillae; trunk); in the Department of Zoological Museum, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

Paratype: PD-G 9530, ♀, 90 mm long, with 109 leg-bearing segments, with developed gonopods; in ethanol 70%; collected together with the holotype, same date and locality, originally labelled ML01a1; entire; in the Department of Biology, University of Padova.

Type locality. Vietnam: Vinh Phuc province: Ngoc Thanh commune: Me Linh Station for Biodiversity: 21°23'42"N, 105°42'48"E; 150 m a.s.l.; secondary forest.

Diagnosis. A *Vinaphilus* species with the following characters: body length up to > 8 cm; head about as long as wide, lacking transverse suture; antenna about 3 times as long as the head, with intermediate articles about as long as wide; most clypeal setae located in a broad subtriangular medial area, a few other setae close to the mid-point of the anterior margin and in 2 anterolateral groups; intermediate labral projections darker, shorter and closer to each other than lateral projections; forcipular coxosternite > 1.5 times as wide as long, with incomplete chitin-lines; trochanteroprefemur much wider than long; tarsungulum ca 2 times as long as the trochanteroprefemur, with finely serrate internal margin; poison calyx elongated; trunk tergites and sternites wider than long; around 109 pairs of legs, with 2 accessory spines; paired pore-fields of the sternites gradually changing from circular to longitudinally elongated along the trunk, but missing on the first and the prepenultimate leg-bearing segment; medial pore-field of the sternites subcircular, present also on the first and the prepenultimate leg-bearing segment; most of the coxal pores covered by the metasternite of the ultimate leg-bearing segment, a few anterior pores on the lateral side of coxopleuron and a single posterior pore isolated on the ventral side; metasternite of the ultimate leg-bearing segment subtrapezoidal, wider than long; ultimate telopodite ca 2 times as long as the penultimate, with a small terminal spine.

Name derivation. A Latin adjective "*unicus*", to emphasize the unique arrangement of the ventral glandular pores.

Description of holotype. *General features* (Fig. 1A–C). Body 65 mm long, depressed, almost uniformly wide along the trunk (ca 1.3 mm) but slightly narrowing posteriorly (penultimate leg-bearing segment 0.8 mm wide). Color (in ethanol) uniformly light yellow, but head and forcipular segment slightly darker.

Cephalic capsule (Fig. 2A, B). Cephalic plate sub-heptagonal, about as long as wide, lateral margins more distinctly converging anteriorly than posteriorly, posterior margin straight; scutes approximately isometric and up to ca 10 μm; transverse suture absent; setae up to ca 100 μm long. Clypeus ca 2.7 times as wide as long, with lateral margins complete; uniformly areolate, with scutes ca 10 μm wide; no obvious clypeal areas; at least 56 setae, all in the anterior half of the clypeus, most of them in a subtriangular intermediate broad area, ca 9 setae in each of 2 anterolateral smaller areas, and a few other apparently broken setae close to the mid-point of the anterior margin of the clypeus. Pleurites uniformly areolate, with 15–17 setae each. Labral margin slightly concave, with a row of 30 projections, including 8 intermediate denticles that are darker, shorter and closer to each other than the lateral projections.

Antenna (Fig. 1A, B). 14 articles. Entire antenna ca 2.7 times as long as the head width. Intermediate articles about as long as wide. Article XIV ca 2.1 times as long as wide, ca 1.6 times as long as article XIII, and twice the length of intermediate articles. Setae gradually denser from article I to X on the dorsal side, from article I to V on the ventral side, almost completely missing on the ventral-internal side of articles I–V, however uniformly dense in the remaining distal articles. Setae gradually shorter from article I to about V, up to 100 μm long on article I and < 40 μm long on article XIV. Apical sensilla ca 10 μm long, spear-like, without projections, distinctly narrowing at about the mid-length. Club-like sensilla ca 10 μm long, grouped on the distal parts of the internal sides of articles IX–XIV and on the distal parts of the external sides of articles V–XIV. Three longitudinal rows of 1–5 proprioceptive spine-like sensilla at the bases of the antennal articles, approximately dorsal, ventro-internal and ventro-external; rows reduced to 0–1 spine on antennal articles VI, X and XIV. A few sensilla, similar to the apical ones but slightly darker and shorter, up to 5 μm long, on both dorso-external and ventro-internal position, close to the distal margin of articles II, V, IX and XIII.

Mandible (Fig. 2C, D). A single pectinate lamella, with 20–30 elongate teeth. Each tooth about 5 times as long as wide.

First maxillae (Fig. 3A). Coxosternite entire, with setae close to the anterior margin; a pair of lappets, covered with scales. Coxal projection subtriangular, longer than wide, with setae on the ventral side. Telopodite longer than wide, of 2 articles, with setae on the ventral side; a lappet emerging from the basal article and covered with scales.

Second maxillae (Fig. 3B). Coxosternite entire, with anterior margin deeply angulated, metameric pores on the central part of each half and about 7 setae near each of the posterior corners. Telopodite of 3 articles, slightly narrowing towards the tip; a number of setae on each article, most of them on the meso-ventral side; pretarsus ca 0.4 times as long as the distal article, slightly bent, narrowing and slightly spoon-shaped at

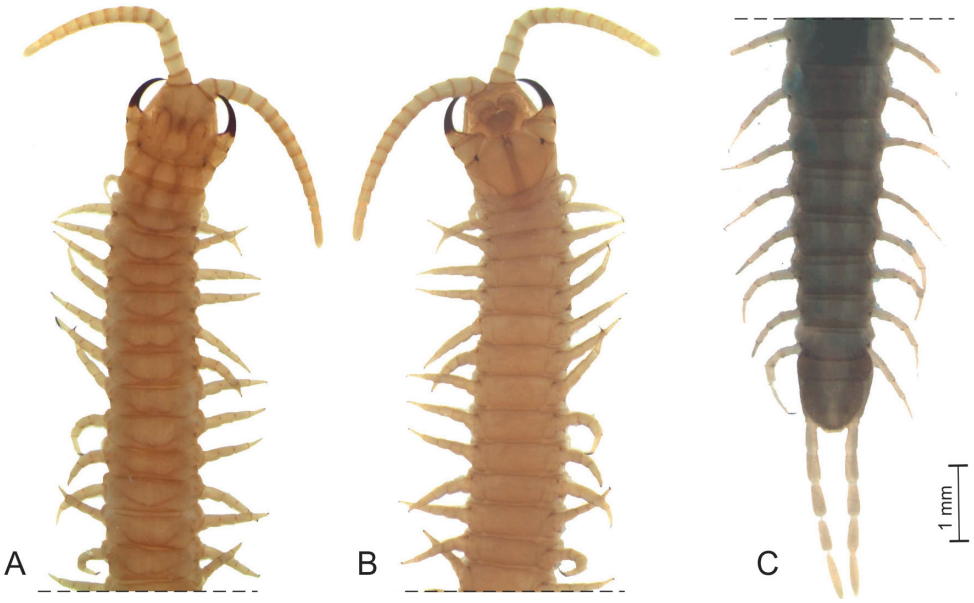

Figure 1. *Vinaphilus unicus* gen. n. sp. n., holotype **A** anterior part of the body, dorsal view **B** anterior part of the body, ventral view **C** posterior part of the body, dorsal view.

the tip, with 7 filaments along the dorsal edge and 7 filaments along the ventral edge; 4 pore-like sensilla on each pretarsus, 1 on the convex side, 3 on the concave side.

Forcipular segment (Fig. 2A, E). Tergite subtrapezoidal, ca 2.8 times as wide as long, with lateral margins strongly converging anteriorly, approximately as wide as the cephalic plate and ca 0.9 times as wide as the following tergite. Exposed part of the coxosternite ca 1.6 times as wide as long; anterior margin with a shallow medial concavity and without denticles; complete coxopleural sutures, entirely ventral, sinuous and diverging anteriorly; chitin-lines incomplete, only visible in the posterior part of the coxosternite. Basal distance between the forcipules ca 0.4–0.5 times of the maximum width of the coxosternite. Trochanteroprefemur ca 1.4 times as wide as long. Intermediate articles distinct. No denticles along the forcipule. Tarsungulum ca 2.8 times as long as wide, ca 2.2 times as long as the trochanteroprefemur; both the external and the internal margins uniformly curved, except for a moderate mesal basal bulge; ungulum not distinctly flattened, internal margin serrated with ca 40 small notches. Elongated poison calyx, ca 5–6 times as long as wide, lodged inside the intermediate forcipular articles.

Leg-bearing segments (Figs 1, 3C, 4). A total of 109 pairs of legs. Metatergite 1 slightly wider than the subsequent one, without pretergite. No paratergites. Walking legs shorter than the width of the trunk; legs of the first pair slightly smaller than the following ones; claws simple, uniformly bent, with 2 accessory spines, the anterior spine reaching at most 20% of the length of the claw, the posterior spine much

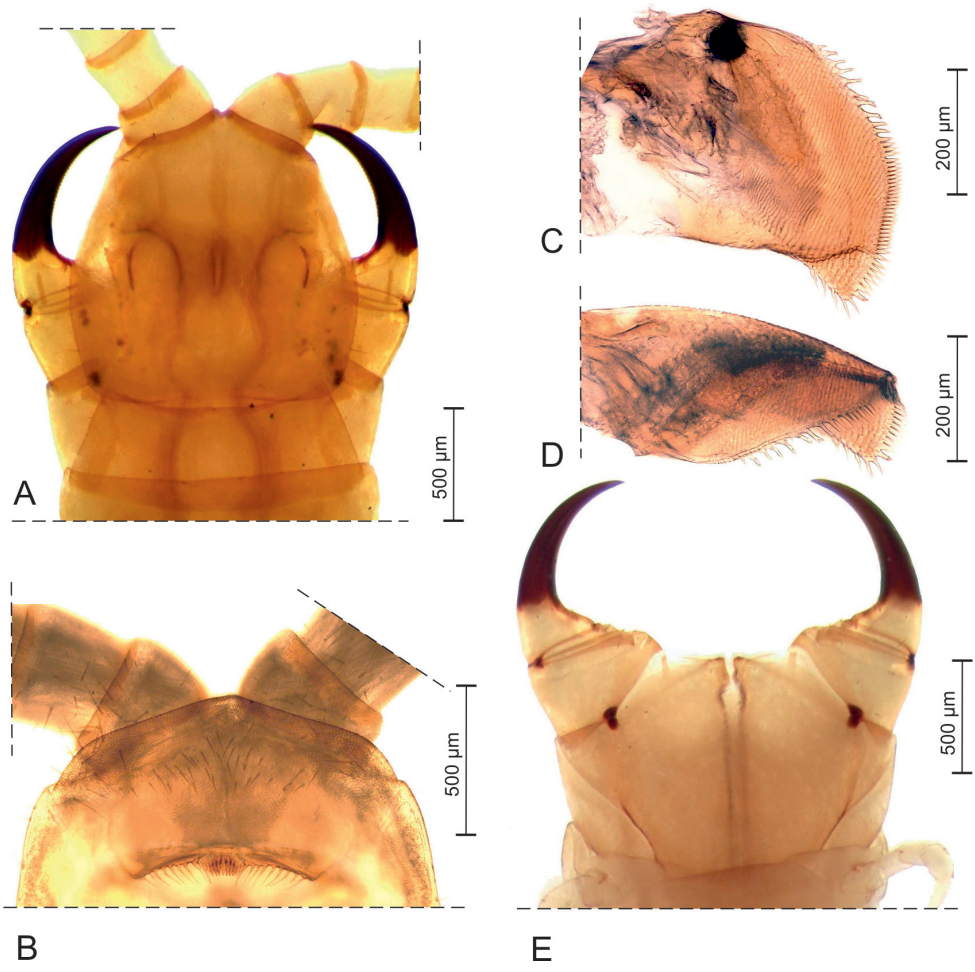

Figure 2. *Vinaphilus unicus* gen. n. sp. n., holotype **A** head and forcipular segment, dorsal view **B** clypeus and labrum, ventral view after removal of forcipules and maxillae **C** left mandible, dorsal view after extraction **D** left mandible, anterior view after extraction **E** forcipular segment, ventral view after removal of head.

shorter. Metasternites ca 2 times as wide as long in the anterior part of trunk, up to 1.3 times as wide as long in the posterior part. Ventral glandular pores densely grouped into 3 separate fields on each metasternite, from the second to the prepenultimate leg-bearing segments: 2 paired fields in the anterior part of the metasternite, close to the lateral margins, subcircular and closer to the anterior corners in the most anterior segments longitudinally slightly elongated and closer to mid-length along the remaining trunk; 1 subcircular medial field approximately in the centre of the metasternite in the anterior part of the body, gradually becoming closer to the posterior margin in the most posterior segments. Only the medial field on the first and the penultimate leg-bearing segment.

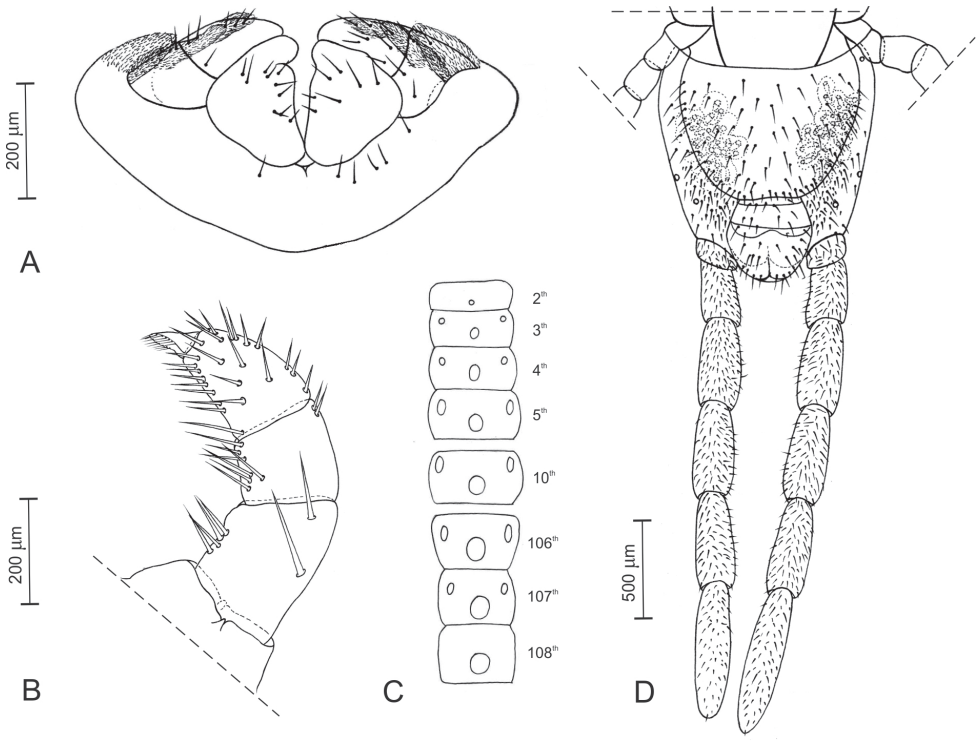

Figure 3. *Vinaphilus unicus* gen. n. sp. n., holotype **A** First maxillae, ventral view after dissection **B** left telopodite of second maxillae, ventral view after dissection **C** sternites of selected leg-bearing segments (indicated by numbers), simplified drawings of pore-fields **D** ultimate leg-bearing segment and postpedal segments, ventral view.

Ultimate leg-bearing segment (Figs 1C, 3D). Pleuropretergite entire, without sulci. Metatergite subtrapezoidal, ca 1.2 times as wide as long, lateral margins convex and converging posteriorly, posterior margin slightly curved. Coxopleuron ca 1.5 times as long as the metasternite. Coxal organs of each coxopleuron opening through about 30 independent pores, mostly clustered and covered by the metasternite, but 2 or 3 pores on the lateral side of the coxopleuron, including 1 pore on the posterior third of the ventral side of the coxopleuron. Metasternite subtrapezoidal, wider than long, anteriorly ca 2.6 times as wide as posteriorly, lateral margins slightly convex and converging backwards; setae denser on 2 broad lateral parts of metasternite, almost absent close to the posterior margin and in a narrower mid-longitudinal stripe. Telopodite approximately 11 times as long as wide, ca 1.8 times as long and ca 1.8 times as wide as the penultimate telopodite; 6 articles; tarsus 2, ca 2.7 times as long as wide and ca 0.7 times as long as tarsus 1; uniformly dense setae, < 45 µm long, on most of the ventral and dorsal sides of the telopodite. Pretarsus absent, a small spine in its place.

Postpedal segments (Fig. 3D). Two gonopods, basally touching, subtriangular, without traces of articulation, covered with setae. Anal organs apparently absent.

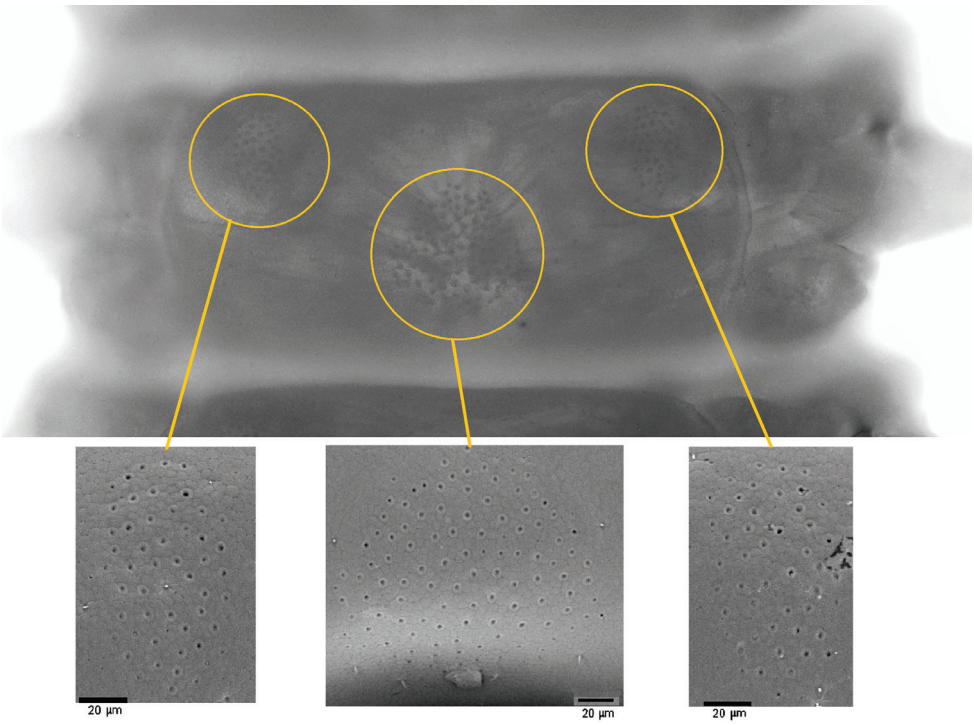

Figure 4. *Vinaphilus unicus* gen. n. sp. n., holotype. Pore-fields of leg-bearing segment 10, ventral view.

Main differences of paratype. Entire antenna ca 2.9 times as long as the head width; intermediate articles ca 1.1 times as long as wide; article XIV ca 2.0 times as long as wide and ca 1.5 times as long as article XIII. Exposed part of the forcipular coxosternite ca 1.7 times as wide as long; tarsungulum ca 1.8 times as long as the trochanteroprefemur. Metasternites ca 3 times as wide as long in the anterior part of trunk, up to 1.4 times as wide as long in the posterior part. About 40 coxal pores on each coxopleuron. Telopodite of the ultimate pair of legs approximately 13 times as long as wide, ca 2.3 times as long and ca 1.4 times as wide as the penultimate telopodite.

Discussion

Taxonomic position

Considering the most recent comprehensive classification of Geophilomorpha (Bonato 2011) and an even more recent tentative reassessment after a phylogenetic analysis (Bonato et al. 2014), the new species described here is confidently recognisable as belonging to the Geophiloidea. This is especially indicated by the unilamellate shape of the mandibles, which is regarded as a major synapomorphy of the superfamily (Bonato et al. 2014).

More precisely, the structure of the labral sclerites, the shape of the labral marginal projections, the presence of filaments on the second maxillary pretarsi and the structure of the female gonopods of the new species are all plesiomorphic characters within the Geophiloidea and are common to all clades basal to the Geophilidae s.l. (Bonato et al. 2014). However, the family-level taxonomy of the basal geophiloids is still uncertain, especially with regards to the taxonomic circumscription of the traditionally recognized family Gonibregmatidae and the recently established Zelanophilidae (Crabill 1963a, 1963b; Bonato 2011; Bonato et al. 2014: table S2). When considering the elongation of the head and the forcipular apparatus, details of the labrum, and the number of legs, the new species is more similar to the species of *Gonibregmatus* and other gonibregmatids such as *Himantosoma* and *Geoporophilus* than to the species of *Zelanophilus* Chamberlin, 1920 and the related *Tasmanophilus* Chamberlin, 1920. While the above mentioned gonibregmatids have stout head, poorly distinct labral sclerites, short and non-denticulate forcipules and more than 90 pairs of legs (Pocock 1899; Silvestri 1919; Attems 1930), the zelanophilids have slightly elongated head, distinct labral sclerites, usually denticulate forcipules and less than 90 pairs of legs (Archey 1922; Crabill 1963a, 1963b). Worth noting is that the geographical provenance of the new species (north-eastern part of the Indochinese peninsula) is well within the distribution range of the above mentioned gonibregmatids (from the Indian peninsula, through the Indochinese peninsula, the Malay Archipelago and New Guinea, to the Fijian islands; see Appendix), whereas zelanophilids have been reported so far only from Australia, Tasmania, and New Zealand (Bonato 2011, Bonato et al. 2014).

Considering all gonibregmatids so far reported from Asia, including nominal species here recognized for the first time in Gonibregmatidae (see Appendix), we can conclude that the new species differs in at least some major characters that are regarded of high diagnostic value at the species level (Table 2).

Similarly, with regard to all previously described genera of Asiatic gonibregmatids, including synonyms and other uncertain gonibregmatids (see Appendix), the new species departs from the morphology of all their type species, as well as from the range of variation among the congeneric species, in some major anatomical characters that are commonly considered diagnostic at the genus level (Table 1). The new species does not fit any other genus especially for the unique arrangement of ventral pore-fields (see below) and the unusual arrangement of the coxal pores. Actually, the distribution of the coxal pores somehow resembles the condition found in all species of *Himantosoma* and *Disargus* and in the type species of *Geoporophilus*. In the three latter genera, however, the pores are aggregated on both the meso-ventral and the meso-dorsal sides, and are hardly covered by the adjacent metasternite, which is relatively smaller in comparison with the coxopleura (Pocock 1890; Silvestri 1919).

The new species departs also from all other genera of basal geophiloids inhabiting other continents. In detail, it differs from *Madageophilus* Lawrence, 1960 (a single species from Madagascar, recently referred to Gonibregmatidae; Appendix) in the elongation of the head, the arrangement of the ventral pore-fields and the absence of claws on the ultimate legs (cf. Lawrence 1960). It differs from *Australiophilus* Verhoeff, 1925

Table 2. Main differences between *Vinaphilus unicus* gen. n. sp. n. and all other species of Gonibregmatidae reported from Asia (see Appendix). Only reliable sources of morphological information were considered (indicated in the Appendix).

Species	Body		Head	Labrum		Second maxillae		Forcipular segment	Anterior part of trunk				Ultimate leg–bearing segment				
	Max length (mm)	Pairs of legs	Distinct transverse suture	Mid-part	Marginal projections	Telopodite: articles	Pretarsus: filaments	Complete chitin–lines	Paratergites	Pincer-like claws	Sternite: anterior pore-fields	Sternite: posterior pore-fields	Anterior extent of coxopleura	Coxal pores: arrangement	Ultimate/penultimate telopodite length	Tarsal articles	Claw
V. unicus sp. n.	90	109	–	Wide, concave	+	3	+	–	–	–	2, narrow	1, narrow	Penultimate legs	Almost only meso-ventral, covered	>>1	2	–
'B.' robustus	58	95	–	Wide, concave	+	?	+	–	?	?	0	0	?	Ventral+lateral+?dorsal	?	2	–
Di. striatus	38	69	+	?	?	?	?	?	?	?	1, narrow	1–2, wide	Penultimate legs	Ventral+lateral+dorsal, denser mesally	1	1?	+
Ds. coloratus	48	73	?	Wide, concave	+	3	+	?	–	?	1, wide	2, wide	Penultimate legs	Ventral+lateral+dorsal	1	2	+
E. hamatus	85	121?–123–125	+	Wide, concave	+	3	+	+	–	+	2, narrow	1, wide	Antepenultimate legs	Ventral+lateral+dorsal	>>1	2	–
E. meinerti	130	103–127–129?	–	Wide, concave	+	3	+	+	–	+	2, narrow	2, wide	Penultimate legs	Ventral+lateral+dorsal	>>1	2	–
Ge. angustus	55	107	–	Wide, concave	+	3	+	–	–	–	2, narrow	2, narrow	Penultimate legs	Ventral+dorsal, denser mesally	>>1	?	?
Go. anguinus	130	115–129	+	Narrow, convex	+	3	–	+	+	–	1, wide	1, wide	Antepenultimate legs	Ventral+lateral+dorsal	1	2	–
Go. cumingii	125	161	–	?	?	2?	?	–	+	–	0	0	Antepenultimate legs	Ventral+lateral+dorsal	>>1	2	–
Go. fijianus	150	177	+	Convex	?	?	?	?	?	?	?	?	?	?	1	?	?
Go. insularis	97	131	–	?	?	?	?	?	+	?	?	?	Antepenultimate legs	Ventral+lateral+dorsal	>>1	2	–
Go. olivaceus	110	99–113	–	Narrow, concave	+	3	–	?	+	–	1, wide	1, wide	Antepenultimate legs	Ventral+lateral+dorsal	?	?	–
Go. plurimipes	unknown	191	–	Convex	?	?	?	?	?	?	?	?	?	?	>>1	?	?
H. bidivisum	45	79	–	?	?	3	+	–	–	–	1, narrow	2, wide	Penultimate legs	Ventral+dorsal, denser mesally	1	2	+
H. porosum	42	59–61	–	Concave	+	3	+	–	–	–	1, narrow	1, wide	Penultimate legs	Ventral+dorsal, denser mesally	1	2	+
H. typicum	69	57–81	–	Concave	+	3	+	–	–	–	1, narrow	2, wide	Penultimate legs	Ventral+dorsal, denser mesally	1	2	+
L. varians	48	55–73	?	Wide, concave	–	3	+?	–	?	?	2	2	Ultimate legs	Ventral+lateral+?dorsal	?	2	–
S. serangodes	90	131–135	?	?	–	3	+	+	?	+	0	1, wide	Penultimate legs	Ventral+lateral+dorsal	>>1	2	–
T. malaccanus	96	93–107	?	Wide, concave	+	3	+	–	–	?	2, wide	1, wide	Penultimate legs	Ventral+lateral+dorsal	?	2	–

(two species from Australia and New Zealand, respectively; variously classified in Geophilidae, Gonibregmatidae or Zelanophilidae; Crabill 1963a; Bonato 2011; Bonato et al. 2014) in the general structure of the labrum, the arrangement of the ventral pore-fields and the coxal pores, and the absence of claws on the ultimate legs (cf. Verhoeff 1925; Crabill 1963a). It differs also from the American genera traditionally separated in the families Eriphantidae (*Eriphantes* Crabill, 1970) and Neogeophilidae (*Neogeophilus* Silvestri, 1918 and *Evallogeophilus* Silvestri, 1918), which have been recently hypothesised to be strictly related to Gonibregmatidae s.s. (Bonato et al. 2014). In particular, the new species differs from *Eriphantes* especially in the general structure of the labrum and the forcipules, the shape of the second maxillae and the arrangement of the ventral pore-fields (cf. Crabill 1970), whereas it differs from the Neogeophilidae in the general body shape and the structure of the first maxillae and the ultimate legs (cf. Silvestri 1918; Crabill 1961).

Arrangement of sternal glands

As far as known, *Vinaphilus unicus* gen. n. sp. n. is unique among all other gonibregmatids, as well as all centipedes at large, in the arrangement of the ventral pore-fields (Figs 3C, 4).

Ventral glands secreting sticky material are present along the body trunk in most of the geophilomorph centipedes. These glands open through the cuticle of almost all metasternites and often also on the coxae of the adjacent walking legs. The functions of the secretions are largely unknown, but are expected to have a role in deterring predators (Minelli 2011). The arrangement of the glands and the associated pores along the body is conveniently described as a modular longitudinal pattern: all or most glandular pores are grouped in one or more distinct clusters (pore-fields) on the ventral side of each leg-bearing segments. Extent, shape and position of the pore-fields are similar between adjacent leg-bearing segments, with some gradual variation across the longitudinal series of segments. The pattern of pore-fields is highly variable between species and is known to have been highly evolvable in the geophilomorph subclade Adesmata (Turcato et al. 1995).

The particular arrangement of pore-fields in *Vinaphilus unicus* gen. n. sp. (two sublateral paired fields anterior to a single narrow medial field; Fig. 3C) only partially resembles those found in some other gonibregmatids and some geophilids, where distinct anterior and posterior pore-fields may co-occur in single sternites. However, two anterior sublateral paired fields are never associated with a posterior single narrow medial field, rather with either a transverse very broad field (e.g. *Eucratonyx*; Ribaut 1912; Attems 1929) or two paired fields (e.g. *Geoporophilus*; Silvestri 1919). On the other hand, a posterior single narrow medial field is never associated with two anterior sublateral paired fields, rather with another single narrow medial field (*Eriphantes*; Crabill 1970).

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Appendix

Species of Gonibregmatidae

Species are listed in alphabetic order. Species traditionally separated in the families Neogeophilidae and Eriphantidae are not considered.

***Eucratonyx hamatus* Pocock, 1899**

Original description – Pocock 1899: 67, fig. 2c.

Other sources for morphology – Ribaut 1912: 285, figs 1-19. Attems 1914: 121, figs 14-21. Attems 1929: 342, figs 304-306. Bonato et al. 2011: 23.

Reported specimens – ≥7.

Type locality – "New Britain" [Papua New Guinea].

Geographical records – Indonesia: Ambon Island (Attems 1927); Dabra, in New Guinea (Chamberlin 1939); "Seltutti", in Aru Islands (Ribaut 1912). Papua New Guinea: Madang (Attems 1914); New Britain (Pocock 1899); Ralum, in New Britain (Attems 1914); New Ireland (Bonato et al. 2011).

***Eucratonyx meinerti* (Pocock, 1889)**

Original description – Pocock 1889: 289, fig. 1, as *Himantarium Meinerti* [sic].

Other sources for morphology – Pocock 1891: 426, fig. page 427, as *Himantarium meinertii* [sic]. Pocock 1899: 66, fig. 2. Silvestri 1919: 104, fig. 38, as *Eucratonyx meinertii* [sic]. Bonato et al. 2011: 23, figs 5, 6d, 7i–l. Koch and Edgecombe 2012: 28, 48, figs 20–21. Bonato et al. 2014: figs 2b, 8b.

Reported specimens – >12.
Type locality – "Sullivan Island" [=Lanbi Kyun, Myanmar].
Geographical records – Myanmar: Great Coco Island (Pocock 1891, as *Himantarium meinertii* [sic]); Lanbi Kyun, in Mergui Archipelago (Pocock 1889, as *Himantarium Meinerti* [sic]); Little Coco Island (Silvestri 1919, as *Eucratonyx meinertii* [sic]); Mawlamyine (Pocock 1891); Palon (Pocock 1891); Reef Island, near Dawei (Pocock 1891). Thailand: Doi Inthanon (Bonato et al. 2011).

***Disargus striatus* (Pocock, 1890)**
Original description – Pocock 1890: 248, fig. 4, as *Himantarium striatum*.
Other sources for morphology – none.
Reported specimens – 1.
Type locality – "Madras" [=Chennai, India].
Geographical records – India: Chennai (Pocock 1889).
Taxonomic notes – The species was originally included provisionally in *Himantarium* (Pocock 1890), later in *Himatosoma* by Pocock (1891) and then invariantly in a distinct monotypic genus *Disargus* since Cook (1896).

***Dschangelophilus coloratus* Verhoeff, 1937**
Original description – Verhoeff 1937: 226, figs 26–31.
Other sources for morphology – none.
Reported specimens – 1.
Type locality – "Telom Valley, Pahang" [Malaysia].
Geographical records – Malaysia: Telom Valley, in Peninsular Malaysia (Verhoeff 1937).
Taxonomic notes – The species was originally classified in Geophilidae in a distinct monotypic genus *Dschangelophilus* Verhoeff, 1937. It was suspected to belong to Zelanophilidae by Bonato et al. (2014). It is here confidently recognised in Gonibregmatidae for the first time, based on the following characters that were described and/or illustrated by Verhoeff (1937): stout head, presence of filaments on the second maxillary pretarsi, short and non-denticulate forcipules, separate female gonopods.

***Geoporophilus angustus* Silvestri, 1919**
Original description – Silvestri 1919: 107, fig. 39.
Other sources for morphology – none.
Reported specimens – 1.
Type locality – "Sumatra: Indragiri" [=Indragiri river, Indonesia].
Geographical records – Indonesia: Indragiri river, in Sumatra (Silvestri 1919).
Taxonomic notes – While *Geoporophilus angustus* was recognized in Gonibregmatidae since Bonato (2011), another species from Sumatra originally classified in the genus *Geoporophilus*, namely *Geoporophilus aporus* Attems, 1930, is here recognised as actually belonging to the family Oryidae and not to Gonibregmatidae.

Even though the mandible was described by Attems (1930) as bearing a single pectinate lamella (as found in Gonibregmatidae, but not in Oryidae), many other characters (shape of labrum, structure of first maxillae, shape of ultimate leg-bearing segment, absence of coxal pores) are common to the Oryidae, and especially to the genus *Orphnaeus* Meinert, 1870, and not to Gonibregmatidae. As a consequence, we propose to recognise *Geoporophilus aporus* Attems, 1930 as a provisionally valid species in the genus *Orphnaeus*, with the name *Orphnaeus aporus* (Attems, 1930) comb. n.

***Gonibregmatus anguinus* Pocock, 1899**

Original description – Pocock 1899: 65, fig. 1.

Other sources for morphology – Attems 1914: 119, figs 1–12. Attems, 1926: figs 427, 429, 430, 431, 432. Attems, 1929: 337, figs 295–299. Koch and Edgecombe 2012: 26, 48, figs 18–19. Bonato et al. 2014: fig. 3a.

Reported specimens – ≥11.

Type locality – "New Britain" [Papua New Guinea].

Geographical records – Indonesia: Jayapura, in New Guinea (Attems 1914). Papua New Guinea: Cape Mimias, in New Ireland (Bonato et al. 2014); New Britain (Pocock 1899); Ponam Island (Attems 1927); Ralum, in New Britain (Attems 1914).

***Gonibregmatus cumingii* Newport, 1843**

Original description – Newport 1843a: 181.

Other sources for morphology – Newport 1843b: 502. Newport, 1845: 434, figs 11–14 of pl. 33, fig. 12 of pl. 40. Newport, 1856: 86. Haase 1887: 113, fig. 118.

Reported specimens – 2.

Type locality – "Philippine Islands".

Geographical records – Philippines: unknown locality (Newport 1843a); Manila (Elera 1895).

***Gonibregmatus fijianus* Chamberlin, 1920**

Original description – Chamberlin 1920: 34.

Other sources for morphology – none.

Reported specimens – 3.

Type localities – "Nadarivatu" [Fiji]; "Vanua Ava" [=Vanuava, Fiji].

Geographical records – Fiji: Nadarivatu, in Viti Levu (Chamberlin 1920); Vanuava, in Kadavu Island (Chamberlin 1920).

***Gonibregmatus insularis* Pocock, 1894**

Original description – Pocock 1894: 318.

Other sources for morphology – none.

Reported specimens – 1.

Type locality – "Island of Saleyer" [sic] [=Selayar Island, Indonesia].

Geographical records – Indonesia: Selayar Island (Pocock 1894).

***Gonibregmatus olivaceus* Attems, 1930**
Original description – Attems 1930: 170, figs 83–89.
Other sources for morphology – none.
Reported specimens – ≥3.
Type locality – "Swela, Ost-Lombok" [=Suela, Indonesia].
Geographical records – Indonesia: Suela, in Lombok (Attems 1930).

***Gonibregmatus plurimipes* Chamberlin, 1920**
Original description – Chamberlin 1920: 33.
Other sources for morphology – none.
Reported specimens – 1.
Type locality – "Lomati" [Fiji].
Geographical records – Fiji: Lomati, in Kadavu Island (Chamberlin 1920).

***Himantosoma bidivisum* Silvestri, 1919**
Original description – Silvestri 1919: 101, fig. 37, as *Himantosoma typicum* var. *bidivisa*.
Other sources for morphology – none.
Reported specimens – 1.
Type locality – "Barkul" [India].
Geographical records – India: Barkul, in Odisha (Silvestri 1919).
Taxonomic notes – This species was originally described as a variety of *Himantosoma typicum* Pocock, 1891 by Silvestri (1919). It was later cited as a subspecies of *H. porosum* Pocock, 1891 by Attems (1938, 1953) and it was recently listed at the species rank by Tran et al. (2013) but without comments. We maintain it provisionally as a separate species for consistency with the common taxonomic practice in Gonibregmatidae, because of obvious morphological differences from both *H. typicum* (shape of ventral pore-fields and arrangement of coxal pores, when controlling for body size) and *H. porosum* (number of legs, shape of ventral pore-fields and arrangement of coxal pores). The name is available according to Article 45.6.4.1 (ICZN 1999). Specimens from Vietnam identified by Attems (1938) as *H. porosum bidivisum* are tentatively assigned to *H. porosum* according to the reported number of legs, as also consistent with the geographical provenance.

***Himantosoma porosum* Pocock, 1891**
Original description – Pocock 1891: 431, fig. page 431.
Other sources for morphology – Silvestri 1895: 719. Attems 1903a: 65, fig. 1. Attems 1903b: 287, figs 7–10. Attems 1914: figs 24–27. Silvestri 1919: 101, fig. 36, as *Himantosoma typicum* var. *tridivisa*.
Reported specimens – ≥8.
Type locality – "Moulmein" [=Mawlamyine, Myanmar].
Geographical records – Indonesia: Cibodas, in Java (Attems 1903a); Sirambi, in Sumatra (Silvestri 1895). Myanmar: Mawlamyine (Pocock 1891). Vietnam: Cap Varella

(Attems 1938, as *Himantosoma porosum bidivisum*); Cau Da (Attems 1938); Deo Ca Pass (Attems 1938); Suoi Dau (Attems 1938).

Taxonomic notes – A variety *tridivisa* was originally described for *Himantosoma typicum* Pocock, 1891 by Silvestri (1919) based on a specimen previously identified by the same Silvestri (1895) as *H. porosum*. It was rarely cited as a subspecies of either *H. porosum* (Attems 1938, 1953) or *H. typicum* (Wang 1967). The name is available according to Article 45.6.4.1 (ICZN 1999). It is here synonymized under *H. porosum* [= *H. typicum tridivisum* syn. n.] because the only putative differences with the former species (relative size of coxopleura and number of coxal pores; Silvestri 1919) are explained by expected interindividual variation associated with difference in body size and possibly age.

***Himantosoma typicum* Pocock, 1891**

Original description – Pocock 1891: 429, fig. page 429.

Other sources for morphology – Pocock 1889: 289, fig. 3, as *Himantarium indicum*. Silvestri 1919: 100, fig. 35. Silvestri 1924: 72. Crabill 1969: 38.

Reported specimens – 8.

Type locality – "Moulmein" [=Mawlamyine, Myanmar].

Geographical records – India: Siju Cave, in Assam (Kemp 1924; Silvestri 1924). Myanmar: Kadan Kyun, in Mergui Archipelago (Pocock 1889, as *Himantarium indicum*); Mawlamyine (Pocock 1891); Mergui Archipelago (Silvestri 1919). Thailand: Sakunotayan waterfall (Bonato et al. 2014).

Taxonomic notes – The two syntypes of *Himantosoma typicum* were indicated both from "Moulmein" [=Mawlamyine] by Pocock (1891: 429), whereas a specimen from "King Island" [=Kadan Kyun] had been previously misidentified by Pocock (1889: 289) as *Himantarium indicum* Meinert, 1886 [currently *Polyporogaster indica*] and later referred to *H. typicum* by the same Pocock (1889: 289).

***Sogophagus serangodes* (Attems, 1897)**

Original description – Attems 1897: 476, figs 1–4, as *Geophagus serangodes*.

Other sources for morphology – Attems 1914: figs 13, 22–23. Attems 1929: 343, fig. 307. Crabill 1969: 38.

Reported specimens – 2.

Type locality – "Soah Konorah" [=Soakonora, Indonesia].

Geographical records – Indonesia: Soakonora, in Halmahera (Attems 1897, as *Geophagus serangodes*).

Taxonomic notes – The species was originally classified in Geophilidae in a distinct monotypic genus *Geophagus* Attems, 1897. The latter name was replaced with *Sogophagus* since Chamberlin (1912) because it was a junior homonym of a genus of Coleoptera. The species was suspected to belong to *Eucratonyx* by Bonato et al. (2011).

***Tweediphilus malaccanus* Verhoeff, 1937**

Original description – Verhoeff 1937: 225, figs 18-25.

Other sources for morphology – Verhoeff 1941: 87. Wang and Tang 1965: 444.

Reported specimens – ≥4.

Type localities – "Gunong Brinchang" [=Gunung Brinchang, Indonesia] and "Telom Valley bei Gunong Siku" [=Telom Valley, near Gunung Siku, Indonesia].

Geographical records – Malaysia: Gunung Brinchang, in Peninsular Malaysia (Verhoeff 1937); Telom Valley, near Gunung Siku, in Peninsular Malaysia (Verhoeff 1937).

Taxonomic notes – The species was originally classified in Geophilidae in a distinct monotypic genus *Tweediphilus* Verhoeff, 1937 and its taxonomic position was never discussed subsequently. It is here confidently recognised in Gonibregmatidae for the first time, based on the following characters that were described and/or illustrated by Verhoeff (1937, 1941): stout head, weakly defined labrum with elongate projections, presence of filaments on the second maxillary pretarsi, short and non-denticulate forcipules, separate female gonopods.

Species possibly belonging to Gonibregmatidae

Species are indicated with the name currently in use.

'*Brachygeophilus' robustus* Attems, 1953

Original description – Attems 1953: 143; figs 10, 11.

Other sources for morphology – none.

Reported specimens – 1.

Type locality – "Xieng Kuang" [=Xiangkhouang, Laos].

Geographical records – Laos: Xiangkhouang (Attems 1953).

Taxonomic notes – The species was originally classified in the geophilid genus *Brachygeophilus* Brölemann, 1909 and its taxonomic position was not revised after that *Brachygeophilus* was synonymised under *Geophilus* Leach, 1814 (since Foddai et al. 1995). The species was suspected to belong to Gonibregmatidae by Bonato et al. (2016), but its taxonomic position cannot be resolved based on the published information.

***Luangana varians* Attems, 1953**

Original description – Attems 1953: 143, figs 7–9.

Other sources for morphology – none.

Reported specimens – ≥2.

Type locality – "Luang Prabang" [=Louangphabang, Laos].

Geographical records – Laos: Louangphabang (Attems 1953).

Taxonomic notes – The species was originally classified in Geophilidae in a distinct monotypic genus *Luangana* Attems, 1953, but it was suspected to belong to Goni-

bregmatidae by Bonato et al. (2016). Its taxonomic position cannot be resolved based on the description and illustrations provided by Attems (1953). Actually, the original description was published posthumously and may be suspected to be actually composite, i.e. based on specimens belonging to different species, as suggested by the very different numbers of legs reported for the type specimens (55 and 73 pairs of legs).

***Madageophilus pauliani* Lawrence, 1960**

Original description – Lawrence 1960: 50, fig. 16.

Other sources for morphology – none.

Reported specimens – 1.

Type locality – "réserve naturelle de l'Andohahela" [=Andohahela National Park, Madagascar].

Geographical records – Madagascar: Andohahela National Park (Lawrence 1960).

Taxonomic notes – The species was originally classified in Geophilidae in a distinct monotypic genus *Madageophilus* Lawrence, 1960, but it was tentatively listed under Gonibregmatidae by Bonato (2011). Its taxonomic position cannot be resolved based on the original description and illustration provided by Lawrence (1960). In particular, the shape of the labrum, of the second maxillary pretarsi and of the pore-fields are suggestive of Gonibregmatidae, however the elongation of the head would be unprecedented for the family.

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DATA PAPER

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Molecular evidence for cryptic species in the common slug eating snake *Duberria lutrix lutrix* (Squamata, Lamprophiidae) from South Africa

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Abstract

We examined the impact of climatic fluctuations on the phylogeographic structure of the common slug eating snake (*Duberria lutrix lutrix*) throughout its distribution in South Africa. The evolutionary history within the taxon was examined using partial DNA sequence data for two mitochondrial genes (ND4 + cyt *b*) in combination with a nuclear locus (SPTBN1). Phylogenetic relationships were investigated for both the combined mtDNA and total evidence DNA sequence data. In addition, population and demographic analyses together with divergence time estimations were conducted on the combined mtDNA data. Topologies derived from the combined mtDNA analyses and the total evidence analyses were congruent and retrieved five statistically well-supported clades, suggesting that *Duberria l. lutrix* represents a species complex. The five clades were generally allopatric, separated by altitudinal barriers and characterised by the absence of shared mtDNA haplotypes suggesting long term isolation. Divergence time estimations indicate that the diversification within the *D. l. lutrix* species complex occurred during the Plio/Pleistocene as a result of climatic fluctuations and habitat shifts for the species. A taxonomic revision of the *D. l. lutrix* species complex may be required to delineate possible species boundaries.

Keywords

snakes, Afrotropical, alpha taxonomy, phylogenetics, altitudinal barriers, southern Africa

Introduction

Climatic oscillations are thought to be responsible for inducing dramatic impacts on the habitat and eco-physiological characteristics promoting cladogenesis (Hewitt 2000, 2004; Daniels et al. 2007, 2009; Barlow et al. 2013; Engelbrecht et al. 2013). The effects of climatic impacts on terrestrial biota vary considerably depending on latitude, longitude, habitat and the topographic heterogeneity of the environment (Hewitt 2000, 2004). Northern temperate continental areas experienced significant Pliocene/Pleistocene climatic changes whilst many biomes closer to the equator were reduced in size due to increased aridity and the expansion of arid environments (Hewitt 2000, 2004). These northern hemispherical climatic conditions resulted in noticeable recent cladogenesis for numerous species (Clark et al. 1999; Hewitt 2000, 2004; Lisiecki and Raymo 2007). In contrast, the impact of climatic changes on speciation in southern hemisphere terrestrial biota remains less studied, with reptiles being particularly neglected (Hewitt 2004; Barlow et al. 2013). Reptiles, as ectotherms, are particularly sensitive to temperature fluctuations and are thus ideal organisms with which to test the impact of climate ameliorations on phylogeographic patterning (Santos et al. 2012; Martínez-Freiría et al. 2015).

The serpent fauna of South Africa contains 116 species, of which 29 are endemic (Bates et al. 2014). There is a paucity of evolutionary studies of South African snakes, limiting our understanding of the patterns and processes that resulted in the contemporary population genetic structure. A phylogeographic study on the widespread African puff adder, *Bitis arietans*, revealed the presence of six refugial areas that existed during the last glacial maximum (LGM) occurring along the west coast, south west coast, southeast coast, in the northern regions, as well as two occurring in the central northern part of South Africa (Barlow et al. 2013). Declining temperatures and increased aridification associated with climatic oscillations are suspected to be causal to the isolation among the *B. arietans* clades (Barlow et al. 2013). One of southern Africa's most widely distributed snake species is the Common Slug Eater, *Duberria lutrix lutrix* (Branch 1998; Spawls et al. 2002; Uetz et al. 2019). *Duberria lutrix lutrix* is a molluscivorous, viviparous, non-venomous, small-bodied snake (Branch 1998). The species occurs in savannah, grassland, coastal bushveld and fynbos habitats (Branch 1998; Bates et al. 2014) where it prefers mesic areas (Branch 1998; Rabiega 2013; Bates et al. 2014). Four *Duberria* species (*D. lutrix*, *D. rhodesiana*, *D. shirana* and *D. variegata*) and five subspecies (*D. l. lutrix*, *D. l. abyssinica*, *D. l. atriventris*, *D. l. basilewskyi* and *D. l. currylindhali*, are currently recognised (Wallach et al. 2014; Uetz et al. 2019). The taxonomic status of these subspecies remains dubious, and some of these subspecies likely represent full species. *Duberria l. lutrix* is the only subspecies that occurs in South Africa, ranging in distribution from the coastal belt fringes of the Western Cape, Eastern Cape, KwaZulu-Natal, Gauteng, Mpumalanga and Limpopo provinces while allopatric, presumably relictual populations, are restricted to the interior in the Klein Karoo and Free State Province of South Africa (Branch 1998, 2002; Rabiega 2013; Bates et al. 2014).

The distribution range of *Duberria l. lutrix* in South Africa is bisected by several large mountain ranges, including the Cape Fold Mountains and the Great Drakens-

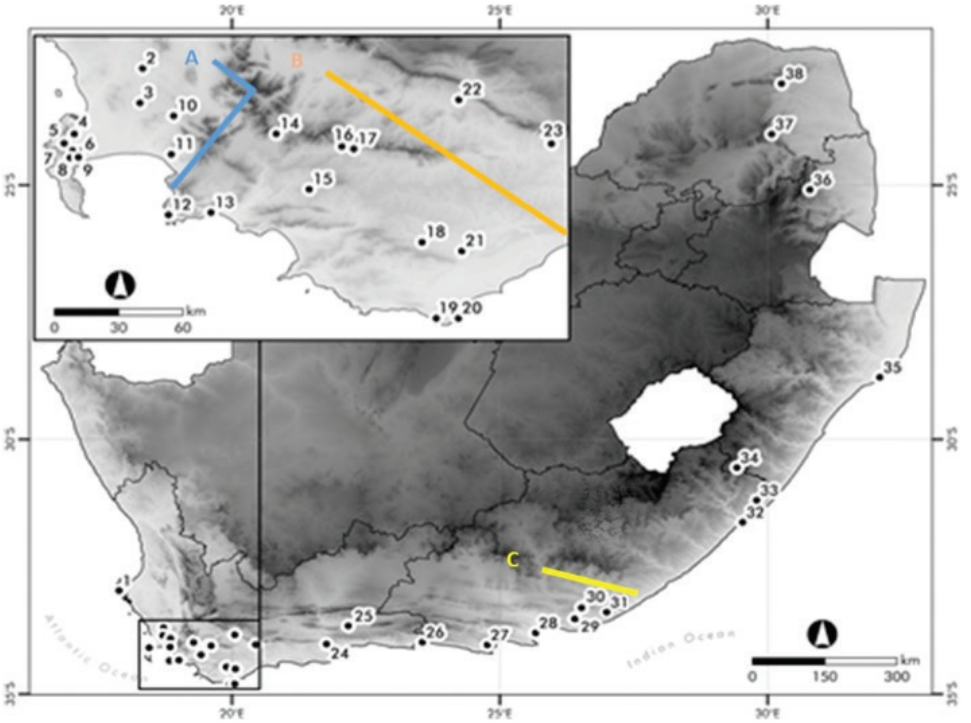

Figure 1. Known biogeographic breaks for co-distributed animal species in South Africa, including the **A** Hottentots Holland Mountains (blue) **B** The Breede River Valley (red) and **C** the Bedford gap (Yellow). The *Duberria l. lutrix* localities sampled throughout South Africa during the present study. Locality numbers correspond to the sample site numbers in Table 1.

berg escarpment (Partridge and Maud 1987, 2000; Partridge 1997) and low-lying xeric corridors. The habitat availability of the species in the Western Cape Province would have undergone significant climatic shifts from mesic conditions in the Miocene to enhanced arid conditions during the Pliocene/Pleistocene, possibly impacting habitat availability for this mesophylic species and the contraction of populations to high-lying mountainous refugia (Daniels et al. 2007; Engelbrecht et al. 2013; Diedericks and Daniels 2014). Xeric areas could potentially act as dispersal barriers for a small-bodied snake species with a preference for mesic environments (Hewitt 2004). The distribution of *D. l. lutrix* overlaps with several phylogeographic breaks for co-distributed lizard species (Fig. 1) (Daniels et al. 2007; Engelbrecht et al. 2013; Diedericks and Daniels 2014). A xeric biogeographic barrier known as the Bedford gap exists which separates the eastern and south-eastern part of the Cape Floristic Region (CFR) between East London and Port Elizabeth (Lawes 1990). This biogeographic gap is characterised by the intrusion of a sub-desert biome resulting in a semi-arid climate and is thought to have existed since the late Pliocene, resulting in unfavourable habitat for *D. l. lutrix* (Lawes 1990; Lawes et al. 2007).

Considering the results observed in phylogeographic studies of other co-distributed reptile species, we postulate that *D. l. lutrix* exhibits similar patterns of genetic differentiation across its distribution in South Africa. The objective of the present study is twofold. First, to examine the phylogeographic relationships within *D. l. lutrix* in South Africa, and to explore the possible impact of climatic changes on the phylogeographic patterning of the species. Second, to examine the presence of possible cryptic lineages within the taxon. Firstly, we hypothesize that climatic induced evolutionary changes during the Plio/Pleistocene promoted cladogenesis in the species. Secondly, we hypothesize that discrete lineages are present within *D. l. lutrix*.

Methods and materials

Sample collection

Road killed specimens or tissue of *Duberria l. lutrix* were obtained from the South African National Biodiversity Institute tissue bank (SANBI – Cape Town, South Africa) and from several private collections and road kills. Road killed specimens or tissue samples were preserved in absolute ethanol and refrigerated at 4 °C. A total of 87 *D. l. lutrix* specimens were collected from 38 localities across South Africa, covering most of the subspecies distribution range (Fig. 1, Table 1). An additional *Duberria* specimen from Uganda was donated by the Californian Academy of Sciences (CAS), USA. Specimens collected during the present study were deposited in the Port Elizabeth Museum Reptile Collection (PEM R), Eastern Cape Province, South Africa (Table 1).

DNA extraction, PCR amplification and sequencing

DNA was extracted from ethanol preserved muscle or liver tissue biopsies. A MacheryNagel DNA extraction kit was used for the DNA extraction, following the manufacturer's protocol. Extracted DNA was stored at -20 °C until needed for the polymerase chain reaction (PCR). Three gene regions were targeted using the PCR, these included two mitochondrial (mtDNA) loci: nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) using the primer pairs listed in Arévalo et al. (1994) and Barlow et al. (2013) respectively; while for cytochrome b (cyt *b*) the primer pairs listed in Burbrink et al. (2000) and Ruane et al. (2015) were used; for the nuclear locus, β-spectrin nonerythrocytic intron 1 (SPTBN1) the primers pairs listed in Ruane et al. (2014) were used (see Table 2 for details of the primer pair combinations). The ND4 and cyt *b* loci have been extensively used in snake phylogeographic studies, while the nuDNA locus has been demonstrated to be a variable nuclear marker in other snake species (Burbrink et al. 2000; Barlow et al. 2013; Ruane et al. 2014, 2015). All specimens were sequenced for the two mtDNA loci, while a single sample per locality was sequenced for the nuDNA locus.

Table 1. A list representing the locality, number of samples (*N*), province, coordinates and number of individuals selected for each gene of *Duberria lutrix lutrix* samples collected. The sample site number corresponds to Fig. 1. An additional sample from Vidal et al. (2008) is indicated with a ‡. PEM = Port Elizabeth Museum, SANBI = South African National Biodiversity Institute.

Sample number	Locality	*N*	Museum / SANBI tissue no	Province	Coordinates	Genbank Accesion numbers		
						ND4	cyt b	SPTBN1
1	Jacobs Bay	1	PEM R22493	Western Cape	32°58'8.27"S, 17°53'27.35"E	MK518189	MK518103	MK518271
2	Klipheuwel	2	unaccessioned	Western Cape	33°41'46.42"S, 18°43'26.97"E	MK518249–50	MK518108–09	MK518274
3	Kraaifontein	1	unaccessioned	Western Cape	33°50'57.39"S, 18°42'46.34"E	MK518200	MK518117	No Sequence
4	Kirstenbosch	3	SANBI 11300, 1450, 2785	Western Cape	33°59'10.89"S, 18°26'12.16"E	MK518190–92	MK518104–06	MK518272
5	Vlakkenberg	1	SANBI 4547	Western Cape	34°1'38.81"S, 18°23'37.87"E	MK518180	MK518093	MK518287
6	Bergvliet	1	unaccessioned	Western Cape	34°3'27.89"S, 18°27'6.26"E	MK518177	MK518089	MK518261
7	Tokai	1	SANBI 4545	Western Cape	34°3'37.40"S, 18°25'44.61"E	MK518232	MK518157	MK518285
8	Silvermine	1	SANBI 4550	Western Cape	34°5'29.69"S, 18°25'12.32"E	MK518219	MK518136	No Sequence
9	Lakeside	1	SANBI 1703	Western Cape	34°5'22.09"S, 18°27'14.02"E	MK518202	MK518119	MK518277
10	Stellenbosch	4	PEM R22494-97	Western Cape	33°54'23.90"S, 18°51'17.47"E	MK518227–31	MK518145–48	MK518282
11	Somerset West	8	PEM R22498-505	Western Cape	34°4'36.34"S, 18°50'40.88"E	MK518220–26, 51	MK518137–44	MK518281
12	Pringle Bay	4	PEM R22506-509	Western Cape	34°20'43.89"S, 18°49'58.08"E	MK518213–16	MK518130–33	No Sequence
13	Kleinmond	1	unaccessioned	Western Cape	34°20'6.03"S, 19°0'44.03"E	MK518248	MK518107	MK518273
14	Villiersdorp	5	PEM R22510-514	Western Cape	33°59'8.86"S, 19°17'10.18"E	MK518234–38	MK518159–63	MK518286
15	Caledon	1	PEM R22515	Western Cape	34°13'51.55"S, 19°25'31.35"E	MK518178	MK518091	MK518263
16	Genadendal	1	PEM R22516	Western Cape	34°2'29.91"S, 19°33'45.08"E	MK518181	MK518094	MK518265
17	Greyton	3	PEM R22517-519	Western Cape	34°3'8.18"S, 19°36'47.06"E	MK518182–84	MK518096–98	MK518266
18	Napier	6	PEM R22520-525	Western Cape	34°27'59.60"S, 19°53'59.71"E	MK518203–08	MK518120–25	MK518278
19	Agulhas	3	unaccessioned	Western Cape	34°48'11.21"S, 19°57'35.67"E	MK518243–45	MK518078–80	MK518259
20	Struis Bay	1	unaccessioned	Western Cape	34°45'42"S, 20°2'26.90"E	MK518252	MK518149	MK518283
21	Bredasdorp	1	unaccessioned	Western Cape	34°30'20.84"S, 20°4'2.12"E	MK518246	MK518090	MK518262
22	Ashton	8	PEM R22526-533	Western Cape	33°50'4.56"S, 20°3'17.09"E	MK518169–76	MK518081–88	MK518260
23	Swellendam	7	PEM R22524-540	Western Cape	34°1'45.81"S, 20°26'42.59"E	MK518253–58	MK518150–56	MK518284
24	Herbertsdale	1	SANBI 10847	Western Cape	34°1'1.25"S, 21°46'0.25"E	MK518185	MK518099	MK518267
25	Oudtshoorn	1	SANBI 2879	Western Cape	33°39'37.07"S, 22°10'24.69"E	MK518210	MK518127	MK518280
26	Natures Valley	1	SANBI 4558	Western Cape	33°58'50.40"S, 23°33'22.79"E	MK518209	MK518126	MK518279

Sample number	Locality	N	Museum / SANBI tissue no	Province	Coordinates	Genbank Accesion numbers		
						ND4	cyt b	SPTBN1
27	Humansdorp	1	SANBI 8108	Eastern Cape	34°1'59.61"S, 24°45'59.39"E	MK518188	MK518102	MK518270
28	Port Elizabeth‡	1	Vidal et al. (2008)	Eastern Cape	33°47'52.79"S, 25°40'18.74"E	FJ404356	FJ494305	No Sequence
29	Hope Fountain	1	SANBI 4474	Eastern Cape	33°31'27.29"S, 26°24'3.91"E	MK518187	MK518101	MK518269
30	Grahamstown	1	unaccessioned	Eastern Cape	33°17'59.89"S, 26°31'37.88"E	MK518247	MK518095	No Sequence
31	Port Alfred	1	SANBI 400	Eastern Cape	33°35'35.60"S, 26°53'3.55"E	MK518211	MK518128	No Sequence
32	Port St John	1	SANBI 12180	Eastern Cape	31°37'45.67"S, 29°32'11.45"E	MK518212	MK518129	No Sequence
33	Kwancele	1	PEM R22541	Eastern Cape	31°11'41.74"S, 29°47'46.19"E	MK518201	MK518188	MK518276
34	Kokstad	7	PEM R22542-548	KwaZulu-Natal	30°33'14.70"S, 29°25'38.49"E	MK518193–99	MK518110–16	MK518275
35	High Water	1	SANBI 5307	KwaZulu-Natal	28°46'52.79"S, 32°5'54.60"E	MK518186	MK518100	MK518268
36	Sabie	2	PEM R 22549-550	Mpumalanga	25°5'19.90"S, 30°47'31.58"E	MK518217–218	MK518134–35	No Sequence
37	Wolkberg	1	unaccessioned	Limpopo	24°00'04.3"S, 30°04'37.4"E	MK518242	MK518164	MK518288
38	Entabeni	1	SANBI 1942	Limpopo	23°0'27.66"S, 30°15'49.80"E	MK518179	MK518092	MK518264
39		1	CAS 204338	Uganda	–	MK518233	MK518158	No Sequence

Table 2. List of the primer pairs and their respective reference used during the present study on *Duberria lutrix lutrix*.

Locus	Protein coding	Primer name and sequence	Primer reference
ND4	Yes	ND4: 5'-ACC TAT GAC TAC CAA AAG CTC ATG TAG AAG C-3'	Arévalo et al. (1994)
		H12763V: 5'-TTC TAT CAC TTG GAT TTG CAC CA-3'	Barlow et al. (2013)
cyt *b*	Yes	L14910: 5'- GAC CTG TGA TMT GAA AAA CCA YCG TTG T-3'	Burbrink et al. (2000)
		LycodryasG3R: 5'-TGG AAT GGR ATT TTR TCG AT-3'	Ruane et al. (2015)
SPTBN1	Yes	SPTBN1F APR-2010: 5'-TTGGTC GAT GCC AGT TGT A-3'	Ruane et al. (2014)
		SPTBN1R APR-2010: 5'-CAG GGT TTG TAA CCT KTC CA-3'	Ruane et al. (2014)

All PCR amplification was performed using standard protocols. PCR conditions were as followed: 94 °C for 4 min., 94 °C for 30 sec., the annealing temperature of the primers varied between 48 °C to 50 °C for 35 sec., 72 °C for 40 sec., for 35 cycles and a final extension at 72 °C for 10 min. A list of the six primer pairs used are provided in Table 2. PCR products were visualized using a 1% agarose gel that contained a 1% ethidium bromide solution. Following successful amplification of a locus, a BioFLUX gel purification kit was used to purify the amplicons, following the manufacturer's protocol. The gel purified PCR amplicons were sequenced at the Central Analytical Facility (CAF), at Stellenbosch University, using an ABI 3700 automated DNA sequencer.

Phylogenetic analysis

The mtDNA and nuDNA sequences were aligned in CLUSTAL W (Larkin et al. 2007) using the default settings. The two protein-coding mtDNA (ND4 and cyt *b*) loci were examined for the presence of pseudogenes by converting the DNA sequence to amino acids to detect the presence of stop codons. Since all loci on the mtDNA were linked, the two mtDNA loci were combined for the phylogenetic analysis. For ND4 and cyt *b*, 740 bp and 610 bp fragment was respectively sequenced for the 87 *D. l. lutrix* specimens. Sequences were deposited in GenBank (Table 1). The combined mtDNA data set yielded a total of 1350 bp. The DNA substitution models obtained in jModelTest 2.0 (Darriba et al. 2012) are provided in Suppl. material 1. In addition, ND4 and cyt *b* sequences for *D. variegata*, as well as an additional specimen of *D. lutrix*. sp from Kenya were downloaded from GenBank and included in the phylogenetic analysis (Kelly et al. 2008; Vidal et al. 2008). For the SPTBN1, the older samples failed to amplify, hence these were coded as missing during the combined analyses. For the nuDNA SPTBN1, a 760 bp fragment was amplified for 30 *D. l. lutrix* specimens and sequences were deposited in GenBank (accession numbers in Table 1). For the nuclear SPTBN1 locus, allelic heterozygotes were inferred using PHASE (Stephens et al. 2001; Stephens and Scheet 2005). PHASE implements a Bayesian method for reconstructing haplotypes from nuclear sequences that include multiple heterozygous base sites within individuals. To estimate allele frequencies, PHASE was run five times. The run with the best goodness-of-fit to an approximate coalescent model was retained, resulting in two nuclear haplotype sequences of alleles per individual.

The combined mtDNA data set was subjected to a Bayesian inference (BI). The BI analysis was performed in MrBayes v3.2.6 (Ronquist et al. 2012; Huelsenbeck et al. 2016), sampling every 10,000 generations for a total of 10 million generations. Convergence and burn-in were determined using Tracer v1.6 (Rambaut et al. 2014). Nodes were considered well supported when they had a posterior probability (p*P*) of > 0.95. All trees were visualised using TreeGraph 2 (Stöver and Müller 2010). For the combined DNA analysis, a single specimen per locality was used of each of the three loci and the same phylogenetic approaches listed above were followed to reconstruct evolutionary relationships. In addition, we also performed a maximum parsimony (MP) analyses on the combined mtDNA data and the total evidence data sets. MP analyses were executed in PAUP*4 v. beta 10 (Swofford 2002). For the MP analyses, trees were generated using the heuristic search option with tree bisection and reconnection (TBR branch swapping using 100 random taxon additions). Phylogenetic confidence in the nodes recovered from MP was estimated by bootstrapping (Felsenstein 1985) analysing 1000 replicates of the data set. Only bootstrap values >75% were regarded as statistically well supported (Felsenstein 1985).

Both Vidal et al. (2008) and Kelly et al. (2009) demonstrated that the two snake species *Amplorhinus multimaculatus* and *Ditypophis vivax* are sister to *Duberria*. Hence the former two species were used as outgroups. Mitochondrial DNA sequences for the latter two species were downloaded from GenBank whereas the nuDNA locus was

coded absent. Uncorrected sequence divergence values for both the ND4 and the cyt *b* loci were calculated in PAUP*4 version beta 10 (Swofford 2002). We did not combine the two mtDNA to calculate the sequence divergence value since we would not be able to compare this value with other phylogeographic studies since most authors perform this analysis on loci individually.

Population and demographic analysis

Haplotype networks were constructed for the combined mtDNA data, as well as for the nuclear SPTBN1 using TCS version 1.3 using a 95% connection limit (Clement et al. 2000). An analysis of molecular variance (AMOVA) was conducted in ARLEQUIN v3.5.2.2 (Excoffier and Lischer 2010) using the combined mtDNA data. The preliminary analyses of the combined mtDNA topology revealed the presence of five clades, hence a hierarchical AMOVA was performed; 1) across all sample localities and; 2) for haploclade one detected using the combined mtDNA data set. The remaining four clades were not analysed further in AMOVA since the low sample sizes limited any statistical inferences. Two neutrality test using Fu's *Fs* (Fu 1997) and Tajima's D (Tajima 1989) were calculated in ARLEQUIN using 10,000 permutations.

Divergence time estimations

We used BEAST v1.8.3 (Drummond et al. 2012) on the mitochondrial data set to determine the age of divergent events within *Duberria l. lutrix*. No fossil calibrations points are available for *Duberria*. The genus *Duberria* belongs to the subfamily Pseudoxyrhophiinae which is part of the family Lamprophiidae. The Lamprophiidae is in turn sister to the Elapidae and both being sister to the superfamily Colubroidea. Hence published mutation rates from the superfamily were used in the divergence time estimation (Figueroa et al. 2016; Hsaing et al. 2015 For the two mtDNA loci (ND4 and cyt *b*) a strict substitution rate of 1.34% (SD=0.251) per million years was used (Daza et al. 2009) after having checked that the relaxed log-normal clock's standard deviation approached zero. The mutation rate for the nuclear marker is unknown, hence this marker was excluded from the divergence time estimation. We ran the analyses under the substitution model as inferred above (TrN+Gamma), unlinked between both mitochondrial genes, and under a coalescent prior with constant population size, as the resulting tree described within species relationships. We ran the Markov chain for 50 million iterations and sampled every 10,000 iteration. Tracer v1.6 (Rambaut et al. 2014) was used to assess chain convergence to ensure minimal autocorrelation between iterations (effective sample size > 2000 for all sampled parameters) and to determine the burn in (10% of samples). TreeAnnotator in BEAST was used to determine a maximum clade credible tree.

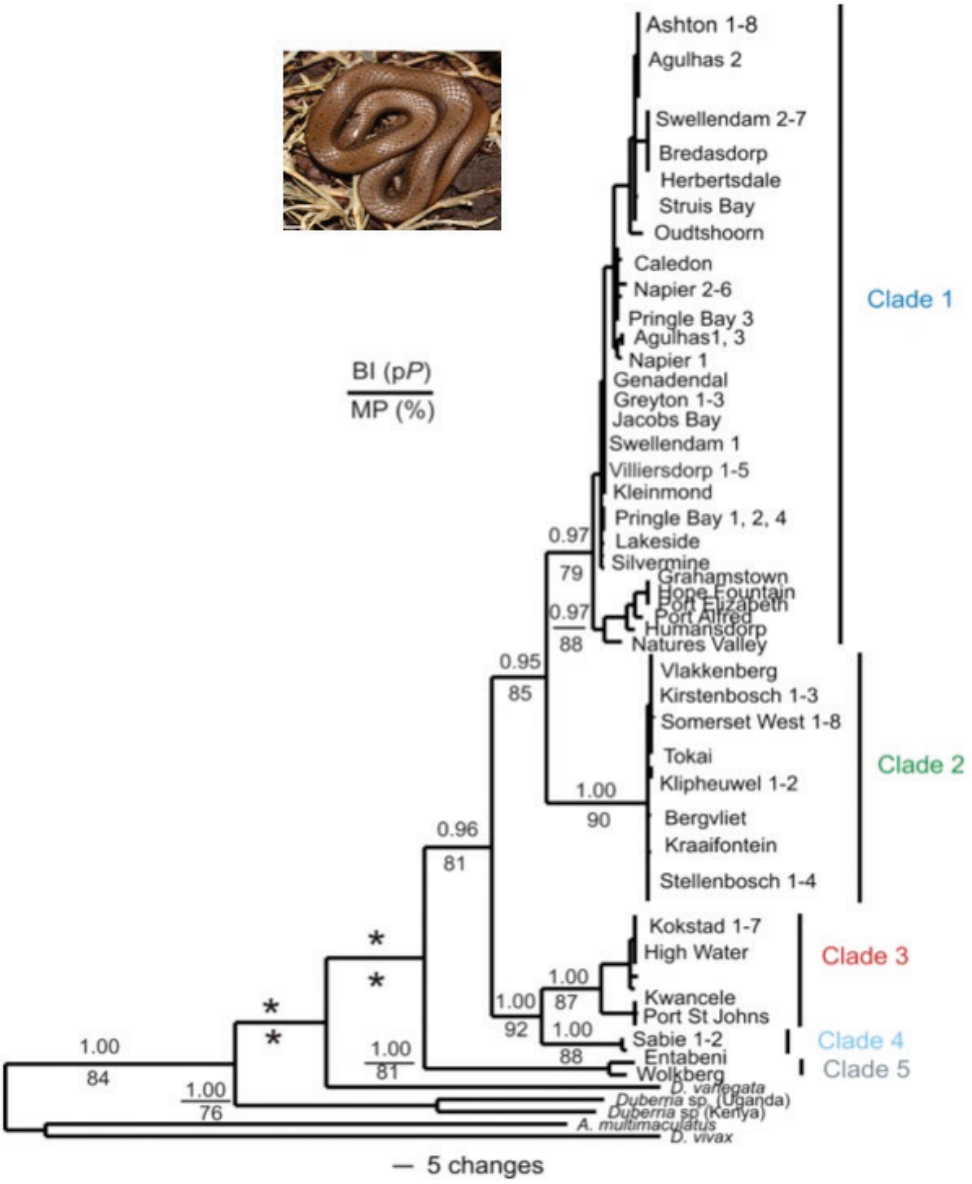

Figure 2. A Bayesian inference topology derived from the combined mtDNA analyses (ND4 + cyt *b*) amongst the South African *Duberria l. lutrix*. The posterior probability value (p*P*) values are presented above each node. Only p*P* values >0.95 are shown. Values below each node are bootstrap values for MP. Only bootstrap values >75% are shown. An asterisk (*) below or above a node indicates the absence of statistical support. The insert shows the typical *Duberria lutrix lutrix*.

Results

Combined mtDNA analyses (*ND4* and *cyt* b)

The MP and BI analyses retrieved near identical tree topologies, hence only the BI topology is shown and discussed. For MP, of a total of 1350 characters, 278 characters were found to be parsimony informative, and recovered 167 trees with a tree length of 597 steps with a consistency index (CI) of 0.59 and a retention index (RI) of 0.88. The BI topology (Fig. 2) retrieved the two East African specimens (Kenya and Uganda) of *Duberria* as basal, while *D. variegata* appeared sister to a South African clade of *D. l. lutrix*, with low statistical support for the monophyly of the clade. Within *D. l. lutrix*, five geographically discrete, statistically well-supported clades were detected (>75%/>0.95 p*P*). Clade one consisted of specimens that occurred predominantly from above the Hottentots Holland Mountains, Agulhas plain and Overberg, the Cape Peninsula and the south-eastern Cape and adjacent interior, and was sister to clade two. Clade two comprised specimens from below the Hottentots Holland Mountains, including the Cape Peninsula and Boland region. Clade three was comprised of specimens from the Eastern Cape coast and samples from interior of KwaZulu-Natal Province, and was sister to clade four. Clade four comprised two specimens from Mpumalanga (Sabie), while clade five comprised two specimens exclusive to the Limpopo Province (Entabeni and Wolkberg).

Population genetic analysis using the combined mtDNA

For the ND4 locus the maximum uncorrected sequence divergence between the first and second clades was 1.68%. Between the second and third clade the maximum uncorrected sequence divergence was 3.84%. Between the third and the fourth clades the maximum uncorrected sequence divergence was 5.95%. Finally, the maximum uncorrected sequence divergence between clades four and five was 6.75% (Suppl. material 1: Table S1). For cyt *b* locus the maximum uncorrected sequence divergence between the first and second clades was 0.98%. Between the second and third clade the maximum uncorrected sequence divergence was 2.62%. Between the third and the fourth clades the maximum uncorrected sequence divergence was 3.77%. The maximum uncorrected sequence divergence between clades four and five was 6.01% (Suppl. material 2: Table S2). The ND4 locus revealed slightly higher levels of uncorrected sequence divergence values during the present study in comparison to the cyt *b* locus.

A total of 35 haplotypes were retrieved for the 87 *Duberria l. lutrix* specimens using the combined mtDNA (Fig. 3). For details of the haplotype distribution consult the Suppl. material 1: Table S3. Five haplogroups were retrieved, revealing a pattern congruent with the combined mtDNA topology (Fig. 2). The AMOVA results among all 38 sample localities revealed that 94.26% (Va = 15.89; df = 37; SS = 1394.37) of the variation occurred among sample sites, while 5.74% (Vb = 0.96; df = 49; SS = 47.44) of the variation occurred within sample sites. These results are indicative of marked genetic differentiation, a result that is corroborated by the marked Φst (0.94) as well as the high

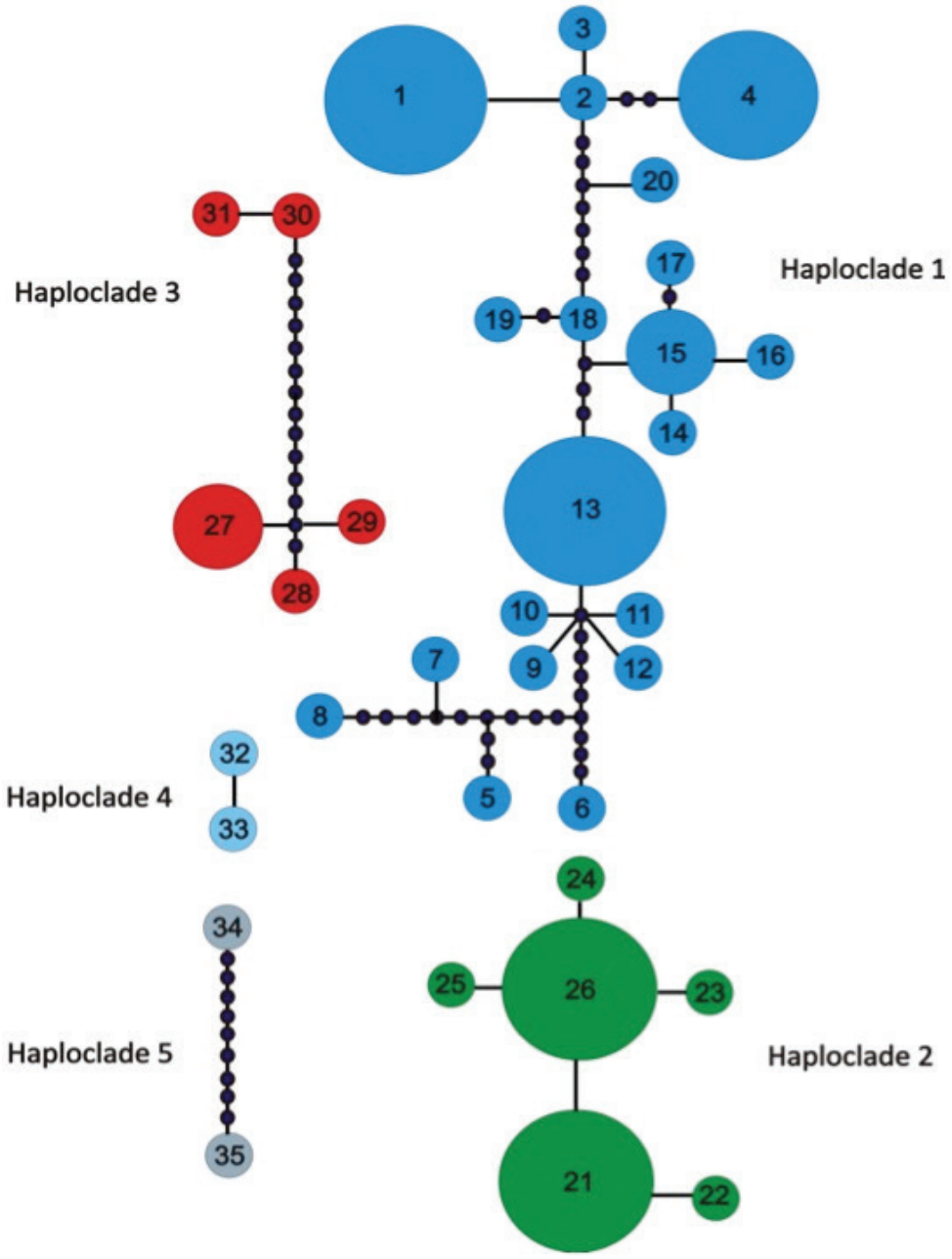

Figure 3. A minimum spanning network derived from combined mtDNA (ND 4 + cyt *b*) analyses demonstrating the five haploclades for *Duberria l. lutrix*. The number inside the boxes correspond to the haplotypes in Table 3. The closed black dots represent unsampled or missing haplotypes.

F_{ST} values among sample localities that were statistically significant for 56 combinations, ranging from 0.05 to 0.99 (results not shown). Haploclade one (Fig. 3) consisted of 20 haplotypes comprising samples from above the Hottentots Holland Mountains,

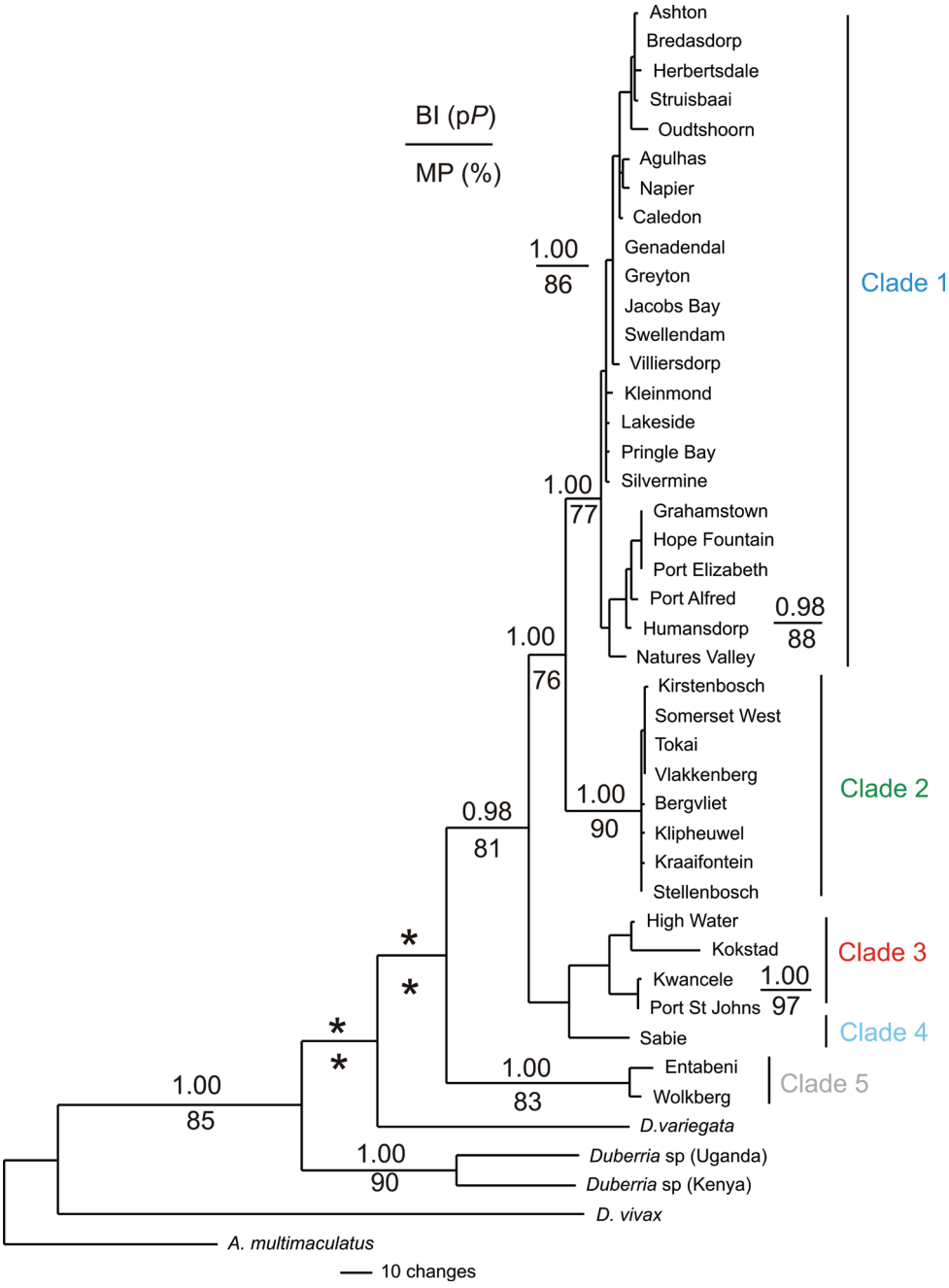

Figure 4. A Bayesian inference topology for the total evidence data sets (ND4 + cyt *b* + SPTBN1) amongst the South African *Duberria l. lutrix* species complex. The posterior probability value (p*P*) values are presented above each node. Only p*P* values >0.95 are shown. Values below each node are bootstrap values for MP. Only bootstrap values >75% are shown. An asterisk (*) below or above a node indicates the absence of statistical support.

Agulhas plain and the Cape Peninsula separated by ten unsampled / missing mutations from samples from the south-eastern Western Cape, with 76.46% of the variation occurring among sample localities, (Va = 3.07; df = 22, SS = 167.63), 23.58% occurred within sample localities (Vb = 0.94; df = 29; SS = 27.53), with a high Φst (0.74) as well as high F_{ST} values among sample localities that were generally statistically significant. The remaining four haploclades, two, three, four and five respectively (corresponding to the identical clades observed in Fig. 2) contained fewer than five haplotypes, hence we did not undertake any further statistical analyses due to the low sample sizes.

Fu's *Fs* values were positive for Agulhas, Kokstad, Pringle Bay and Swellendam and indicate an excess of intermediate polymorphisms due to recent population bottlenecks or balancing selection, however none of these were statistically significant. Two of the Fu's *Fs* values were negative for Somerset West and Napier indicating an excess of low frequency polymorphisms consistent with population expansions or positive directional selection. However, only the Somerset West population was statistically significant ($P < 0.02$). The remaining sample localities had a Fu's *Fs* of zero. For Tajima's D, five sample localities, Swellendam, Pringle Bay, Napier, Somerset West and Kokstad were negative, while only Napier and Kokstad were statistically significant ($P < 0.05$). The remaining sample localities has a Tajima's D of zero. Negative Tajima's D indicates an excess of low frequency polymorphism, population expansion or purifying selection.

SPTBN1

Ten haplotypes were retrieved for the 30 specimens using the TCS analyses (network not shown). For a list of the sample localities per haplotype consult Suppl. material 2. Three haploclades were retrieved. Haploclade one contained six haplotypes from all the remaining sample localities (from clades 1, 2, 3 and 4; Fig. 2). Haploclade two contained a single haplotype from Oudtshoorn (clade 1; Fig. 2). Haploclade three contained three haplotypes from localities in the KwaZulu-Natal (Kokstad) and the Limpopo provinces (Wolkberg and Entabeni) (from clades five and three respectively; Fig. 2).

Total evidence phylogeny (*ND4*, cyt *b* + SPTBN 1)

The combined mtDNA and nuDNA sequence data yielded a total of 2110 bp. The MP and the BI analyses retrieved highly congruent tree topologies, hence only the BI topology is shown. For the MP analyses, 293 characters were found to be parsimony informative, tree length of 627 steps, 730 trees, with CI = 0.58 and RI = 0.77. The total evidence BI topology (Fig. 4) was congruent with the combined mtDNA topology (Fig. 2). A monophyletic *Duberria* was retrieved with the two East African *Duberria* specimens (*D. l. atriventris*) form a basal split, while *D. variegata* was sister to a clade containing all the *D. l. lutrix* samples from South Africa. These five clades were also evident in the combined mtDNA topology (Fig. 2).

Divergence time estimate based on total mtDNA data

Clade five diverged from the remaining *D. l. lutrix* clades 3.42 Mya (4.13 Mya to 2.72 Mya; 95% HPD). These divergences dates fall into the Pliocene and Pleistocene epochs. Clade four diverged from clades one, two and three occurred 2.05 Mya (1.34 Mya to 2.95 Mya; 95% HPD). The divergence between clade three from clades one and two occurred 1.23 Mya (0.74 Mya and 1.81 Mya; 95% HPD). Cladogenesis between clades one and two occurred 0.46 Mya (0.27 Mya and 0.69 Mya; 95% HPD) (Suppl. material 3).

Discussion

Biogeographic affinities

The phylogeographic results demonstrated the presence of five mtDNA clades across the sampled distribution range of *Duberria l. lutrix* in South Africa, implying that the snake represents a species complex. Furthermore, these five clades were characterised by the absence of shared mtDNA haplotypes and marked sequence divergences values for both the ND4 and cyt *b* loci, suggesting possible genetic isolation and limited dispersal. However, the nuclear DNA sequences data failed to retrieve patterns congruent with the mtDNA data, hence it is not possible to exclusively imply the presence of five possible taxa within the *Duberria l. lutrix* species complex, and requires further taxonomic delineation. In addition, the divergence time estimates suggest that cladogenesis in the *D. l. lutrix* species complex occurred during the Plio/Pleistocene epochs, a period that was characterised by increased aridification throughout South Africa, resulting in the contraction of mesic adapted species. Our results reflect the impact of Plio/Pleistocene climatic driven fragmentation on a snake species resulting in possible cladogenesis. The latter result is in line with what has been reported for the widely distributed puff adder in South Africa (Barlow et al. 2013).

During the late Miocene, climatic profiles changed dramatically, resulting in decreasing levels of precipitation and marked aridification, a trend that was enhanced in the Plio/Pleistocene (Siesser 1980; Marlow et al. 2000; Cowling et al. 2009; Engelbrecht et al. 2013). Furthermore, during the Plio/Pleistocene epochs, coastal regions experienced dramatic marine transgressions that have been estimated to vary between 150–200 meters in certain regions (Partridge and Maud 1987, 2000; Partridge 1997). These events likely resulted in the extinction of low-lying terrestrial taxa characterised by low vagility and habitat specificity and the contraction of animals to high-lying refugial areas along the coastal belt mountains and the adjacent interior. More recently, during periods of glacial maxima in the Holocene, the interior of South Africa is thought to have become more inhospitable to ectotherms, due to low winter temperatures and reduced precipitation levels. These factors possibly caused mesic adapted organisms such as *D. l. lutrix* to seek more favourable habitat along the high-lying

mountainous coastal regions of the Cape Fold and Drakensberg Mountains (Barlow et al. 2013; Tolley et al. 2014). During the Last Glacial Maximum coastal areas would have had exposed areas of continental shelf, off the current south-west and western coasts of South Africa, due to the lowering sea levels. This would have provided favourable habitat, as well as, acting as dispersal corridors for many species (Schreiner et al. 2013). During the interglacial period these corridors would have been inaccessible due to the rising of sea levels. Rapid changes in elevation can provide significant biogeographic barriers to the dispersal of ectotherms; this is evident when one compares the geographic topology of clades one and three. Clade one extends from the western coast of the Cape Peninsula above the Hottentots Holland Mountains range until just before the south-eastern Cape coastline whilst clade three occurs below the Hottentots Holland mountain range extending into the Cape Flats. The rapid changes in elevation between the two clades limits the dispersal of *D. l. lutrix*. Similar phylogeographic breaks have been observed in other co-distributed reptile species (Daniels et al. 2007, 2009; Tolley et al. 2009, 2014; Barlow et al. 2013). Climatic fluctuations would have altered environmental conditions during the Plio/Pleistocene allowing for dispersal of *D. l. lutrix* around these mountain ranges due to the changes in sea levels. Further evidence for isolation induced by climatic conditions during the Plio/Pleistocene can be found between clades one and two. Clade one occurs predominately throughout the western half of the Greater Cape Floristic Region (GCFR), namely the Cape Peninsula, above the Hottentots Holland mountain ranges and throughout the Agulhas plains and Klein Karoo, whilst clade two occurs within the eastern half of the GCFR, along the south-eastern Cape coastline and interior. Potentially, the reason for the genetic isolation between the two clades may be due to changes in the climatic conditions across the GCFR. The western and eastern sections of the GCFR are characterised by distinct rainfall regimes with the western half being characterised by a winter rainfall, while the eastern half is characterised by aseasonal and/or summer rainfall regime (Siesser 1980; Cowling et al. 2009; Tolley et al. 2009). It has been hypothesised that the division between some clades of reptiles corresponds to the changes in the rainfall regimes (Tolley et al. 2009, 2014). This is further corroborated by clades two and four which are found in the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces. The Bedford gap is situated between the two clades. However, it is uncertain whether the genetic isolation is due to a combination of the xeric conditions and changes in rainfall patterning or simply due to one of the two variables. As rainfall patterns change and environments become more xeric, the minimum annual temperature of the area decreases, which can potentially limit the dispersal capabilities, as well as, survival capabilities of ectotherms. Furthermore, this would explain why *D. l. lutrix* has not dispersed along the western coastline of South Africa where the average annual precipitation and minimum annual temperatures are lower. This can be further evaluated by observing the effects that the Breede River xeric corridor had on the phylogenetic patterning of *D. l. lutrix*. When examining the topology of the phylogenetic trees (Figs 2, 4) the Breede River xeric corridor did not display any pronounced impact on the phylogeographic patterning. This observation favours the

change in rainfall patterning as a possible source for the genetic isolation observed within *D. l. lutrix*. However, climatic fluctuations during interglacial periods would have lessened the impact that this xeric valley had on the dispersal of this species as precipitation levels changed. Finally, the extinction of intermediary haplotypes, possibly due to the climatic fluctuations, in widely distributed species with low dispersal capabilities and gene flow may have resulted in these pronounced phylogenetic gaps. Widespread sampling of *D. l. lutrix* is required to affirm or reject these inferences.

Although the climatic fluctuation would have forced species to retreat into refugia (Barlow et al. 2013; Tolley et al. 2014), evidence for the relictual populations, which are proposed to be restricted to the interior in the Klein Karoo and Free State are not corroborated by our results. The haplotype network for the nuclear marker SPTBN1 showed the Klein Karoo locality to be isolated from the other localities in the western and south-eastern Cape coastline and interior. However, this can be potentially biased; as firstly, the SPTBN1 is a protein coding locus and secondly, the mutation rates for nuclear markers in ectotherms are much slower than the mitochondrial markers, limiting inferences derived from them.

Cryptic diversity and taxonomy

The combined mitochondrial and nuclear data set retrieved five clades that show evidence for geographically distinct *Duberria lutrix lutrix* lineages. The mtDNA data shows, high levels of uncorrected sequence divergence values. Glaw et al. (2007) reported that within the Malagasy *Geodipsas infralineata* using the cyt *b* marker uncorrected sequence divergence between the two clades ranged between 4.7–4.8%. Similarly, Ruane et al. (2017) using morphology and DNA sequencing (of the cytochrome oxidase one locus) observed two clades within the widespread Malagasy snake *Mimophis mahfalensis*. It is noteworthy, that while the phylogenetic affinities within the Lamprophiidae has recently received attention (Vidal et al. 2008; Portillo et al. 2018), phylogeographic studies remain limited. The latter observation suggests that species diversity among widespread species in the family may have resulted in an underestimation of alpha taxonomic diversity.

These sequence divergence values are similar to values observed in other snake lineages within Colubroidea and the Malagasy Pseudoxyrhophiinae that are considered genetically different (Feldman and Spicer 2002; Gou et al. 2011; Kindler et al. 2013; Ruane et al. 2015). This indicates that the respective clades might potentially be composed of cryptic species; however, we are cautious of the pitfalls of using these divergence estimations as exclusive evidence for species boundaries. Our results partially support the observation by Branch (2002, 2003), that the clade to which specimens from Port St Johns belong may represent a cryptic lineage. However, considering the sparse sampling of *D. l. lutrix* during the present study this might be an underestimation of species diversity. We advocate larger sample sizes per sample locality and a more comprehensive geographic sampling of the species range throughout South

Africa coupled with more sensitive nuclear DNA markers, such as microsatellites or single nucleotide polymorphisms (SNP's) to examine patterns of biparental gene flow. However, it is frequently difficult to obtain large sample sizes for snakes, specifically for phylogeographic studies. Similar observation has been made in other studies on snakes (Martínez-Freiría et al. 2015).

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Supplementary material 1

Uncorrected ("p") distance matrix for the ND4 locus for all the *Duberria lutrix lutrix* samples used during the present study

Authors: Kyle Kulenkampff, Francois Van Zyl, Sebastian Klaus, Savel R. Daniels

Data type: molecular data

Link: https://doi.org/10.3897/zookeys.838.32022.suppl1

Supplementary material 2

Uncorrected "p" distances for the cyt b for the *Duberria lutrix lutrix* sampled during the present study

Authors: Kyle Kulenkampff, Francois Van Zyl, Sebastian Klaus, Savel R. Daniels

Data type: molecular data

Link: https://doi.org/10.3897/zookeys.838.32022.suppl2

Supplementary material 3

Distribution of the 35 haplotype frequency for the combined mtDNA loci sequence data for the 87 *Duberria lutrix lutrix* specimens

Authors: Kyle Kulenkampff, Francois Van Zyl, Sebastian Klaus, Savel R. Daniels

Data type: molecular data

Explanation note: The haplotype numbers (N) corresponds to the numbers on the minimum spanning network (Fig. 3).

Link: https://doi.org/10.3897/zookeys.838.32022.suppl3