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On the identity of *Chamaedrilus glandulosus* (Michaelsen, 1888) (Clitellata, Enchytraeidae), with the description of a new species

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Abstract

The taxonomy of *Chamaedrilus glandulosus* (Michaelsen, 1888) s. l., most commonly known previously as *Cognettia glandulosa*, is revised. A recent molecular systematic study has shown that this taxon harbours two cryptic, but genetically well separated lineages, each warranting species status. In this study these two lineages are scrutinized morphologically, on the basis of Michaelsen's type material as well as newly collected specimens from Central and Northern Europe. *Chamaedrilus glandulosus* s. s. is redescribed and *Ch. varisetosus* **sp. n.** is recognized as new to science. The two species are morphologically very similar, differing mainly in size, but seem to prefer different habitats, with *Ch. glandulosus* being a larger aquatic species, and *Ch. varisetosus* being smaller and mainly found in moist to wet soil.

Keywords

Cognettia, *Chamaedrilus*, cryptic species, Oligochaeta, taxonomy

Introduction

In 1888 Michaelsen described an enchytraeid worm, *Pachydrilus sphagnetorum* var. *glandulosus* Michaelsen, 1888, as a variant of *P. sphagnetorum* Vejdovský, 1878. The description was based on material from the banks of the Bille and Elbe rivers in Hamburg, northern Germany. These two taxa were then transferred to *Marionina* Michaelsen, 1890 (in Pfeffer 1890), and *P. sphagnetorum* var. *glandulosus* was considered a good species, *Marionina glandulosa*, separate from *M. sphagnetorum* (Michaelsen 1900). Later Friend (1919) assigned both species to *Chamaedrilus* Friend, 1913, an action seldom noticed by subsequent authors. For instance, when Nielsen and Christensen (1959) established *Cognettia*, they transferred *Marionina glandulosa* to their new genus without considering its previous placement in *Chamaedrilus*. Nielsen and Christensen's (1959) concept of *Cognettia* came to embrace a number of terrestrial and freshwater enchytraeids and until recently it has been widely accepted. However, as noted by Schmelz and Collado (2010) and now more closely investigated by ourselves (Martinsson et al. 2014), *Cognettia* is indeed a junior synonym to *Chamaedrilus*. For details about the complex taxonomical history and a formal revision of *Chamaedrilus*, see Martinsson et al. (2014).

Several cryptic forms have been found within well-known morphology-based taxa of former *Cognettia* (Martinsson and Erséus 2014). The morphospecies *Chamaedrilus sphagnetorum* s. l. was found to be a non-monophyletic assemblage of at least four species; these have been revised and described by Martinsson et al. (2014). The taxon *Ch. glandulosus*, on the other hand, traditionally distinguished from *sphagnetorum* by the possession of secondary septal glands and longer spermathecal ectal ducts (Nielsen and Christensen 1959), was shown by both nuclear and mitochondrial DNA evidence to consist of two separately evolving lineages in Northern Europe. These two lineages appeared as sister species, i.e., representing a monophyletic group (Martinsson and Erséus 2014). According to Christensen (1959) *Ch. glandulosus* s. l. reproduces both by fragmentation and parthenogenetically, but the eggs must be activated by spermatozoa for normal development (Christensen 1961). However it is still possible that at least one of the two cryptic species occasionally reproduces biparentally. Uniparental reproduction makes species delimitation problematic, in particular when referring to the biological species concept (Mayr 1942). However, as discussed by Martinsson and Erséus (2014), asexual organisms form distinct clusters and can be delimited using the unified species concept by de Queiroz (2007). According to this concept, the sole requirement of a species is that it is a separately evolving metapopulation lineage, and criteria (e.g. morphological differences, reproductive isolation, or gene tree monophyly) from any of the more traditional species concepts can be used to delimit the lineages. The greater the number of criteria supporting a divergence, the stronger the case is for speciation, but, even a single piece of evidence, if properly substantiated, may be enough to establish lineage separation.

The aim of this study is to revise the taxonomy of *Chamaedrilus glandulosus* s. l. by delimiting *Ch. glandulosus* s. s., with the designation of a lectotype, and describing *Ch. varisetosus* sp. n.

Material and methods

This study is based on two syntypes of *Pachydrilus sphagnetorum* var. *glandulosus* Michaelsen, 1888, from the original syntype series of ten, borrowed from the Zoological Museum of Hamburg University (ZMUH), Germany, of which one is here designated as lectotype, plus material analysed by Martinsson and Erséus (2014), and new specimens collected in northern and central Europe. A list of all examined specimens, with locality data and GenBank accession numbers for DNA-barcodes is given in Table 1.

Newly collected specimens were DNA-barcoded using the cytochrome c oxidase subunit I (COI) marker, as described by Martinsson and Erséus (2014); DNA was extracted from a few posterior-most segments of each worm, using Epicentre QuickExtract DNA Extraction Solution 1.0, following the manufacturer's instructions, while the rest of the specimen was used for morphological studies, i.e., as a voucher. All new barcodes were matched with COI sequences of *Cognettia glandulosa* 'A' and 'B' from Martinsson and Erséus (2014). For tissue samples of the over 100 years old syntypes, newly designed primers were tested to amplify a short part of COI, as well as a fragment of the ribosomal 16S mtRNA gene, respectively, but these attempts were unsuccessful.

Unless otherwise mentioned in the descriptions, all information refers to the studied material only, in that the two taxa treated in this paper have previously been classified as one and the same species. Michaelsen's syntypes were first studied as temporary mounts in glycerol. The newly designated lectotype was then stained with paracarmine and permanently mounted in Canada balsam on a slide as outlined by Erséus (1994), and so were all other voucher specimens (including the types of *Ch. varisetosus* sp. n.). All measurements and observations were made on preserved and somewhat compressed animals under a compound microscope (Leitz Laborlux K). As the posterior parts of the specimens were used for DNA extraction, the body size is arbitrarily given as the length of the 20 anteriormost segments and the width in segment XII (latter representing not clitellum but general body width). This size estimate was used also in Martinsson et al. (2014). In the descriptions, body measurements are given as the range followed by the mean ± 1 standard deviation. Differences in size between the two species were visualised with boxplots (Fig. 1, where asterisks denote the outliers), and tested by using two-sided t-tests performed in SPSS v. 22 (SPSS Inc., Chicago). Sketches were drawn using a camera lucida and used as templates for producing digital illustrations with Adobe PhotoShop.

The geographical distributions consider the origin of our material as well as that of COI barcode matches in BOLD (Barcoding of Life Data Systems, Ratnasingham and Hebert 2007). The Barcode Index Numbers (BIN) (Ratnasingham and Hebert 2013) are given under Remarks, for respective species. The BIN system clusters the sequences to produce operational taxonomic units that are assumed to closely correspond to species (http://www.boldsystems.org).

All specimens studied, including new types, are deposited in the Swedish Museum of Natural History (SMNH), Stockholm, the University Museum Bergen (UMB),

Table 1. List of material included in this study, with specimen identification numbers, voucher numbers, collection data, GPS coordinates, and GenBank accession numbers for COI barcodes. Voucher numbers in bold indicate type specimens, barcode numbers in bold are newly generated sequences. Locality data are given in the form: country, province, municipality and locality; GPS coordinates are given as decimal degrees. CZ = Czech Republic, FIN = Finland, GER = Germany, NOR = Norway, SWE = Sweden.

Species	Spm. nos.	Museum voucher nos.	Sexual maturity	Collection locality	Coordinates		Leg.	Coll. date	Barcode Acc. nos.
					N	E			
Ch. glandulosus		**ZMUH V 429a**	mature	**GER. Hamburg**, Hamburg, Bille River bank	53.54	10.09	W. Michaelsen	Pre 1888	-
Ch. glandulosus		**ZMUH V 429b**	immature	**GER. Hamburg**, Hamburg, Bille River bank	53.54	10.09	W. Michaelsen	Pre 1888	-
Ch. glandulosus	CE2011	SMNH133613	immature	**SWE. Västergötland**, Vårgårda, Lången Lake littoral	57.9973	12.5868	C. Erséus	Jun 30 2006	KF672372
Ch. glandulosus	CE2841	SMNH133614	immature	**SWE. Öland**, Borgholm, Räpplinge, stream	56.8195	16.9444	A. Ansebo, L. Matamoros & C. Erséus	Jun 13 2007	KF672374
Ch. glandulosus	CE2887	SMNH133615	submature	**SWE. Södermanland**, Vingåker, Låttern Lake littoral	59.0854	16.0426	C. Erséus	Jul 30 2007	KF672375
Ch. glandulosus	CE2888	SMNH133616	submature	**SWE. Södermanland**, Vingåker, Låttern Lake littoral	59.0854	16.0426	C. Erséus	Jul 30 2007	KF672376
Ch. glandulosus	CE2889	SMNH133617	immature	**SWE. Södermanland**, Vingåker, Låttern Lake littoral	59.0854	16.0426	C. Erséus	Jul 30 2007	KF672377
Ch. glandulosus	CE2890	SMNH133618	immature	**SWE. Södermanland**, Vingåker, Låttern Lake littoral	59.0854	16.0426	C. Erséus	Jul 30 2007	KF672378
Ch. glandulosus	CE2891	SMNH133619	immature	**SWE. Södermanland**, Vingåker, Låttern Lake littoral	59.0854	16.0426	C. Erséus	Jul 30 2007	KF672379
Ch. glandulosus	CE8510	SMNH133620	immature	**SWE. Lappland**, Kiruna, Abisko, marsh pond	68.3485	18.9719	D. Fontaneto	Jul 6 2007	JN260143
Ch. glandulosus	CE10655	SMNH133612	immature	**FIN. Keski-Suomi,** Jyväskylä, Alvajärvi Lake littoral	62.315	25.730	H. Saarikoski	Fall 2009	JN260270
Ch. glandulosus	CE17761	SMNH142041	immature	**SWE. Södermanland**, Vingåker, Hjälmaren Lake littoral	59.133	15.814	C. Erséus	Jul 27 2012	**KP878475**
Ch. glandulosus	CE17806	SMNH142042	immature	**SWE. Södermanland**, Vingåker, Hjälmaren Lake littoral	59.133	15.814	C. Erséus	Jul 27 2012	**KP878476**
Ch. glandulosus	CE18516	SMNH142043	submature	**SWE. Västergötland**, Lerum, Aspen Lake littoral	57.7656	12.2525	C. Erséus & B. Williams	Jun 1 2013	-
Ch. glandulosus	CE18517	SMNH142044	mature	**SWE. Västergötland**, Lerum, Aspen Lake littoral	57.7656	12.2525	C. Erséus & B. Williams	Jun 1 2013	**KP878477**
Ch. glandulosus	CE18518	SMNH142045	immature	**SWE. Västergötland**, Lerum, Aspen Lake littoral	57.7656	12.2525	C. Erséus & B. Williams	Jun 1 2013	**KP878478**
Ch. glandulosus	CE20212	SMNH142046	immature	**NOR. Östfold**, Halden, Enningdalselva River	58.9099	11.5210	C. Erséus	Oct 12 2013	**KP878474**
Ch. varisetosus	CE2634	SMNH133600	immature	**SWE. Öland**, Borgholm, S Greda, sandy soil	56.9929	16.8765	A. Ansebo, L. Matamoros & C. Erséus	Jun 12 2007	KF672367
Ch. varisetosus	CE2931	SMNH133601	immature	**SWE. Öland**, Borgholm, Egby, peaty soil	56.8621	16.8539	A. Ansebo, L. Matamoros & C. Erséus	Jun 12 2007	KF672368
Ch. varisetosus	CE4027	SMNH133602	immature	**SWE. Skåne**, Ystad, Nyvangsskogen, wet soil	55.5606	13.8239	C. Erséus	May 31 2008	KF672369
Ch. varisetosus	CE4028	SMNH133603	immature	**SWE. Skåne**, Ystad, Nyvangsskogen, wet soil	55.5606	13.8239	C. Erséus	May 31 2008	KF672370
Ch. varisetosus	CE6626	SMNH133604	immature	**SWE. Uppland**, Vallentuna, Brottby,peaty soil	59.5477	18.2467	C. Erséus	Jun 4 2009	KF672371

Species	Spm. nos.	Museum voucher nos.	Sexual maturity	Collection locality	Coordinates		Leg.	Coll. date	Barcode Acc. nos.
					N	E			
Ch. varisetosus	CE9376	SMNH133605	immature	**SWE. Medelpad**, Timra, Söråker, forest soil	62.5235	17.4782	C. Erséus	Jun 8 2010	KF672424
Ch. varisetosus	CE9517	SMNH133606	immature	**SWE. Lappland**, Kiruna, Björkliden, peat	68.4262	18.3509	C. Erséus	Jun 12 2010	JN260194
Ch. varisetosus	CE9524	SMNH133607	immature	**SWE. Lappland**, Kiruna, Björkliden, river	68.4277	18.4448	C. Erséus	Jun 12 2010	KF672425
Ch. varisetosus	CE9525	SMNH133608	immature	**SWE. Lappland**, Kiruna, Björkliden, river	68.4277	18.4448	C. Erséus	Jun 12 2010	JN260195
Ch. varisetosus	CE9526	SMNH133609	immature	**SWE. Lappland**, Kiruna, Björkliden, river	68.4277	18.4448	C. Erséus	Jun 12 2010	JN260282
Ch. varisetosus	CE9536	SMNH133610	immature	**SWE. Lappland**, Kiruna, Kiruna, forest soil	67.8546	20.2173	C. Erséus	Jun 13 2010	JN260198
Ch. varisetosus	CE9581	SMNH133611	immature	**SWE. Lappland**, Vilhelmina, Klimpfjäll, grassland soil	65.0621	14.8066	C. Erséus	Jun 15 2010	JN260206
Ch. varisetosus	CE11485	SMNH142026	immature	**SWE. Västergötland**, Lerum, Almekärr, wet soil	57.7614	12.2706	C. Erséus & A. Achurra	Apr 23 2011	**KP878462**
Ch. varisetosus	CE18904	SMNH142027	immature	**NOR. Telemark**, Hjartdal, Kovstulheia, stream	59.8182	8.7222	C. Erséus & B. Williams	Jun 13 2013	**KP878463**
Ch. varisetosus	CE19031	SMNH142028	immature	**NOR. Telemark,** Kviteseid, Kviteseid, wet forest litter	59.3532	8.5196	C. Erséus & B. Williams	Jun 13 2013	**KP878460**
Ch. varisetosus	CE19052	**ZMBN99905**	mature	**NOR. Buskerud**, Hol, Örtedalsåna River, wet moss	60.4866	7.8562	C. Erséus	Aug 10 2013	**KP878464**
Ch. varisetosus	CE19113	SMNH142029	immature	**NOR. Buskerud**, Hol, Geilo, forest soil	60.5329	8.2113	C. Erséus	Aug 11 2013	**KP878465**
Ch. varisetosus	CE19117	SMNH142030	immature	**NOR. Buskerud**, Hol, Geilo, forest soil	60.5329	8.2113	C. Erséus	Aug 11 2013	**KP878466**
Ch. varisetosus	CE19677	SMNH142031	immature	**NOR. Sör-Tröndelag**, Tydal, Langsvola, litter	62.8388	11.805	C. Erséus	Aug 14 2013	**KP878467**
Ch. varisetosus	CE19716	SMNH142032	immature	**NOR. Sör-Tröndelag**, Röros, Hitterdalen, stream bank	62.6060	11.6599	C. Erséus	Aug 15 2013	**KP878461**
Ch. varisetosus	CE19749	SMNH142033	immature	**NOR. Sör-Tröndelag**, Röros, Doktortjönna Lake shore	62.5763	11.3745	C. Erséus	Aug 15 2013	**KP878468**
Ch. varisetosus	CE19818	**ZMBN99906**	submature	**NOR. Hedmark**, Engerdal, Nymoen, wet moss	61.6569	11.8164	C. Erséus	Aug 15 2013	**KP878469**
Ch. varisetosus	CE19819	**SMNH Type-8732**	submature	**NOR. Hedmark**, Engerdal, Nymoen, wet moss	61.6569	11.8164	C. Erséus	Aug 15 2013	**KP878470**
Ch. varisetosus	CE19823	SMNH142034	immature	**NOR. Hedmark**, Engerdal, Nymoen, wet moss	61.6569	11.8164	C. Erséus	Aug 15 2013	**KP878471**
Ch. varisetosus	CE19831	SMNH142035	immature	**NOR. Hedmark**, Engerdal, Nymoen, wet moss	61.6569	11.8164	C. Erséus	Aug 15 2013	**KP878472**
Ch. varisetosus	CE19832	SMNH142036	immature	**NOR. Hedmark**, Engerdal, Nymoen, wet moss	61.6569	11.8164	C. Erséus	Aug 15 2013	**KP878459**
Ch. varisetosus	CE20021	SMNH142037	immature	**NOR, Östfold**, Hvaler, Asmalöy, dry soil	59.0630	10.9396	C. Erséus	Sep 22 2013	**KP878473**
Ch. varisetosus	CE20046	SMNH142038	immature	**NOR. Östfold**, Fredikstad, Trosvik, litter on clay	59.2364	10.9012	C. Erséus	Sep 23 2013	**KP878479**
Ch. varisetosus	SM171	SMNH142039	immature	**CZ. NW Moravia**, Okres Šumperk, Králický Sněžník, moss in stream	50.1499	16.8624	K. Elliott & S. Martinsson	Jun 15 2013	**KP878457**
Ch. varisetosus	SM172	SMNH142040	immature	**CZ. NW Moravia**, Okres Šumperk, Králický Sněžník, moss in stream	50.1499	16.8624	K. Elliott & S. Martinsson	Jun 15 2013	**KP878458**

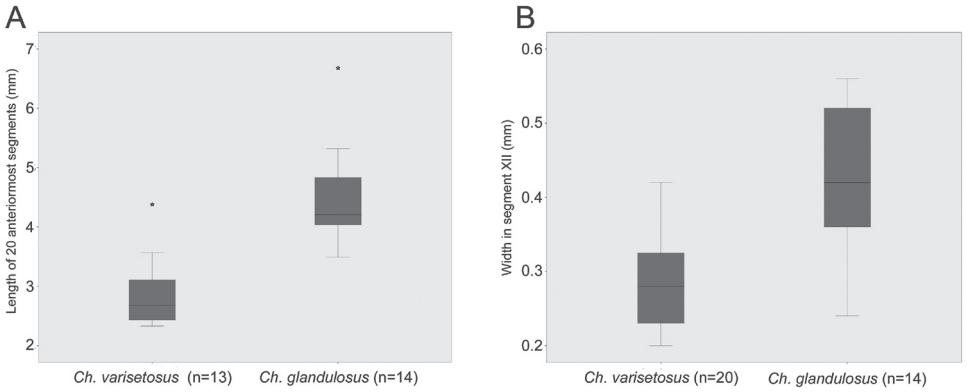

Figure 1. Boxplots showing differences in body size between *Chamaedrilus glandulosus* (Michaelsen, 1888) sensu stricto and *Ch. varisetosus* sp. n. **A** Length of 20 anteriormost segments **B** Width in segment XII. Both differences are significant (two-sided t-tests; P = 1.5E-5 and P = 5.5E-5, respectively).

Norway, and the Zoological Museum Hamburg (ZMUH), Germany; all COI barcodes are deposited in GenBank (see Table 1).

Taxonomy

***Chamaedrilus glandulosus* (Michaelsen, 1888), sensu stricto**
Fig. 2

Pachydrilus sphagnetorum var. *glandulosus* Michaelsen, 1888: 490, plate 23, fig. 2a–c.
Marionia sphagnetorum var. *glandulosa*; Michaelsen 1889: 29.
Marionina glandulosa; Michaelsen 1900: 74.
Chamaedrilus glandulosus; Friend 1919: 174, partim.
Enchytraeoides glandulosa; von Bülow 1955: 257.
Cognettia glandulosa; Nielsen and Christensen 1959: 43, fig. 30, partim; Schmelz and Collado 2010: 79, partim.
Cognettia glandulosa B; Martinsson and Erséus 2014.

Lectotype. ZMUH V 429a, mature anterior part, in alcohol, leg. W. Michaelsen, date not given (before 1888).

Type locality. GERMANY: Hamburg, banks of Bille River, in detritus ("*Billeufer, im Detritus*") (N 53.54°, E 10.09°).

Paralectotype. ZMUH V 429b, immature specimen, in alcohol; same collection data as for lectotype.

Additional type material (not studied). Paralectotypes ZMUH V 429b, 8 specimens in alcohol, same collection data as for lectotype.

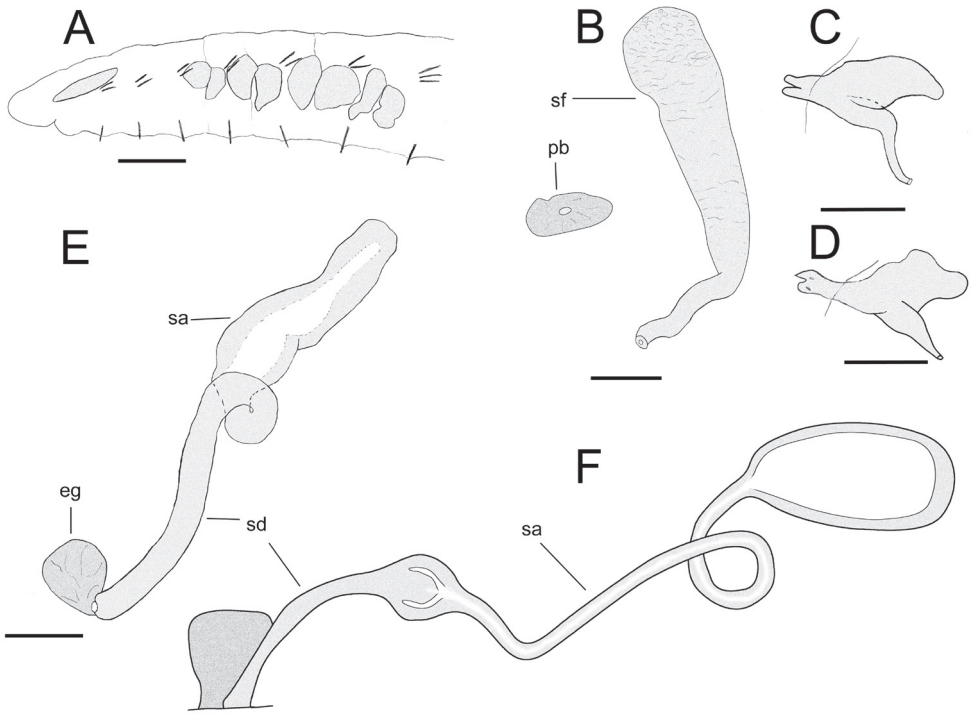

Figure 2. *Chamaedrilus glandulosus* (Michaelsen, 1888) sensu stricto. **A** Anterior part of body (immature specimen) in lateral view, indicating chaetal distribution and the size, shape and number of pharyngeal glands **B** Sperm funnel, ental tract of vas deferens and penial bulb, to show their relative size proportions **C** Nephridium at septum 8/9, lateral view **D** Nephridium at septum 10/11, lateral view **E** Spermatheca **F** Spermatheca redrawn from Michaelsen (1888). Abbreviations: eg = ectal gland; pb = penial bulb; sa = spermathecal ampulla; sd = spermathecal duct; sf = sperm funnel. Scale bars: 200 μm (**A**); 50 μm (**B–E**).

Other material. See Table 1. In total 15 specimens, of which one from Finland, one from Norway and 13 from Sweden (whereof one mature and three submature). All specimens except one are DNA barcoded (Table 1).

Diagnosis. Can be separated from all other European species of *Chamaedrilus* except *Ch. varisetosus* by its unique combination of 2–4 pairs of well-developed secondary pharyngeal glands, two chaetae per lateral bundle in preclitellar segments, and three chaetae in all other bundles, spermathecae with comparatively long ectal ducts, and genitalia shifted forward 3–4 segments (in relation to normal placement in Enchytraeidae). No characters completely separate this species from *Ch. varisetosus* sp. n., but specimens of *Ch. glandulosus* are usually larger and have only two chaetae in the lateral bundles of preclitellar segments, whereas *Ch. varisetosus* usually has three chaetae in lateral bundles of III-V. Furthermore, *Ch. glandulosus* is found in aquatic habitats only (i.e. submerged under water for most of the time), whereas *Ch. varisetosus* is found in both aquatic and terrestrial habitats; so far we have not found them occurring together.

Description. EXTERNAL CHARACTERS: Size: length of 20 anteriormost segments 3.49-6.68 mm, mean 4.55±0.87 (n=11); body width in XII 0.24–0.56 mm, mean 0.42±0.10 (n = 14). Chaetae sigmoid without nodulus, 60–100 μm long, chaetal formula 2,(3)-3:3-3, with 3 lateral chaetae per bundle from VII-IX; in sexually mature specimens, ventral chaetae, or both ventral and lateral chaetae, missing in the segment bearing male pores (VIII or IX). In the sexually mature and submature specimens examined, clitellum poorly developed.

INTERNAL CHARACTERS: Brain concave posteriorly, 160–210 μm long. Pharyngeal glands 3–4 primary pairs; 2–4 pairs of well-developed secondary glands (Fig. 2A), secondary glands behind the first pair of primary glands sometimes missing. Dorsal blood vessel arising in XVI–XX. First pair of nephridia present at 7/8–8/9; nephridia with efferent duct originating antero-ventrally, close to septum; anteseptale consisting of funnel only; postseptale elongate (Fig. 2C–D). Chloragogen cells granulated; 35–55 μm long. Coelomocytes granulated, round to oval, 25–30 μm long.

Seminal vesicle distinct and unpaired in one specimen (CE18516), poorly developed in all other mature or submature specimens. Other genitalia paired. Sperm funnel about 200 μm long, tapering, 25 μm wide basally, 50 μm wide proximally; collar 55–60 μm wide. Spermatozoa on collar in a few mature/submature worms. Vas deferens long, simple, with several loops, about 12 μm wide. Penial bulb poorly developed, about 25 μm wide, 60–65 μm long (Fig. 2B). Male pores in VIII or IX. Spermathecae paired; pores located slightly below lateral chaetae; ectal duct smooth, 240 μm long, about 17 μm wide; ectal gland 35–40 μm in diameter; ampulla oval, about 150 μm long, not attached to oesophagus (Fig 2E); sperm in ampulla of lectotype only. Spermathecae confined to V or entering into VI.

Habitat and distribution. Occurs in freshwater habitats, in sand and gravel bottoms in lakes and small streams, and climbing on vegetation and dead wood in water. Barcoded specimens document occurrence in Finland, Germany, Norway and Sweden, but the species is probably more widely distributed, not only in Europe. For instance, *Ch. glandulosus* s. l. has also been reported from North America: the records by Nurminen (1973) and Healy (1996) are insufficiently described and cannot even tentatively be assigned to any of the two species, and the records by Schlaghamerský (2013) and Schlaghamerský et al. (2014) are likely to be *Ch. varisetosus*, see under Habitat and distribution for that species.

Biology. Seems to reproduce mainly parthenogenetically; specimens with developing genitalia are found from June to July (Sweden).

Remarks. Michaelsen (1888; 1900) described this species as sturdier than *Ch. sphagnetorum*, with 2 chaetae per preclitellar lateral bundle and three chaetae in all other bundles. This together with the fact that Michaelsen's type material was collected at an aquatic site makes us confident that our new material is conspecific with Michaelsen's species. Michaelsen (1888) described the spermathecae *in vivo* as very long ("they often project, in spite of much meandering, up to the segment VII") and the ampullae to consist each of an ectal enlargement followed by a long connecting tube and an expanded ental chamber (Fig 2F). In our new material the spermathecae seem to be either not fully developed or much con-

tracted after fixation: they show simple oval ampullae, not differentiated into ectal and ental compartments. In the mature lectotype we can only follow the spermathecae to what we interpret as the ampullar ectal enlargement. *Chamaedrilus glandulosus* is larger than *Ch. varisetosus* described below. Both the length of the 20 anteriormost segments (P = 1.5E-5) and the width in segment XII (P = 5.5E-5) differ significantly between the two species (Fig. 1).

This species is represented in BOLD by BIN: AAT8923.

***Chamaedrilus varisetosus* sp. n.**
http://zoobank.org/BEA27C2F-484B-465A-AA06-034E84F0FF20
Fig. 3

Chamaedrilus glandulosus; Friend 1919: 174, partim.
Cognettia glandulosa; Nielsen and Christensen 1959: 43, fig. 30, partim; Schmelz and Collado 2010: 79, partim.
Cognettia glandulosa A; Martinsson and Erséus 2014.

Holotype. ZMBN99905, CE19052, mature, anterior part, COI barcode acc. no. KP878464, leg. Christer Erséus, Aug 10, 2013.

Type locality. NORWAY: Buskerud, Hol, at Örtedalsåna River (S of Haugastöl), elevation 1,075 m above sea level (N60.4866°, E7.8562°).

Paratypes. ZMBN99906, CE19818, submature, anterior part, COI barcode acc. no. KP878469; **NORWAY: Hedmark**, Engerdal, Nymoen at Femundelva (Trysilelva) River, at Nordre Husfloen Farm (N61.6569°, E11.8164°), leg. Christer Erséus, Aug 15, 2013. SMNH type-8723, CE19819, submature, anterior part, COI barcode acc. no. KP878470. Same collection data as for the other paratype.

Other material. See Table 1. Twenty-seven immature specimens, of which 2 from the Czech Republic, 12 from Norway, and 13 from Sweden, all DNA-barcoded.

Etymology. The species is named after the variation in numbers of chaetae in the lateral preclitellar bundles.

Diagnosis. The new species can be separated from all other European species of *Chamaedrilus* except *Ch. glandulosus* s. s. by its unique combination of 3–4 pairs of well-developed secondary pharyngeal glands, two chaetae in most lateral bundles in preclitellar segments, and three chaetae in all other bundles, spermathecae with comparatively long ectal ducts, and genitalia shifted forward 3–4 segments (in relation to normal placement in Enchytraeidae). No characters completely separate this species from *Ch. glandulosus,* but specimens of *Ch. varisetosus* are generally smaller, have shorter chaetae and smaller internal organs, and usually have a few preclitellar lateral bundles with three chaetae (*Ch. glandulosus* constantly has two chaetae per lateral bundle in preclitellar segments). Furthermore, *Ch. varisetosus* is mainly found in moist to wet soils, whereas *Ch. glandulosus* is only found in aquatic habitats.

Description. EXTERNAL CHARACTERS: Size: length of 20 anteriormost segments 2.33–4.38 mm, mean 2.89±0.59 (n = 13); body width in XII 0.20–0.42 mm,

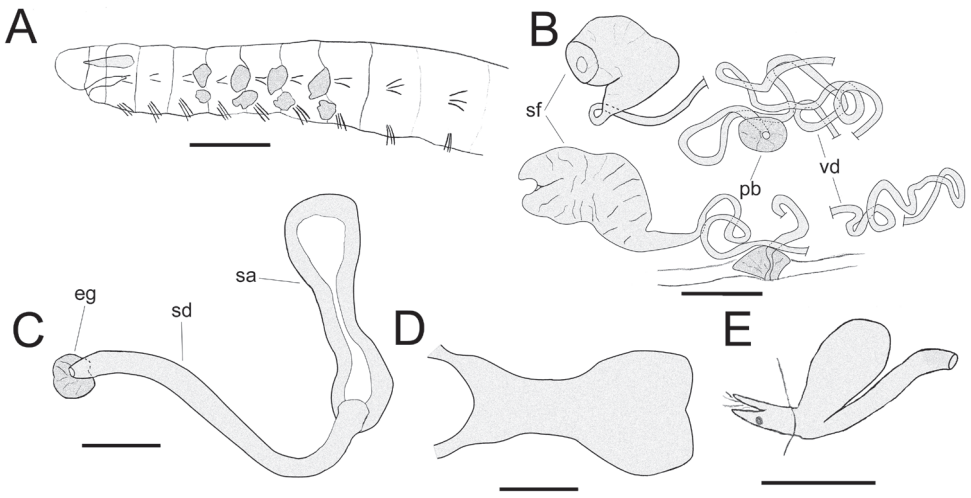

Figure 3. *Chamaedrilus varisetosus* sp. n. **A** Anterior part of body (immature specimen) in lateral view, indicating chaetal distribution and the size, shape and number of pharyngeal glands **B** Male genitalia of a mature worm with male pores in segment VIII **C** Spermatheca **D** Brain, dorsal view **E** Nephridium at septum 10/11, lateral view. Abbreviations: eg = ectal gland; pb = penial bulb; sa = spermathecal ampulla; sd = spermathecal duct; sf = sperm funnel; vd = vas deferens. Scale bars: 200 μm (**A**); 50 μm (**B–E**).

mean 0.28±0.07 (n = 20). Chaetae sigmoid without nodulus, 50–60 μm long, chaetal formula 2,3-(2),3:3–3; most specimens with 3 chaetae in lateral bundles of III(or IV)-V and 2 chaetae in the other lateral preclitellar bundles, but some specimens have 2 chaetae in all preclitellar lateral bundles; in sexually mature specimens, chaetae missing in the segment bearing male pores (VIII or IX). In the mature and submature specimens examined, clitellum only developed (but poorly) in the segment bearing the male pores and ½ a segment posterior and anterior to that segment.

INTERNAL CHARACTERS: Brain slightly concave posteriorly, concave anteriorly, 125–140 μm long, about twice as long as broad (Fig. 3D). Pharyngeal glands, 3–4 primary pairs; 3–4 pairs of well-developed secondary glands (Fig. 3A), secondary glands behind the last pair of primary glands sometimes missing. Dorsal blood vessel arising in XIII–XVII, rarely in XI or XVIII. First pair of nephridia present at 8/9–11/12; nephridia with efferent duct originating antero-ventrally, close to septum; anteseptale consisting of funnel only; postseptale oval, elongate (Fig. 3E). Chloragogen cells granulated, 20–30 μm long. Coelomocytes finely granulated, round to oval, approximately 20 μm long.

Seminal vesicle unpaired, distinct in all three mature/submature specimens. Other genitalia paired. Sperm funnel about 100 μm long, 40–50 μm wide; collar indistinct, 25–30 μm wide. Spermatozoa not observed on collar. Vas deferens long, with several loops, about 5–7 μm wide. Penial bulb poorly developed, about 25 μm wide, 35–40 μm long (Fig. 3B). Male pores in VIII or IX. Spermathecae paired; pores located slightly below lateral chaetae; ectal duct smooth, 225 μm long, approximately 15 μm wide; ectal gland 25–30 μm in diameter; ampulla about 150 μm long, with ectal enlargement,

followed by a contraction and a tubular to oval ental chamber; no sperm observed in ampulla; ampulla not attached to oesophagus (Fig. 3C). Spermathecae entering into VI.

Habitat and distribution. Found both in aquatic and terrestrial habitats. In freshwater found on stony bottoms in rivers, on land found in both deciduous and coniferous forest as well as in grassland soils. Known from Canada (BOLD record), the Czech Republic, Finland (BOLD record), Norway and Sweden, but may be more widely distributed in Europe and North America. Schlaghamerský's (2013) description of *C. glandulosa* from Michigan fits our description of *Ch. varisetosus.* This and Schlaghamerský's et al. (2014) records from Minnesota and Wisconsin are likely to refer to the same species.

Biology. Parthenogenetic reproduction more limited in time (maturing specimens found in August in Norway) than fragmentation (observed in May-September in Sweden and Norway). Worms with regenerating tails and/or heads rather frequent. This species may correspond to the population studied by Christensen (1959), in which the number of mature worms was high for a short period during the autumn. The variation in number of the lateral chaetae corresponds to that given in the diagnosis by Nielsen and Christensen (1959).

Remarks. This species is represented in BOLD by BIN: AAT9501.

Discussion

The two species treated in this paper, *Chamaedrilus glandulosus* sensu stricto and *Ch. varisetosus* sp. n., are easily separated morphologically from other species of *Chamaedrilus* by a unique combination of characters: the secondary pharyngeal glands are well developed in several segments, there are two chaetae in most preclitellar lateral bundles, but no enlarged chaetae, the genital organs are shifted forwards, and the spermathecae have comparatively long ectal ducts. The two species are morphologically similar and they have therefore been regarded as a single taxon by previous authors (e.g., Nielsen and Christensen 1959; Schmelz and Collado 2010). As demonstrated in the present paper, they can only be separated by their body size, chaetal size (and prevailing number) and, when fully grown, by the proportions of most internal organs. Genetically, however, they are well separated from each other (Martinsson and Erséus 2014), and they are also ecologically separated, with *Ch. glandulosus* found in aquatic habitats, whereas *Ch. varisetosus* is predominantly found in moist to wet soil. Ecological and physiological differences have been found between cryptic lineages in morphospecies of various organisms (e.g. Beauchamp et al. 2002; Feckler et al. 2014; Sattler et al. 2007), and if such lineages are not formally recognized and named, the differences may continue to be overlooked or neglected.

Martinsson and Erséus (2014) found *Chamaedrilus glandulosus* and *Ch. varisetosus* sp. n. to be sister species, nested within a part of the *sphagnetorum*-complex, making the latter non-monophyletic. The *sphagnetorum*-complex also turned out to be morphologically more heterogeneous than *Ch. glandulosus* s. l. (Martinsson et al. 2014), which could probably be, at least partly, explained by its non-monophyly. However,

not even the two morphologically indistinguishable species, *Ch. sphagnetorum* s. s. and *Ch. pseudosphagnetorum* Martinsson et al., 2014 came out as sister species in the phylogenetic study (Martinsson and Erséus 2014).

Without the genetic data, the delimitation of *Ch. glandulosus* and *Ch. varisetosus* would have been much more challenging, all the more so because these worms, like those in the *sphagnetorum* complex, are mostly found sexually immature. It should also be considered that these species, even when mature, actually reproduce uniparentally, as mentioned in the introduction and discussed earlier by Martinsson and Erséus (2014). Uniparental reproduction makes species delimitation harder; however, we still believe this is possible using the unifying species concept (see Introduction). In the present case, we have a combination of genetic, ecological and morphological differences, supporting the split of *Ch. glandulosus* s. l. into two species. It should further be noted that it is not known with certainty if Christensen (1959; 1961) studied both species, or only one of them. As mentioned in the description, *Ch. varisetosus* seems to correspond well with the taxon studied in his 1959 paper and also fits the description given by Nielsen and Christensen (1959). Until the mode(s) of reproduction is (are) studied again for the two species, we cannot exclude the possibility that one or both species may reproduce biparentally, at least occasionally.

Genetic studies discovering cryptic and unnoticed diversity need to be followed by formal taxonomic revision, including careful morphological scrutiny, updated descriptions and species names, if possible based on barcoded types. We believe that an integrative approach, combining genetic and morphological data with as much as possible of ecological and physiological information, will strengthen studies of enchytraeid systematics.

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RESEARCH ARTICLE

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Molecular data for *Crenavolva* species (Gastropoda, Ovulidae) reveals the synonymy of *C. chiapponii*

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Abstract

During fieldwork in Indonesia and Malaysia, eight lots containing 33 specimens belonging to the genus *Crenavolva* (Ovulidae) were collected. Species were initially identified as *C. aureola*, *C. chiapponii*, *C. striatula* and *C. trailli*, respectively. For *C. chiapponii* this is the second record. In contrast to the ecological data available from the original description of this species, it was found in shallow water on a gorgonian host coral, i.e. *Acanthogorgia* sp. A molecular analysis based on COI and 16S mtDNA markers, including sequence data obtained from GenBank, showed that *C. chiapponii* should be considered a junior synonym of *C. aureola* and that previously identified ovulid specimens are probably misidentified.

Keywords

Acanthogorgia, host association, molecular phylogeny, Octocorallia, 16S, COI

Introduction

The nominal taxon *Crenavolva* was introduced as a subgenus by Cate (1973), together with the subgenera *Crenavolva*, *Cuspivolva* and *Serratovolva*. In the most recent overview regarding Ovulidae these three taxa are considered genera (Lorenz and Fehse 2009). At present 18 nominal species are recognized within *Crenavolva* (Rosenberg 2014), most of which are considered rare (Lorenz and Fehse 2009). These species are

considered rare because few specimens have been collected, probably because they occur at depths greater than standard recreational diving depth of c. 30 m and/or are only known from a limited geographical area, usually just the type locality. This also accounts for *C. chiapponii* Lorenz & Fehse, 2009, which is only known from Balicasag Isl., Bohol, Philippines, where specimens were trawled from 70–120 m depth and, therefore, were considered rare and confined to deeper water (Lorenz and Fehse 2009). Like almost all other ovulids, species of *Crenavolva* are associated with octocoral hosts (Schiaparelli et al. 2005; Reijnen 2010) belonging to several families (e.g. Melithaeidae, Ellisellidae, Subergorgiidae and Plexauridae). However, the host species are usually not collected or are disregarded and therefore unknown, which is also the case for *C. chiapponii*.

Molecular data (16S and COI) obtained from *Crenavolva* was used by Meyer (2003) to root the phylogeny of the Cypraeidae. Later, the 16S sequence data were used by Schiaparelli et al. (2005) to produce the first molecular phylogenetic reconstruction of the Ovulidae, which included two *Crenavolva* species: *C.* cf. *rosewateri* (Cate, 1973) and *C. tokuoi* Azuma, 1989. In the present study, material of four additional nominal *Crenavolva* species, amongst other ovulids, have been used to reconstruct a phylogeny. The newly acquired molecular data are for *C. aureola* (Fehse, 2002), *C. chiapponii* Lorenz & Fehse, 2009, *C. striatula* (Sowerby I, 1828) (type species), and *C. trailli* (Adams, 1855). In addition to this phylogenetic reconstruction, data on host species and distributional records are given for this group of rarely recorded ovulid snails.

Materials and methods

Collection and identification

During fieldwork in Indonesia (Halmahera, Ternate; Sulawesi, Lembeh Strait) and Malaysia (Borneo, Semporna and Kudat) specimens of *Crenavolva* species were collected by SCUBA diving (Table 1). The snails and their octocoral hosts were photographed in situ (Fig. 1) whenever possible and subsequently fixed in 80% ethanol. The holotype of *C. chiapponii* was studied at the Muséum national d'Histoire naturelle (MNHN) in Paris. For the identification of the other ovulid species, Cate (1973), Fehse (2002b) and Lorenz and Fehse (2009) were used. For the identification of the host species, microscopy slides of their calcareous skeletal parts (sclerites) were made by dissolving the samples in a 4% solution of household bleach. The residual sclerites were rinsed with tap water followed by demineralised water before mounting on a slide or on a stub for Scanning Electron Microscopy (SEM). Stubs with sclerites were coated with Au/Pd before SEM images were made with a JEOL 6480 LV. Identification of the octocorals to genus level was based on Stiasny (1947) and Fabricius and Alderslade (2001).

Table 1. Specimens used in the analyses, including locality, host, and GenBank accession data.

Collection number	Species	Locality (Locality code)	Coordinates	Date collected	Host species	GenBank Accession number (16S; COI)	Reference
RMNH.MOL.164072	*Crenavolva aureola* (Fehse, 2002)	Malaysia, Semporna, Si Amil Island (SEM.16)	4°19'02.1"N; 118°52'30.7"E	4-12-2010	*Acanthogorgia* sp.	KP033143; KP033151	This publication
RMNH.MOL.164085	*Crenavolva aureola* (Fehse, 2002)	Indonesia, Halmahera, Tidore, N of Desa Rum (TER.18)	0°44'35.8"N; 127°23'06.3"E	4-11-2009	*Acanthogorgia* sp.	KP033144; KP033152	This publication
RMNH.MOL.164209	*Crenavolva aureola* (Fehse, 2002)	Indonesia, Halmahera, Tanjung Ratemu (S of river)(TER.21)	0°54'24.7"N; 127°29'17.7"E	5-11-2009	*Acanthogorgia* sp.	KP033148; KP033156	This publication
RMNH.MOL.164211	*Crenavolva chiapponii* Lorenz & Fehse, 2009	Indonesia, Halmahera, Tanjung Ratemu (S of river)(TER.27)	0°54'44.5"N; 127°29'09.9"E	8-11-2009	*Acanthogorgia* sp.	KP033157	This publication
RMNH.MOL.164217	*Crenavolva chiapponii* Lorenz & Fehse, 2009	Indonesia, Lembeh, Tanjung Kusukusu (LEM.31)	1°27'13.8"N; 125°14'13.0"E	16-2-2012	*Acanthogorgia* sp.	KP033149; KP033158	This publication
RMNH.MOL.164062	*Primovula rosewateri* (Cate, 1973)	Malaysia, Semporna, Kulapuan Island 2, N side (SEM.31)	4°32'07.4"N; 118°50'18.2"E	9-12-2010	*Paratelesto* sp.	KP033142; KP033150	This publication
RMNH.MOL.164186	*Crenavolva striatula* (Sowerby I, 1828)	Malaysia, Sabah, S Pulau Banggi, E Molleangan Besar Island, (TMP.37)	7°05'07.2"N; 117°03'33.8"E	19-9-2012	*Echinogorgia* sp.	KP033146; KP033154	This publication
RMNH.MOL.164144	*Crenavolva trailli* (Adams, 1855)	Malaysia, Sabah, Kalang, (TMP.41)	6°59'48.1"N; 117°03'13.4"E	18-9-2012	*Subergorgia* sp.	KP033145; KP033153	This publication
RMNH.MOL.164189	*Crenavolva trailli* (Adams, 1855)	Malaysia, Sabah, Kalang, (TMP.41)	6°59'48.1"N; 117°03'13.4"E	18-9-2012	*Paraplexaura* sp.	KP033147; KP033155	This publication
-	*Crenavolva* cf. *rosewateri* (Cate, 1973)	Philippines, Bohol, Balicasag Island	-	-	-	AY161394; AY161627	Meyer 2003
-	*Crenavolva tokuoi* Azuma, 1989	Philippines, Bohol, Balicasag Island	-	-	-	AY161390; AY161623	Meyer 2003
-	*Primovula beckeri* (Sowerby III, 1900)	Indonesia, Sulawesi	-	-	-	AJ868555; -	Schiaparelli et al. 2005
-	*Ovula ovum* (Linnaeus, 1758)	Indonesia, Sulawesi, Spermonde Archipelago	-	-	-	AY161399; AY161632	Meyer 2003

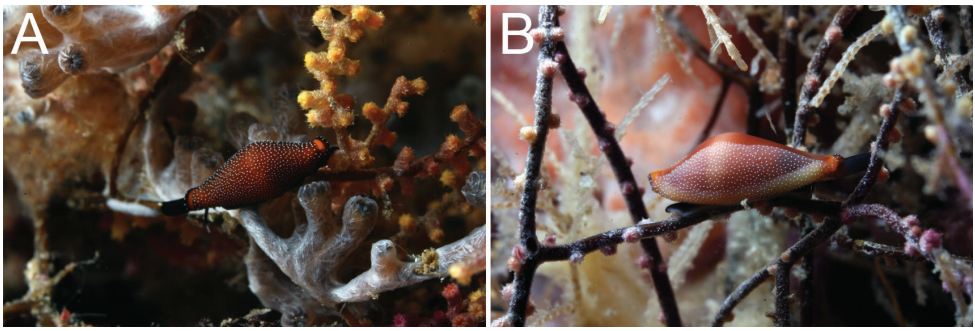

Figure 1. A *In situ* image of *Crenavolva aureola* (Fehse, 2002) (RMNH.MOL.164209) and **B** *C. chiapponii* Lorenz & Fehse, 2009 (RMNH.MOL.164211) on *Acanthogorgia* sp. at Halmahera, Indonesia at 21 m and 17 m depth respectively.

Barcoding Ovulidae

Specimens were barcoded for the COI barcoding region and for additional phylogenetic research also for the 16S marker. Tissue samples obtained from the foot and/or mantle were extracted with the Machery-Nagel DNA extraction kit on a KingFisher Flex. The standard COI barcoding primers by Folmer et al. (1994) and the Palumbi (1996) 16S primers were used. PCR amplification was performed on a C1000 Touch Thermal Cycler (Bio-RAD). Sequencing of the PCR products was performed at Macrogen Europe on an ABI 3730xl Automated Sequencer. Sequences were edited in Sequencher 4.10.1 and aligned with GUIDANCE (Penn et al. 2010) using the MAFFT algorithm (Katoh et al. 2005). Selecting an evolutionary model was done with jModeltest based on the Akaike Information Criterion score. MEGA 6.0.6 (Tamura et al. 2013) was used to perform Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses and to calculate p-distances. Bayesian analyses were performed in MrBayes 3.2.0 (Ronquist and Huelsenbeck 2003). MrBayes was run for 4,000,000 generations with six chains. Data were sampled every 100 generations. Sequence data for *Ovula ovum* (Linnaeus, 1758) from GenBank was used as an outgroup. GenBank data for *Crenavolva* cf. *rosewateri* (Cate, 1973), *C. tokuoi* Azuma, 1989 and *Primovula beckeri* (Sowerby III, 1900) was also included in the phylogenetic analyses.

Results

Collecting and morphology

Eight lots, containing 33 specimens representing four nominal *Crenavolva* species (*C. aureola*, *C. chiapponii*, *C. striatula* and *C. trailli*) were collected in Indonesia and Malaysia (Table 1; Fig. 2). For *C. chiapponii* this is the first record from shallow water. The specimens were assigned to these nominal species based on shell shape (rhomboid, inflated or slender) and the colour bands on the dorsum, which in case of *C. striatula* were

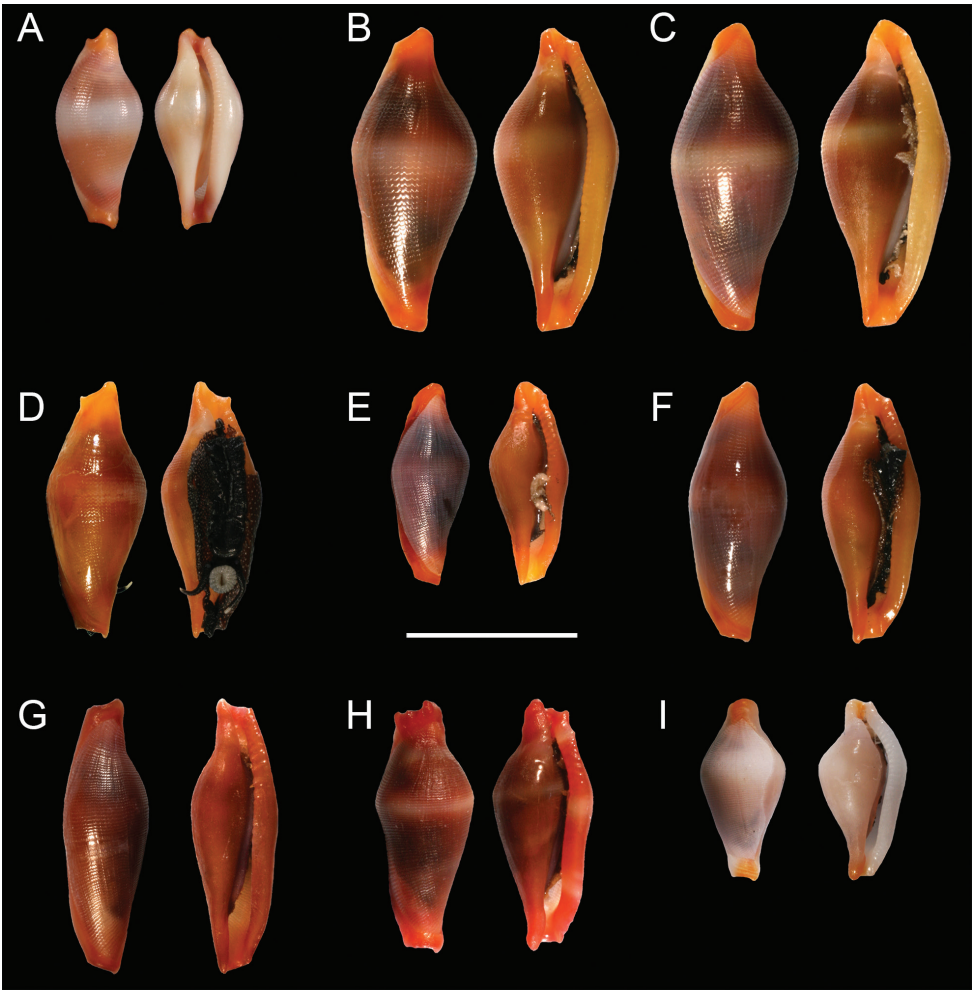

Figure 2. Dorsal and ventral views of shells. **A** Holotype of *Crenavolva chiapponii* Lorenz & Fehse, 2009 (MNHN 21244) **B** *C. chiapponii* Lorenz & Fehse, 2009 (RMNH.MOL.164211) **C** *C. chiapponii* Lorenz & Fehse, 2009 (RMNH.MOL.164217) **D** *C. aureola* (Fehse, 2002) (RMNH.MOL.164085) **E** *C. aureola* (Fehse, 2002) (RMNH.MOL.164072) **F** *C. aureola* (Fehse, 2002) (RMNH.MOL.164209) **G** *C. trailli* (Adams, 1855) (RMNH.MOL.164144) **H** *C. striatula* (Sowerby I, 1828) (RMNH.MOL.164186) **I** *Primovula rosewateri* (Cate, 1973) (RMNH.MOL.164062). Scale bars: 5 mm.

also present on the labrum. For *C. aureola* and *C. chiapponii* the absence or presence of a white dorsal band on the shell is allegedly the most obvious character to distinguish the species. After examination of the illustrations presented by Lorenz and Fehse (2009) and the newly collected material, minor morphological differences (strongly or weakly pronounced dentation, keeling angle, strongly or weakly produced funiculum, position of the widest part of the shell) do not clearly separate between *C. aureola* and *C. chiapponii* and can be considered morphological variation in a single species. The soft tissue

colouration of both *C. aureola* and *C. chiapponii* is very similar (e.g. Fig 1; Lorenz and Fehse 2009: A106, A107 p. 527). Both have a semi-transparent mantle which is entirely covered with small, irregularly placed, white dots, and both have a completely black or white foot, black tentacles with white tips, and a black siphon.

Molecular data

Nine specimens representing five species were sequenced for COI and 16S. For one sample of *C. chiapponii* (RMNH.MOL.164211) the 16S marker could not be amplified. Sequences were concatenated and aligned (GUIDANCE alignment score: 0.965034) which resulted in an alignment length of 1080 base pairs per specimen including indels. Sequences obtained from GenBank are slightly shorter (~40 base pairs), these missing base pairs were coded as 'missing data'. The program jModeltest yielded in HKY+G as most optimal evolutionary model. This evolutionary model was implemented in the Bayesian and ML analysis. The results from the different phylogenetic reconstructions were congruent, therefore only the ML tree is shown (Fig. 3).

In the phylogenetic reconstructions, specimens of *Crenavolva striatula* and *C. tokuoi* form an unresolved trichotomy with the other *Crenavolva* specimens. The two *Primovula* species cluster together and are well-supported sister species to all the *Crenavolva* species (with *C. striatula* as type species for the genus). This implies that the *Crenavolva* species used herein form a monophyletic group. The clustering of two *C. trailli* specimens is highly supported. Another well-supported clade holds three nominal species: *Crenavolva aureola*, *C. chiapponii* and *C.* cf. *rosewateri*. The pairwise p-distances between these three species are very low (16S: 0.2%; COI: 0.7%; concatenated: 0.9%).

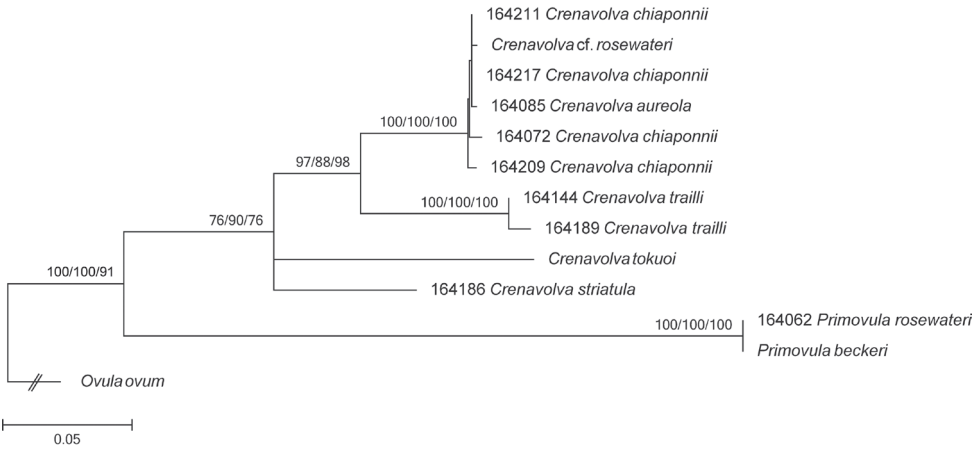

Figure 3. Maximum Likelihood cladogram with support values for the ML/MP/BP analyses. Numbers preceding the species names represent RMNH.MOL. collection numbers of Naturalis Biodiversity Center; species names without numbers are obtained from GenBank for which additional data can be found in Table 1.

In contrast, the sequence divergence between *C. trailli* and the *C. chiapponii* / *C. aureola* clade is almost ten times larger (16S: 5.2%; COI: 8.7%; concatenated: 8.2%). The sequence divergence between the two *C. trailli* specimens (16S: 0.6%; COI: 0.8%; concatenated: 0.8%) is almost equal to that between *C. aureola* and *C. chiapponii*. With the help of the Automatic Barcode Gap Discovery tool (ABGD) (Puillandre et al. 2011), the data were analysed to identify the MOTU's within the dataset. The results of this analysis showed that the barcode gap to identify the different species is 5–6% sequence divergence. This resulted in five groups containing the following species: 1, *C. aureola*, *C. chiapponii*, *C.* cf. *rosewateri*; 2, *C. trailli*; 3, *C. tokuoi*; 4, *C. striatula*; 5, *P. rosewateri*. One of the samples obtained from GenBank, viz. *Crenavolva* cf. *rosewateri* (= *Primovula* cf. *rosewateri*), clusters surprisingly within the clade containing *C. aureola* and *C. chiapponii* and not with the other *Primovula rosewateri* specimen. Instead, *Primovula beckeri* proves to be identical to the newly sequenced specimen of *Primovula rosewateri* from Malaysia.

Octocoral hosts

Almost all Ovulidae species are associated with Octocorallia hosts. By examining the sclerites and the habitus of the host corals, several new host species for ovulids of the genus *Crenavolva* could be identified. An overview of previously identified host species and new records is provided in Table 2. Some of the former host identifications were published with obsolete generic names, and therefore their names in the current literature are also provided. Before *C. chiapponii* was synonymised, *Acanthogorgia* would have been a new host record. Yet, Reijnen (2010) already recorded *Acanthogorgia* sp. as a host for *C. aureola* and therefore it is not a new host record. Morphologically at least two different species of *Acanthogorgia* could be distinguished but these could not be identified since a revision of the family Acanthogorgiidae is lacking.

Table 2. Literature overview of the octocoral hosts of selected *Crenavolva* species including new records. Updated names of the octocoral hosts are provided between parentheses.

Ovulid species	Host genera	Reference
Crenavolva aureola	*Euplexaura*; *Astromuricea* (= *Echinogorgia*); *Acanthogorgia*	Lorenz and Fehse 2009; Reijnen 2010
Crenavolva chiapponii (= *C. aureola*)	*Acanthogorgia*	this publication; Reijnen 2010
Crenavolva striatula	*Ellisella*; *Euplexaura*; *Echinogorgia*	Lorenz and Fehse 2009; Yamamoto 1973; Cumming 1997; Mase 1989;
Crenavolva trailli	*Echinogorgia*; *Anthoplexaura* (= *Astrogorgia*); *Plexauroides* (= *Echinogorgia*); *Euplexaura*; *Subergorgia*	Goh et al. 1999; Mase 1989
Primovula rosewateri	*Subergorgia*; *Dendronephthya*; *Stereonephthya*; *Paratelesto*	Goh et al. 1999; Lorenz and Fehse 2009; this publication
Primovula beckeri	*Acanthogorgia*; *Acabaria* (= *Melithaea*); *Unicella* [sic] (= *Eunicella*); *Lophogorgia* (= *Leptogorgia*)	Schiaparelli et al. 2005; Lorenz and Fehse 2009

Furthermore, examination of the ovulid species and their octocoral hosts revealed that in two instances individuals formerly identified as *C. chiapponii* and *C. aureola* would have co-occurred on the same host coral, in both cases *Acanthogorgia* sp.

Discussion

Based on the molecular data and morphological observations listed above, *C. chiapponii* is considered a junior synonym of *C. aureola*. The systematic account is therefore as follows:

Systematic part

Family Ovulidae Fleming, 1822
Genus *Crenavolva* Cate, 1973

***Crenavolva aureola* (Fehse, 2002)**

Primovula aureola Fehse 2002: 37, pl. 1, fig. 1
Delonovolva formosa. — Gosliner et al. 1996: 136, fig. 469. Not *Delonovolva formosa* (Sowerby II in Adams and Reeve 1848) [= *Cuspivolva formosa* (Sowerby II in Adams and Reeve 1848)]
Primovula sp. — Coleman 2003: 51, fig. (Ovul: 121).
Crenavolva chiapponii Lorenz and Fehse 2009: 69, pl. 74, fig. 7–11.

The occurrence of *C. chiapponii* (= *C. aureola*) on Indonesian shallow water coral reefs would have represented new distribution records, both geographically and bathymetrically, before it was synonymised. However *C. chiapponii* proved to be a junior synonym of *C. aureola* and the new distribution records fall within the distribution range already known for *C. aureola*. Apparently, the dorsal white band and the minor morphological differences in shell shape are not indicative of species-level differences between *C. aureola* and *C. chiapponii*.

Molecular data

The species *Primovula rosewateri* was previously placed in the genus *Crenavolva* by Cate (1973) but Fehse (2002a) moved it to *Primovula*, primarily based on the triangular shape of the funiculum. The results of the molecular analyses (Fig. 3) support this decision. There is great genetic similarity between *C.* cf. *rosewateri* (= *Primovula* cf. *rosewateri*) obtained from GenBank, and *C. aureola*. However, the specimen from GenBank was collected from Balicasag Island, near Bohol, Philippines, which is the

type locality of *C. chiapponii*. This location is approximately 85 km from Mactan Island of Cebu, Philippines which is the type locality of *C. aureola*. It is not unlikely that the so-called *C.* cf. *rosewateri* from GenBank (AY161394 (16S), AY161627 (COI)) was misidentified and actually represents *C. aureola*. Moreover, the newly sequenced specimen of *P. rosewateri* from Malaysia convincingly clusters with *Primovula beckeri*. According to Lorenz and Fehse 2009, *P. beckeri* has an E African distribution and was originally described from South Africa. The specimen obtained from GenBank is from Sulawesi, Indonesia (Schiaparelli et al. 2005). It is therefore unlikely that this sequence represents *P. beckeri* but instead is the quite similar species from the central Indo-Pacific, *P. rosewateri*.

Host species and distribution records

The ranges of the presently discussed species all fit within the Coral Triangle (see Hoeksema 2007) and depend on the ranges of their host species. Species of the genus *Acanthogorgia* are not unique hosts for just *Crenavolva* spp. Reijnen (2010) already mentioned *Acanthogorgia* spp. as a host for *Dentiovula eizoi* Cate & Azuma, 1973 (in Cate 1973) and *D. colobica* (Azuma & Cate, 1971). *Acanthogorgia* species and their ovulid associates are both known to occur from shallow to deep water in the Coral Triangle. In an overview of the Acanthogorgiidae by Stiasny (1947) the deepest record for an *Acanthogorgia* species is 4239 m, collected SE of Seram, Indonesia (*Acalycigorgia densiflora* = *Acanthogorgia densiflora* (Kükenthal & Gorzawsky, 1908). Nevertheless, Stiasny (1947) doubts the identification and compared it to congeneric species which are found in waters not exceeding 400 m depth. As a result Stiasny (1947) doubts the entire record. Therefore the deepest reliable record for an *Acanthogorgia* species in the Malayan Archipelago is 1254 m for *Acanthogorgia multispina* (Kükenthal & Gorzawsky, 1908). The deepest record for *Crenavolva* species is from approximately 1000 m, which is the deepest record for any ovulid species found to date (Lorenz and Fehse 2009).

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and Department of Fisheries Sabah. The MV Celebes Explorer accommodated the research in Semporna. The 2012 Tun Mustapha Park expedition (TMP) was jointly organized by WWF-Malaysia, Universiti Malaysia Sabah (UMS), Sabah Parks and Naturalis Biodiversity Center, the Netherlands. The research permits were granted by the Economic Planning Unit, Prime Minister's Department Malaysia and Sabah Biodiversity Centre. Both expeditions to Malaysia were co-organised by Ms Zarinah Waheed and WWF Malaysia, which was greatly appreciated. Dr Philippe Bouchet and Ms Virginie Heros were so kind to accommodate a visit to Muséum national d'Histoire naturelle (MNHN) in Paris to investigate the Ovulidae (type) collections. Dr Leendert P. van Ofwegen kindly provided help by identifying the octocoral hosts and Sancia van der Meij was a perfect dive buddy and ovulid hunter. Sequencing of the barcoding region of the ovulids was part of one of the barcoding initiatives within the Naturalis Biodiversity Center. Funding for fieldwork was provided by the Jan-Joost ter Pelkwijkfonds and A.M. Buitendijkfonds (Naturalis Biodiversity Center), Malacological Society of Australasia and the Percy Sladen Fund. I also would like to thank Prof Dr Catherine S. McFadden, an anonymous reviewer and Dr Thierry Backeljau for their constructive comments and remarks which improved the manuscript greatly.

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RESEARCH ARTICLE

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A review of Bornean Micronectidae (Hemiptera, Heteroptera, Nepomorpha) with descriptions of two new species from Sabah, Malaysia[1]

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Abstract

Previous research of Bornean Micronectidae Jaczewski, 1924 (pygmy water boatmen) is summarized based on the data from the literature and recent work. All the Bornean micronectids belong to the genus *Micronecta* Kirkaldy, 1897. Descriptions or redescriptions and a key to the eight species, which have so far been found in Borneo are presented, namely *M. decorata* Lundblad, 1933, *M. ludibunda* Breddin, 1905, *M. liewi* **sp. n.**, *M. lakimi* **sp. n.**, *M. lumutensis* Chen, Nieser & Lansbury, 2008, *M. skutalis* Nieser & Chen, 1999, *M. kymatista* Nieser & Chen, 1999) and *M. quadristrigata* Breddin, 1905. The synonyms are indicated under each species. To facilitate identification, illustrations and habitus photos are provided. The faunistic components of Micronectidae in Borneo are discussed from a zoogeographic point of view.

Keywords

Hemiptera, Micronectidae, *Micronecta*, new species, key, Borneo

[1] Result of the Scientific Expedition to Kinabalu-Crocker Range in September, 2012, organized jointly by the Sabah Parks, Malaysia and the Naturalis Biodiversity Centre, The Netherlands.

Introduction

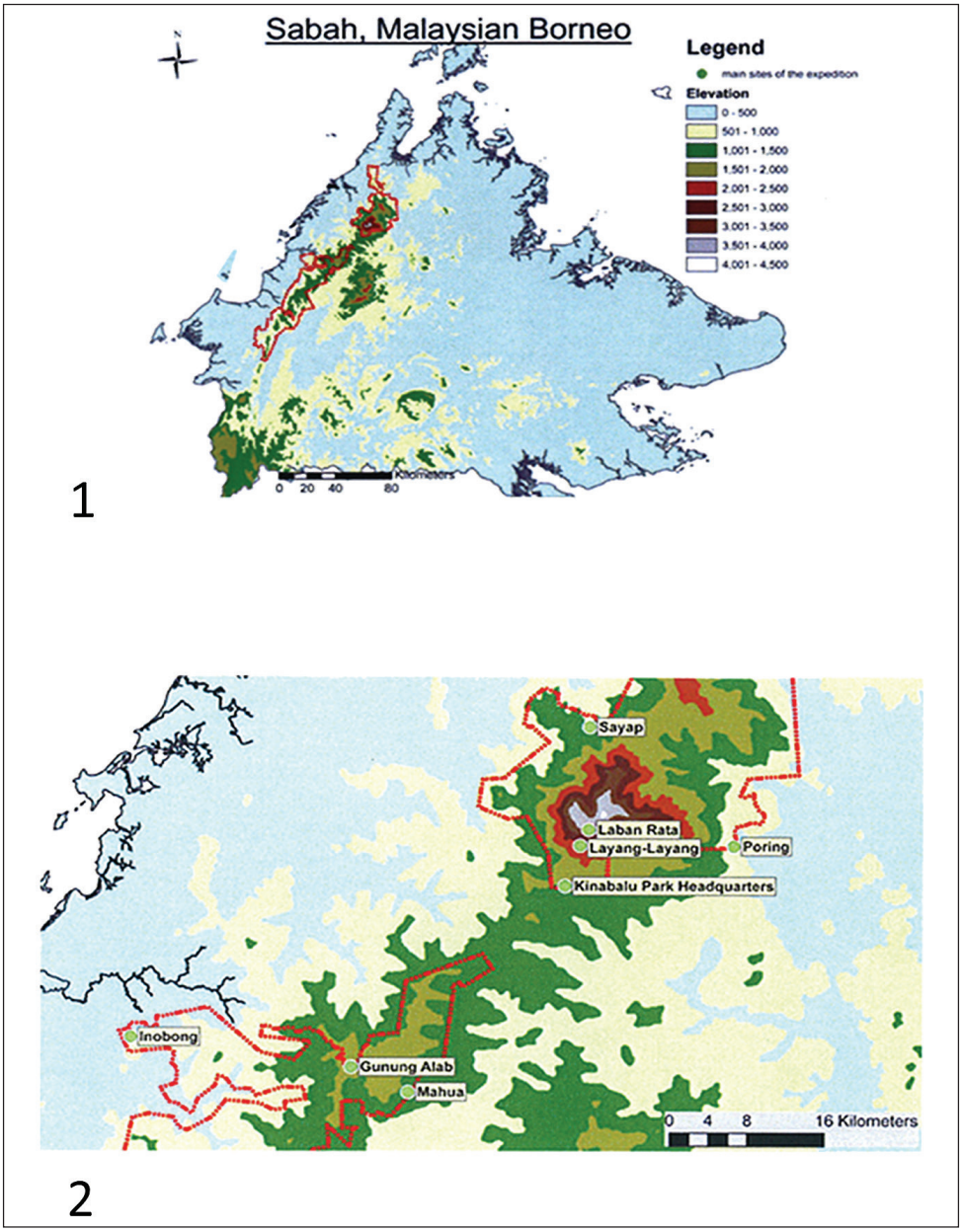
The Scientific Expedition to Mount Kinabalu–Crocker Range in September 2012 (http://kinabalu-expedition.blogspot.nl/), organized jointly by Sabah Parks, Malaysia and the Naturalis Biodiversity Centre (NBC), The Netherlands, offered us an opportunity to collect water bugs at several substations in the Parks. The result has led to a better understanding of the water bug fauna in the area, including the discovery of several undescribed species. As a result of the expedition, a review of the Sabah Micronectidae is presented. For locations and the ground plan of the Sabah Parks, see Figs 1–4.

The Micronectidae (pygmy water boatmen) belong to the superfamily Corixoidea (Leach, 1815), which is in the infraorder Nepomorpha Popov, 1971. Most species in the Nepomorpha live in water and are characterized by the antennae implanted under the head. In the most obligate aquatic species, their antennae are shorter than the head and not visible in dorsal view. Within Nepomorpha, the Corixoidea are recognized by the broadly triangular, unsegmented rostrum, although transverse grooves are present in most species (Fig. 5). The abdominal structure in males is strongly modified in Micronectidae as in other Corixoidea taxa, with segments V–VIII asymmetrical (Figs 10, 11). The male genitalic structures (Figs 11, 12) are similar to those of *Sigara* Fabricius, 1775, in the Corixidae: Corixinae. The females have a unique spermatheca (Fig. 13) by having a large distal seminal receptacle among water bugs (Larsen 1938, and Pluot-Sigwalt, personal communication).

Micronectids are small bugs with a body length less than 5 mm. The Bornean species are all less than 3.5 mm long. Most species of Micronectidae occur in the tropics and subtropics, with only a few found in temperate or cold climates of the Palaearctic Region. The Micronectidae can be easily separated from Corixidae (Leach, 1915) by the following characteristics: scutellum exposed, not covered by the pronotum, and the absence of ocelli. Micronectids are usually found in shallow stagnant or virtually stagnant habitats. Most species seem to prefer an open sandy or clayey bottom with little or no plant debris. In our experience, they can be especially numerous in shallow edges of ponds with sandy bottoms in temperate regions, and in open shallow pools of stream beds with sandy bottoms in tropical areas (Figs 98, 99).

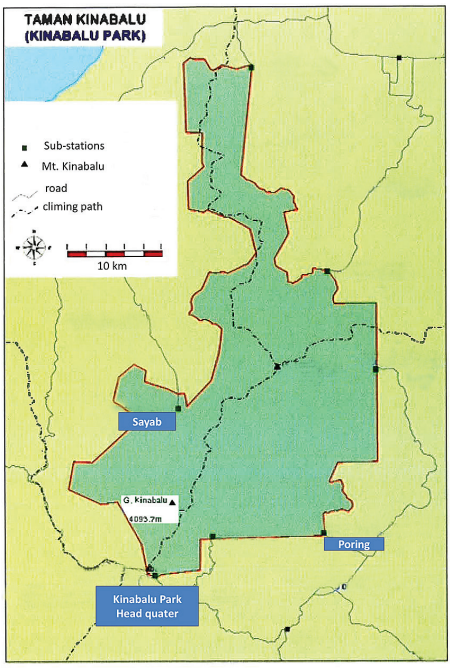
The history of Bornean Micronectidae

Although the history of studying of micronectids can be traced back to Linnaeus more than 200 years ago, the Bornean fauna of Micronectidae remains poorly known. Wróblewski (1968) speculated that *Micronecta decorata* Lundblad, 1933 might be present in Borneo. Only the recent expeditions to Borneo by NCB Naturalis have led to the first confirm records of micronectids on the island. Three species were found in 1999 (*M. ludibunda* Breddin, 1905; *M. kymatista* Nieser & Chen, 1999; *M. skutalis* Nieser & Chen, 1999); and later *M. lumutensis* Chen et al., 2008 was described from

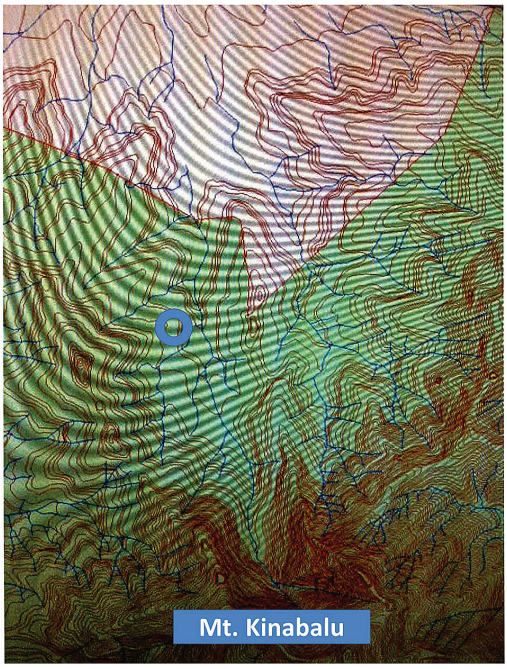


Figures 1–2. 1 Map of Sabah, (from Kitaura et al. 2003), indicating the excursion area in the Sabah Parks in 2012 **2** The substations in Crocker Range and Mt. Kinabalu National Parks (from Kitaura et al. 2003), with indications the localities of the samples. Credit: Sabah Parks.

Kalimantan. The last expedition in 2012 exploring the mountainous areas of the Sabah Parks added two species new to science, one species new to Borneo, and the confirmation of *M. decorata* on the island.



3 Overview of the Sabah Parks



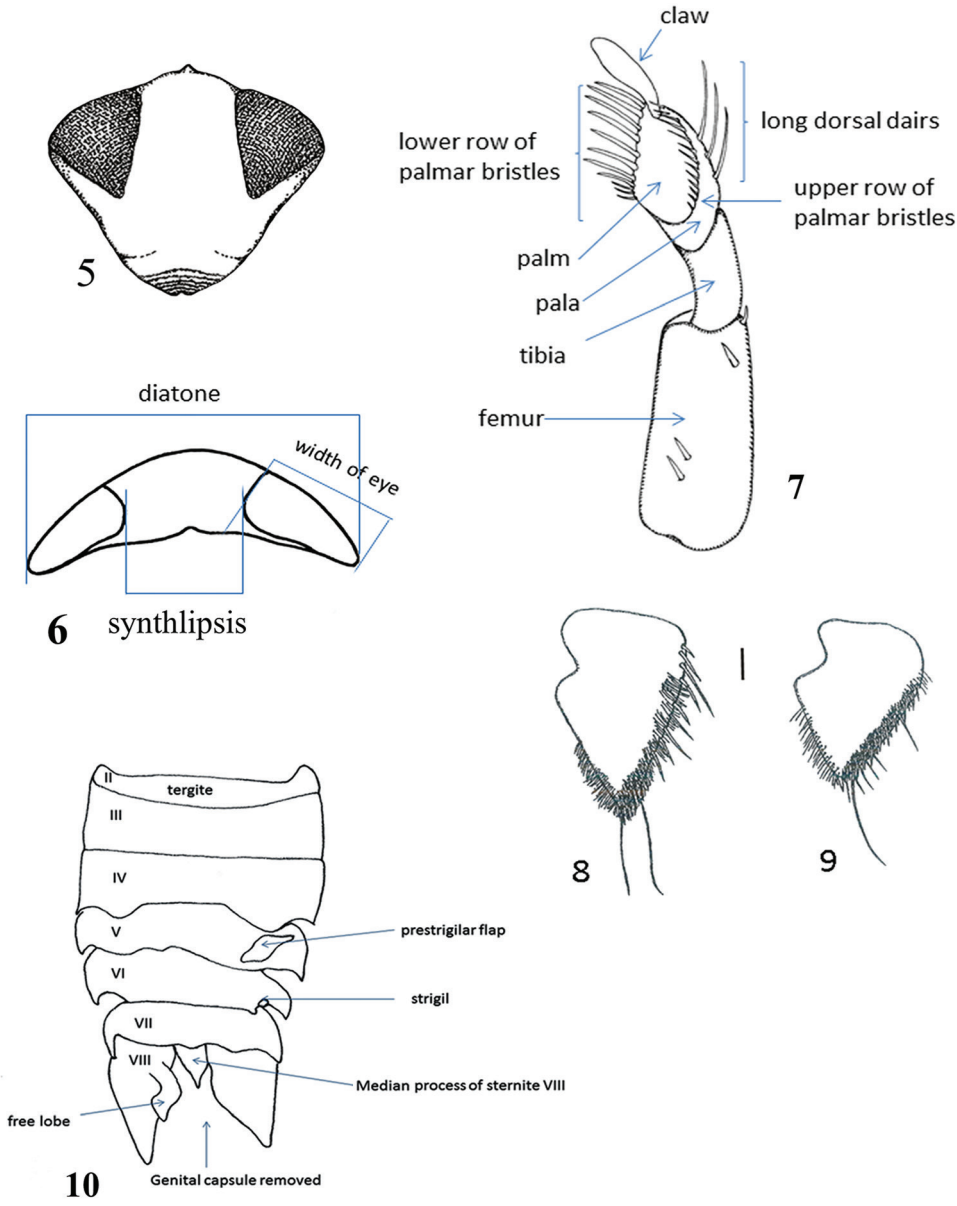
4 Streams around Sayab substation

Figures 3–4. 3 The area of Sabah Parks, with indications of the sampling area **4** The rivers and streams around the Sayab Substation, with the indication of the sampled sites. Credit: Sabah Parks.

Material and methods

Mount Kinabalu gives rise to five catchments (Wong and Philipps 1996). Sungai Silau–Silau and its tributary, the small stream of Carson Falls, originate in the Headquarters (Figs 2–3) area and flow into the Sungai Liwagu, which originates on the south slope near Headquarters and discharges into the Labuk River, which flows eastward into Labuk Bay north of Sandakan. Likewise, the Sungai Kipungit at Poring (Figs 2–3) ultimately discharges into Sungai Labuk. Our samples CN1268, CN1270, CN1271, CN1272 and CN1274 are from this catchment. The Sungai Kadamaian originates up-mountain from Kampong Kiau, and its tributary Sungai Kematis up-mountain from Kampong Sayap (Figs 2–4). The Sungai Kadamaian flows northwestward past Kota Belud into the South China Sea. Samples CN1262, CN1263, CN1264, and CN1275 are from the Sungai Kadamaian catchment area. Finally Sungai Kibambang and Sungai Mahua, which originate in the Crocker Range (Fig. 2) flows into Sungai Pegalan which joins Sungai Padas before draining into Brunei Bay. Samples CN1277, CN1278, CN1279, CN1281, CN1283, CN1285, CN1286, CN1288, and CN1289 are from the Sungai Pegalan catchment area.

The specimens obtained in Sabah were collected with a hand net, unless otherwise indicated in the material examined sections. The number of net sweeping or the time



Figures 5–10. 5–7, 10 *Micronecta* sp. diagrammatical illustrations of morphological terms used in the text: **5** head in frontal view **6** head in dorsal view **7** fore leg **8–9** *Micronecta* spp. right part of tergite VIII of males, in dorsal view, scale 0.1 mm: **8** *M. kymatista* Nieser & Chen, 1999 **9** *M. quadristrigata* Breddin, 1905 **10** *Micronecta* sp. schematic dorsal view of male abdominal segments.

spent one locality was not standardized. We usually collected in a given locality until three subsequent netting hauls did not yield any additional species. When unusual specimens were collected, an additional effort was made to collect a longer series. Most

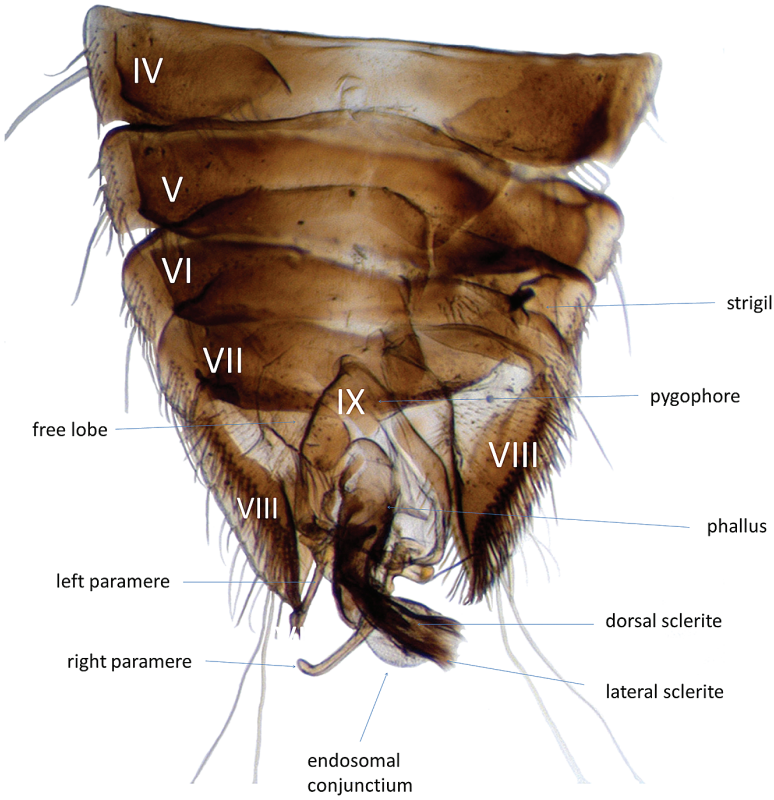


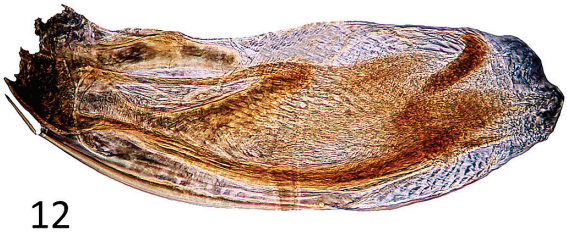
Figure 11. *Micronecta* sp., male, abdomen in dorsal view.

studied specimens are preserved in 96% ethanol, but some were mounted on carton labels or on microscopic slides.

To facilitate working with the key and better understanding the descriptions, three diagrammatic figures (Figs 6, 7, 10) and a photograph (Fig. 11) of the male genitalic structures of *Micronecta* sp. are provided. Anatomical abbreviations and terms used in species descriptions are indicated in Figs 5–13.

Specimens were studied by using a binocular (Zeiss Stemi 2000) and a compound microscope (Olympus BX51). Measurements are in mm, based on five specimens of each sex from the series (including the holotype, if available) and presented as a size range. Ocular index is 2S/ (D–S). Photographs were taken with Zeiss Discovery V12 SteRIO, lens Zeiss Plan Apo S 1.0×, FWD 60 mm, and, if necessary, were further processed using Adobe Photoshop CS6. Line illustrations were made using a binocular Zeiss Stemi 2000 with a camera lucida.

The studied specimens from several museum collections were mainly caught at light. The holotypes of the newly described species are placed in the Naturalis Biodiversity Centre (RMNH); the remaining material collected in Sabah is divided over NCTN, NMPC, RMNH, and ZCSM.



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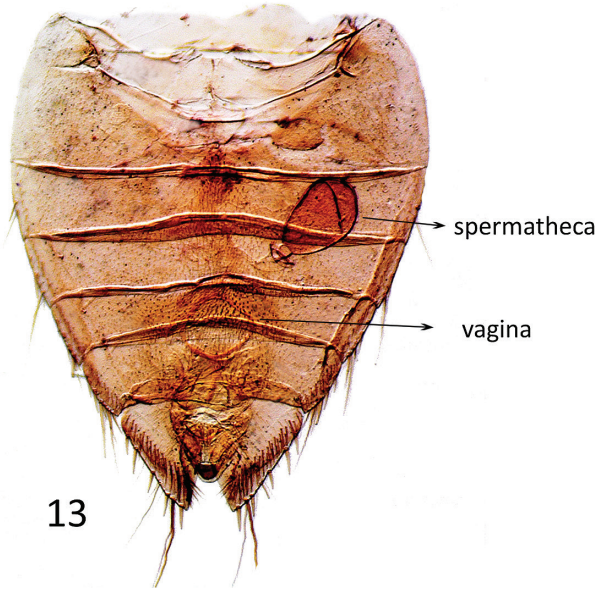

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Figures 12–13. 12 *Micronecta* sp., male phallus **13** *Micronecta* sp. female abdominal segments in ventral view, indicating the genitalia structures.

The following acronyms of museum collections are used:

NCTN Nieser & Chen Collection, Tiel, The Netherlands;
NHMW Naturhistorisches Museum Wien, Vienna, Austria;
NMPC National Museum (Natural History), Praha, Czech Republic;
RMNH Naturalis Biodiversity Centre, Leiden, The Netherlands;
ZCSM Zoological Collection of The Sabah Parks, Sabah, Malaysia;
ZMHB Museum für Naturkunde der Humboldt Universität zu Berlin, Bereich Zoologisches Museum, Berlin, Germany;
ZMUH Zoologisches Institut und Zoologisches Museum, Universität Hamburg, Hamburg, Germany.

Key to species of *Micronecta* occurring in Borneo (mainly applicable to males)

1 Corium with four solid longitudinal, darker stripes with variation from weak to distinct rings; pronotum typically with a pair of oval rings, varying from virtually absent to distinct (Fig. 12); left paramere with a laterally compressed tip (Fig. 77). Body length 1.9–2.4 mm ..... ***M.* (*Dichaetonecta*) *ludibunda* Lundblad, 1933**

– Corium with or without broken longitudinal stripes; pronotum without darker markings **2**
(Remarks: Some specimens of *M. kymatista* and *M. quadristrigata* may have fairly distinct longitudinal stripes on the corium, but these species have the left paramere with a sickle-shaped apex (Figs 87, 89); and are larger on average with lengths of 2.2–3.1 mm)

2 Smaller species, body length less than 2.0 mm **3**

– Larger species, body length 2.0 mm or more **5**

3 Hemelytra with a broad transverse medium to dark brown band at middle (Figs 17, 26); left paramere with a ribbed apex (Fig. 83). Body length 1.7–1.8 mm ..... ***M.* (*Micronecta*) *liewi* sp. n.**

– Hemelytra without a broad transverse medium to dark brown band; left paramere not ribbed apically. Body length 1.5–1.7 mm **4**

4 Left paramere with a rounded apex and a small indentation at the base of the shaft (Fig. 85). Body length 1.5–1.7 mm ..... ***M.* (*Micronecta*) *skutalis* Nieser & Chen, 1999**

– Left paramere with an indented apex and without a small indentation at the base of the shaft (Fig. 79). Body length 1.5 mm ..... ***M.* (*Micronecta*) *lumutensis* Chen, Nieser & Lansbury, 2008**

5 Free lobe of tergite VIII straight, with a sinuate apical margin (Fig. 70); apex of left paramere not sickle-shaped (Fig. 81) **6**

– Free lobe of tergite VIII sinuate with a rounded apical margin (Fig. 72); apex of left paramere sickle-shaped (Fig. 87) **7**

6 Species dark brown; free lobe of tergite VIII apically narrowed (Fig. 70); right paramere apically dilated (Fig. 80). Body length 2.0–2.2 mm ..... ***M.* (*Micronecta*) *lakimi* sp. n.**

– Species light to medium brown; free lobe of tergite VIII apically widened (Fig. 66); apex of right paramere acutely narrowed (Fig. 74). Body length 2.2–2.4 mm ..... ***M.* (*Dichaetonecta*) *decorata* Lundblad, 1933**

7 Apical half of inner margin of right part of tergite VIII with 28–35 marginal hairs caudally arranged in a double or triple row (Fig. 8). Body length 2.8–3.1 mm ..... ***M.* (*Sigmonecta*) *kymatista* Nieser & Chen, 1999**

– Apical half of inner margin of right part of tergite VIII with 20–25 marginal hairs caudally arranged in a single to double row (Fig. 9). Body length 2.2–2.9 mm ..... ***M.* (*Sigmonecta*) *quadristrigata* Breddin, 1905**

Descriptions and redescriptions of the species of *Micronecta* in Borneo

Genus *Micronecta* Kirkaldy, 1897

Type species. *Notonecta minutissima* (Linnaeus, 1758), by original designation.

Subgenus *Dichaetonecta* Hutchinson, 1940

Type species. *Sigara scholtzi* Fieber, 1860, by original designation.

Diagnosis. Male: palar claw usually of moderate size, strigil present, seventh abdominal sternite with one or two strongly developed bristles, prestrigilar flap with a very obtuse tip, left paramere variable but not with a plate-like shaft with sub parallel margins, right paramere elongate.

Micronecta (*Dichaetonecta*) *decorata* Lundblad, 1933

Figs 14, 24, 30, 31, 43, 51, 58, 66, 74, 75, 90

Micronecta decorata Lundblad, 1933: 93–94 (original description).
Micronecta decorata: Wróblewski 1968: 775 (checklist).
Micronecta decorata: Nieser 2000: 287 (key).
Micronecta decorata: Chen et al. 2005: 420 (checklist).

Material examined. THAILAND (new record for Thailand): Chiang Mai Province: Doi Saket, Ban Pong Ao, Kuang River at bridge in road 118, 38 km NE Chiang Mai City, 30.i.2002, leg. P. Chen, N. Nieser, A. Thanyakam & C. Duangsupa, C0220, 19 males 30 females. **Uttaradit Province:** Baan Muangchedton, Lake Naam Pat, 10 km W of Ban Khok town, 10.ii.2002, stagnant ponds downstream of barrage, 10.ii.2002, leg. P. Chen, N. Nieser, A. Thanyakam, C. Duangsupa & W. Jaiyai, C0231, 7 males 13 females. All macropterous (samples stored in ethanol 70%). **MALAYSIA: Sabah (confirmation of occurrence in Borneo):** Kota Belud Dist., Crocker Range Park, Sungai Mahua at substation beside restaurant, 05°47.53'N, 116°24.19'E, 1053 m. a.s.l., 22.ix.2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1283, 1 male and 1 female macropterous. (All are in the collection of NCTN).

Redescription. Macropterous specimens. Generally a medium-sized, (length 2.2–2.4 mm) yellowish-brown species, with darker markings varying from virtually absent (Fig. 14) to quite distinct, medium- brown: a V-shaped stripe on clavus and four interrupted longitudinal stripes on corium (Fig. 24); eyes castaneous to grayish.

Dimensions. Body length: male 2.2–2.3, female 2.2–2.4; width: male 1.01–1.06, female 1.00–1.18; diatone: male 0.77–0.81, female 0.75–0.84; width of pronotum: male 0.82–0.88, female 0.81–0.93; ocular index: male 1.56–1.77, female 1.48–1.65. Body length twice the maximal width (male 2.23/1.04, female 2.33/1.12). Pronotum

slightly wider than head (H/P male 0.80/0.85, female 0.81/0.88), synthlipsis one and half times the posterior width of an eye (S/E male 0.37/0.21, female 0.36/0.24).

Colour. Frons and vertex sordid yellow, eyes castaneous to grayish. Pronotum yellowish-brown, disk without markings except for a distinct yellowish stripe on posterior margin. Hemelytra light brown, with elongate darker marks arranged in four interrupted, longitudinal, brown stripes on corium (Fig. 24). Right membrane slightly paler than corium, without markings; left membrane hyaline. Embolium yellowish brown with three brown spots. Venter, abdomen, thorax, and legs pale yellow. [Our Thai material contains specimens with only a very vague or virtually absent hemelytral pattern. The Borneo specimens show a hemelytral pattern similar to *M. quadristrigata* as stated by Lundblad (1933). Apparently, the hemelytral pattern fades when the specimens are stored in 70% or 96% ethanol].

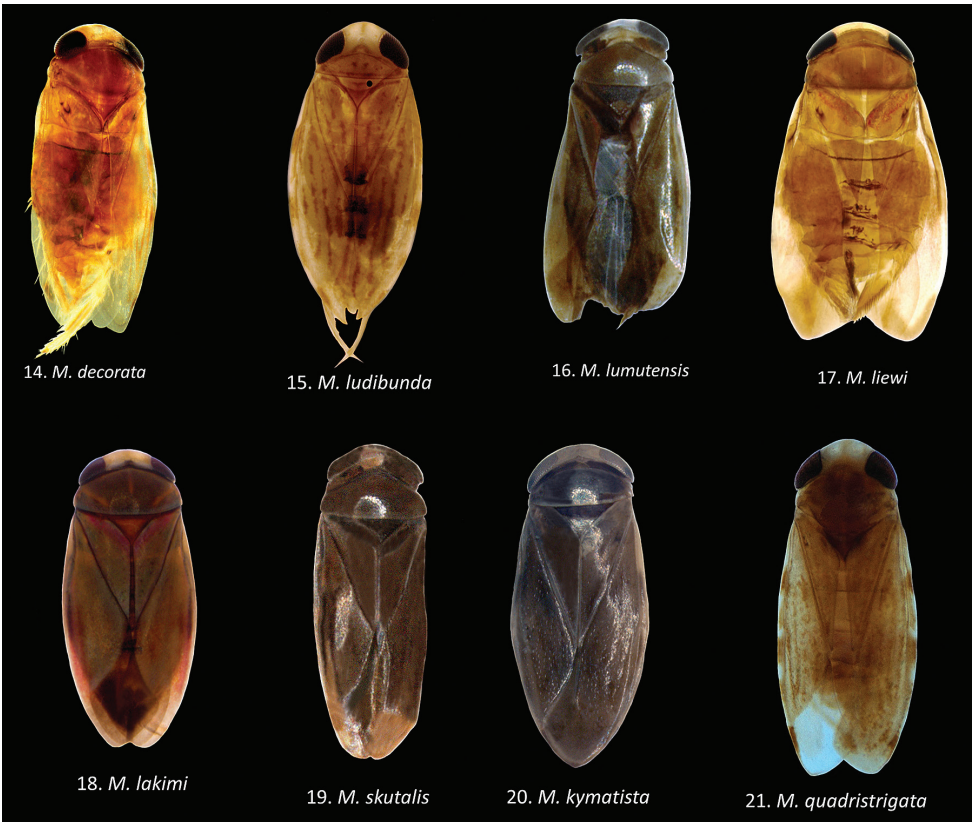
Pronotum. About two and a half times as wide as long (W/L 0.87/0.36), dorsally convex with lateral margins straight and more or less truncate (Fig. 14). Hemelytra smooth, with four shallow, longitudinal grooves on corium, densely beset with small spinules, notably on corium. The right membrane texture same as corium, smooth without grooves or spines. Spines laterally on abdominal segments: V with two short and one longer stout spine; VI with three short and one long spine; VII with two or three short and one long stout spine; VIII with four or five short and one long, stout spine or sometimes without a long spine and two long hair-like bristles.

Legs. Length of segments: fore leg: male: femur 0.28, tibia 0.14, pala 0.15; female: femur 0.31, tibiotarsus 0.30; middle leg: male: femur 0.85, tibia 0.27, tarsus 0.39, claw 0.30; female: femur 0.85, tibia 0.29, tarsus 0.40, claw 0.28; hind leg: male: femur 0.51, tibia 0.40, tarsus I 0.39, tarsus II 0.19, claw 0.13; female: femur 0.52, tibia 0.42, tarsus I 0.37, tarsus II 0.19, claw 0.12. Palmar bristles: 21–23 in upper row, 17–18 in lower row.

Male. Fore femur (Fig. 30), with a pair of pegs on proximal third, a small peg distally, and a larger bristle-like spine sub-distally; pala with four long dorsal hairs. Claw (Fig. 31) parallel sided, with a transverse carina. Dorsum of abdomen: prestrigilar lobe sub-triangular, with a short, obtusely rounded apex (Fig. 43); strigil small, sub-oval, comb with about 45 comparatively distinct teeth (Fig. 51); free lobe of left part of tergite VIII with an expanded apex (Fig. 66), a sinuate apical margin, and 10–15 apical bristles. Left paramere (Fig. 75) with a narrow, apically, slightly dilated shaft and a subapical indentation; right paramere in lateral view (Fig. 74) with an evenly curved, sickle-shaped shaft, apex acutely tapering, basal lobe with about 25 stridulatory ridges. Mediocaudal lobe of sternite VII (Fig. 58) with apical part acutely pointed, with one strongly developed bristle.

Female. Fore femur with the same general arrangement of pegs and setae as in male. The seminal capsule of spermatheca clavate (Fig. 90).

Comparative notes. Males can be recognized by the form of the free lobe of tergite VIII. The palmar claw of the male, with its oblique carina, also is unique but it is often folded into the palm, usually making it difficult to observe.



Figures 14–21. Habitus of *Micronecta* spp., in dorsal view, legs omitted: **14** *M. decorata* Lundblad, 1933, macropterous male, body length 2.38 mm **15** *M. ludibunda* Breddin, 1905, brachypterous male, body length 2.32 mm **16** *M. lumutensis* Chen, Nieser & Lansbury, 2008, paratype, macropterous male, body length 1.40 mm **17** *M. liewi* sp. n., paratype, macropterous male, body length 1.77 mm **18** *Micronecta* (*Micronecta*) *lakimi* sp. n., paratype, macropterous male, body length 2.12 mm **19** *M. skutalis* Nieser & Chen, 1999, paratype, macropterous male (membrane rolled partly inward), body length 1.58 mm **20** *M. kymatista* Nieser & Chen, 1999, paratype, macropterous male, body length 2.80 mm **21** *M. quadristrigata* Breddin, 1905, macropterous male, body length 2.88 mm.

Habitat. We have taken this species several times in Chiang Mai and other northern provinces in Thailand, where it is apparently quite common. Sample C0220 was taken from shallow virtually stagnant water in a wide unshaded river bed with a sandy bottom.

Distribution. Thailand (see above); Malay Peninsula (Wróblewski 1968: record for Malaysia without exact locality; Fernando and Cheng 1974); INDONESIA: Sumatra (Lundblad 1933), Java (Lundblad 1933); and Borneo.

Note. Wróblewski (1968) recorded this species from Borneo with a question mark. His speculation is confirmed here.

Micronecta (*Dichaetonecta*) *ludibunda* Breddin, 1905

Figs 15, 22, 25, 32, 33, 44, 52, 59, 67, 76, 77, 91

Micronecta ludibunda Breddin, 1905a: 57 (original description).
Micronecta ludibunda: Breddin 1905b: 157–158 (extensive description).
Micronecta graphiptera Horváth, 1918: 146 (original description).
Micronecta ludibunda: Lundblad 1933: 95–96 (redescription).
Micronecta inconspicua Lundblad, 1933: 96–98 (original description).
Micronecta striatella Lundblad, 1933: 98–100 (original description).
Micronecta ludibunda: Wróblewski 1968: 765–767 (redescription)
Micronecta ludibunda: Nieser and Chen 1999: 80 (record from Kalimantan Timur)
Micronecta ludibunda: Polhemus and Golia 2006: 531–534 (occurrence in Florida, USA).
Micronecta ludibunda: Tinerella 2008: 29–34 (redescription, extensive synonymy).

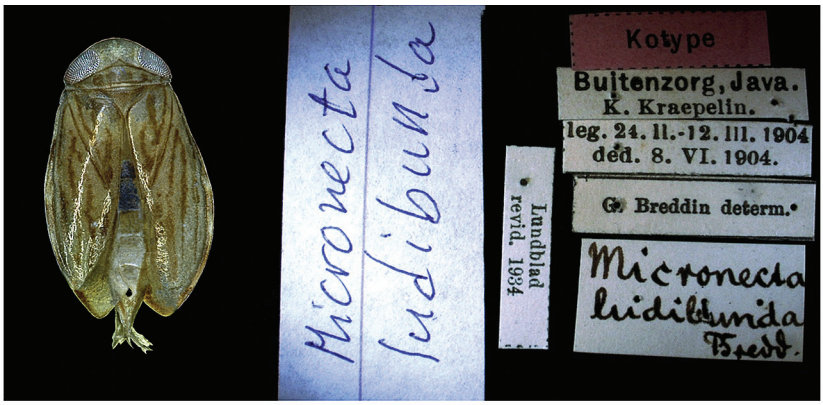
Type material examined. Syntypes, INDONESIA: "Kotype; Buitenzorg (= Borgor) Java, K. Kraepelin; leg. 24.II–12.III.1904, ded.8.VI.1904; Breddin determ.; Lundblad revid. 1934", 2 males 2 females (ZMUH).

Additional material examined. THAILAND: Chon Buri Province: Khao Khaew Open Zoo, ponds, 7.iv.2001, leg. P. Chen, S. Leepitakrat & B. Kavinseksan, 50 males 50 females (sample stored in 70% ethanol in NCTN).

Redescription. Brachypterous and macropterous specimens. Generally a medium-sized (length 1.9–2.4 mm), yellowish-brown, species with four distinct, uninterrupted, longitudinal stripes on corium (Figs 15, 22, 25), and a variable darker pattern on pronotum, typically consisting of a pair of oval rings. Brachypterous and macropterous specimens differ in the development of the pronotum, but the differences between the brachypterous and macropterous morph are less pronounced than in most other species of *Micronecta*.

Dimensions. Body length: brachypterous male 1.9–2.2, macropterous male 2.1–2.3, brachypterous female 1.9–2.3, macropterous female 2.2–2.4; width: male 1.01–1.18, female 1.04–1.22; diatone: male 0.68–0.85, female 0.70–0.87; width of pronotum: male 0.69–0.87, female 0.71–0.92; ocular index: male 1.02–1.18, female 0.87–1.14. Body length twice the maximal width (male 2.06/1.05, female 2.25/1.15). Pronotum slightly wider than head (H/P male 0.76/0.78, female 0.77/0.81), synthlipsis subequal to the posterior width of an eye (S/E male 0.24/0.24, female 0.26/0.28).

Colour. Frons and vertex sordid yellow, eyes castaneous. Pronotum yellowish brown, disk typically with a pair of darker oval rings, varying from nearly absent via fragmented rings to complete; posterior margin with a distinct yellowish stripe. Hemelytra yellowish brown; clavus with a darker, V-shaped, medium-brown stripe; corium typically with four longitudinal, medium-brown, uninterrupted stripes (Figs 15, 22, 25); embolium yellowish with four or five brown spots; right membrane poorly delimited from the corium, with the same colour and texture but without darker stripes; left membrane more distinctly separated from corium, hyaline, and more membranous than corium. Venter, abdomen, thorax, and legs pale yellow.



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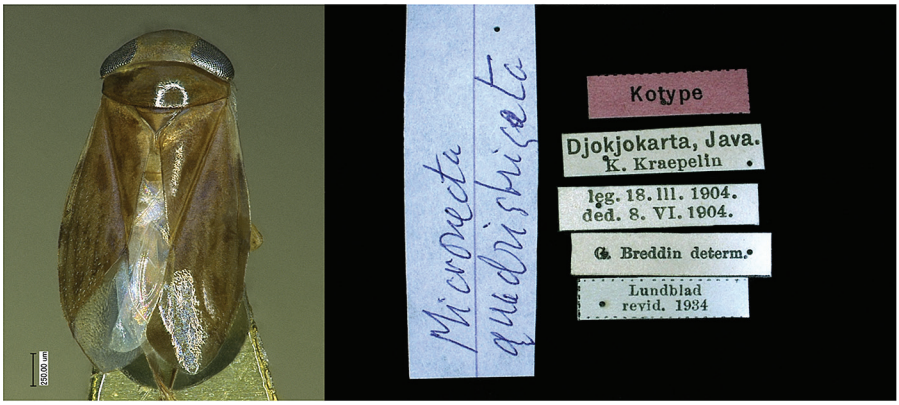

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Figures 22–23. The syntypes in ZMUH, Germany: **22** *Micronecta ludibunda* Breddin, 1905, syntype, brachypterous female, body length 1.80 mm **23** *Micronecta quadristrigata* Breddin, 1905, syntype, macropterous female, body length 2.85 mm.

Pronotum short (Fig. 15), about four times as wide as long (W/L 0.79/0.20); in brachypterous specimens dorsally flat, in macropterous specimens dorsally somewhat convex. Hemelytra smooth, sparsely beset with small spinules, notably on corium. Spines laterally on abdominal segments: V with two short and one longer stout spine; VI with two short, and one long spine; VII with two or three short, and one long stout spine; VIII with five short and one long stout spine, and one long hair-like bristle.

Legs. Length of leg segments: fore leg: male: femur 0.26, tibia 0.14, pala 0.14; female: femur 0.26, tibiotarsus 0.26; middle leg: male: femur 0.70, tibia 0.23, tarsus 0.30, claw 0.25, female: femur 0.76, tibia 0.23, tarsus 0.33, claw 0.26; hind leg: male: femur 0.46, tibia 0.36, tarsus I 0.40, tarsus II 0.13, claw 0.08; female: femur 0.48, tibia 0.37, tarsus I 0.42, tarsus II 0.16, claw 0.08. Palmar bristles: 10 to 11 in upper row, 10 to 11 in lower row.

Male. Fore femur (Figs 32, 33) with a pair of pegs on proximal third and a pair of small pegs distally; pala with three long dorsal hairs. Claw slender and clavate, apex

mucronate. Dorsum of abdomen: prestrigilar lobe (Fig. 44) sub-triangular, with a short, truncate apex; strigil (Fig. 52) small, suboval, comb with about 55 comparatively distinct teeth; free lobe of left part of tergite VIII (Fig. 67) with a slightly expanded apex and 10–15 apical bristles. Left paramere (Fig. 77) with a narrow, more or less parallel-sided shaft, apex laterally compressed, flag-like; right paramere in lateral view (Fig. 76) with an evenly curved shaft and tapering apex, basal lobe not distinctly differentiated from basal part of paramere, with over 50 stridulatory ridges. Mediocaudal lobe of sternite VII (Fig. 59) long, with apical part elongate and obtusely rounded to pointed apically, with or without one to two larger bristles.

Female. Fore femur with the same general arrangement of pegs and setae as in male. The seminal capsule of spermatheca mushroom-shaped (Fig. 91).

Comparative notes. Within Bornean *Micronecta*, this species is easily recognized in both sexes by its distinct linear pattern on the hemelytra (Figs 15, 22, 25).

Distribution. This species with a wide distributional pattern, so far has been reported from: India and Sri Lanka (Hutchinson 1940, Wróblewski 1972), Thailand (Wróblewski 1968), Vietnam (Wróblewski 1967), West Malaysia (Leong 1966), Indonesia (Breddin 1905a, Lundblad 1933, Nieser and Chen 1999), New Guinea and Solomon Islands (Tinerella 2008), and introduced into Florida, U.S.A. (Polhemus and Golia 2006). Nieser and Chen (1999) mentioned one male from Borneo: Kalimantan Timur in NHMW.

Subgenus *Micronecta* Kirkaldy, 1897

Type species. *Notonecta minutissima* Linnaeus, 1758, by original designation.

Diagnosis. Males with palar claw usually relatively large and apically dilated; sternite VIII with three to six (usually four) well-developed bristles; shaft of left paramere usually plate-like with subparallel margins.

Micronecta (*Micronecta*) *lumutensis* Chen, Nieser & Lansbury, 2008

Figs 16, 34, 45, 60, 68, 78, 79, 92

Micronecta lumutensis Chen, Nieser & Lansbury, 2008: 270–272 (original description).

Type material examined. INDONESIA: Kalimantan Timur: Pasir, Gunung Lumut, 2 km E of Rantaulayong, 01°36.36'S, 115°58.38'E, 24.XI.2005, E. Gassó Miracle, EGM25, evergreen rainforest along river, at light, ML 19/21 hrs., 1 male holotype, 1 male and 2 female paratypes, all macropterous (RMNH).

Redescription. Macropterous form. Generally a small (body length 1.5 mm) grayish *Micronecta*, with poorly contrasting markings.

Dimensions. Body length: male 1.48–1.52, female 1.45–1.50; width: male 0.69–0.72, female 0.52–0.54; diatone: male 0.49–0.51, female 0.52–0.54; width of prono-

Figures 24–29. *Micronecta* spp., left forewings, in dorsal view: **24** *M. decorata* Lundblad, 1933 **25** *M. ludibunda* Breddin, 1905 **26** *M. liewi* sp. n., paratype **27** *M. lakimi* sp. n., paratype **28** *M. skutalis* Nieser & Chen, 1999 **29** *M. quadristrigata* Breddin, 1905.

tum: male 0.54–0.57, female 0.56–0.58; ocular index: male 1.77–1.78, female 1.59–1.60. Body length 2.1–2.5 times the maximal width. Pronotum slightly wider than head, synthlipsis wider than the posterior width of an eye (S/E 0.24/0.16).

Colour. Vertex sordid yellow, the frons yellowish with a brown spot, eyes grey, rostrum yellowish with dark brown transverse grooves. Pronotum yellowish brown, disk unmarked, posterior and lateral margins with a yellowish stripe. Hemelytra yellowish brown, apex of clavus darker brown, corium with three interrupted longitudinal brown stripes (Fig. 16), right membrane poorly delimited from the corium, with the same colour and texture as corium but without darker stripes, left membrane more distinctly separated from its corium, hyaline, and more membranous than the corium. Venter of abdomen and thorax grayish, legs yellowish.

Pronotum (Fig. 16) convex dorsally, about two and half times as wide as long (W/L male 0.56/2.1, female 0.57/0.23). Hemelytra smooth, beset with small spinules, notably on corium, arranged in longitudinal rows, and along the membranal suture. Spines laterally on abdominal segments: V with two short and one longer stout spines; VI with two or three short and one intermediate spine; VII with three or four short, one intermediate, and one or two long, stout spines; VIII with five short spines and two long hair-like bristles.

Legs. Length of leg segments: fore leg: male: femur 0.19, tibia 0.07, pala 0.11; female: femur 0.20, tibiotarsus 0.20; middle leg: male and female: femur 0.49, tibia 0.17, tarsus 0.24, claw 0.17; hind leg: male and female: femur 0.31, tibia 0.25, tarsus I 0.26, tarsus II 0.12, claw 0.07. Palmar bristles: about 15 in upper row, about 16 in lower row.

Male. Fore femur (Fig. 34) with a pair of pegs on proximal third, a subdistal peg dorsally, and one or two small pegs distally; pala with three long, dorsal hairs. Claw slender, clavate. Dorsum of abdomen: prestrigilar flap (Fig. 45) with a short, acute apex; strigil small, suboval, at a magnification of 400×, no separate teeth observable;

free lobe of left part of tergite VIII (Fig. 68) more or less parallel-sided, softly curved, with a rounded apex and 9–10 apical bristles. Mediocaudal lobe of sternite VII (Fig. 60) short, acute, with three or four larger bristles. Left paramere (Fig. 79) apically slightly dilated, with an apical impression; right paramere in lateral view (Fig. 78) gradually widened toward apex, basal lobe with about eight stridulatory ridges.

Female. Fore femur with the same general arrangement of pegs and setae as in male. Seminal capsule of spermatheca mushroom-shaped (Fig. 92).

Comparative notes. The small size, with a body length of about 1.5 mm, separates this species from other Bornean species except *M. skutalis*. Males of *M. lumutensis* and *M. skutalis* can be separated by the characters of parameres as given in the key (Figs 78–79, 84–85). In addition, the seminal capsule of *M. lumutensis* is mushroom-shaped (Fig. 92), whereas that of *M. skutalis* is egg- or urn-shaped (Fig. 95). Females can be indentified only by their association with males.

Habitat. The type specimens were collected at light in a mountainous area.

Distribution. Indonesia: Kalimantan Timur (Chen et al. 2008).

Micronecta (*Micronecta*) *liewi* sp. n.

http://zoobank.org/49A37756-016D-4BF7-A0B3-FAE0BAF10C5F

Figs 17, 26, 35, 36, 46, 53, 61, 69, 82, 83, 93, 98

Type material examined. Holotype: male (body length 1.72, in RMNH), MALAYSIA: Sabah, Crocker Range, Inobong Substation, Sungai Kibambangan (Fig. 98), downstream of waterfall, 05°51.28'N, 116°08.41'E, 433 m. a.s.l., 18.ix.2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1277. Paratypes: same data as holotype, 12 males, 17 females. All macropterous (in RMNH, NCTN, NMPC, ZCSM).

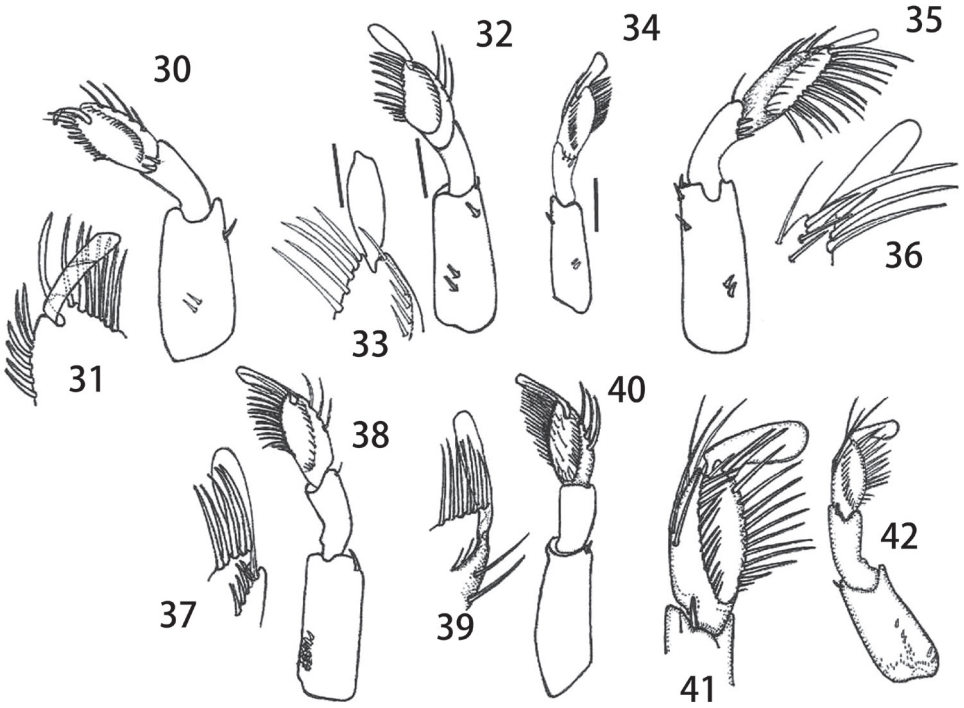
Description. Macropterous form (Fig. 17). Generally, a rather small (body length 1.7–1.8) yellowish to light brown species, with distinct brown markings.

Dimensions. Length: male 1.71–1.79, female 1.72–1.82; width: male 0.89–0.90, female 0.89–0.93; diatone: male 0.65–0.68, female 0.64–0.69; width of pronotum: male 0.71–0.71, female 0.70–0.75; ocular index: male 1.82–2.06, female 1.89–2.18. Body length twice maximal width (male 1.74/0.90, female 1.78/0.91).

Colour. Frons and vertex sordid yellow, eyes dark castaneous. Pronotum and hemelytra sordid yellow to light brown, the hemelytra with a broad transverse medium to dark brown band at middle (Fig. 26), left membrane medium to dark brown. Disk of pronotum unmarked. Venter and thorax sordid yellow, laterally infuscate, abdomen grayish brown, medially variably lighter. Legs pale yellow.

Head slightly narrower than pronotum, synthlipsis 1.7–1.8 times as wide as the posterior margin of an eye.

Pronotum well developed, dorsally convex with lateral margins distinctly straight, and more or less truncate (Fig. 17), slightly over 2.5 times as wide as long (W/L male 0.71/0.26, female 0.72/0.28). Hemelytra (Fig. 26) smooth, beset with extremely small unobtrusive spinules. Spines laterally on abdominal segments: V with one short and



Figures 30–42. *Micronecta* spp., right foreleg in anteroventral view including apex of pala: **30–31** *M. decorata* Lundblad, 1933 **32–33** *M. ludibunda* Breddin, 1905 **34** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **35–36** *M. liewi* sp. n., paratype **37–38** *M. lakimi* sp. n., paratype **39–40** *M. skutalis* Nieser & Chen, 1999 **41** *M. kymatista* Nieser & Chen, 1999 **42** *M. quadristrigata* Breddin, 1905. Scale bars: 0.1 mm (**30, 32, 34, 35, 37, 39, 42**); 0.05 mm (**31, 33, 36, 38, 40, 41**).

one long spine, VI with two short, and two long spines; VII with three or four short and one long spine; VIII with four or five medium long spines and two long hair-like bristles.

Legs. Length of leg segments: fore leg: male: femur 0.24, tibia 0.11, pala 0.12; female: femur 0.23, tibiotarsus 0.22; middle leg: male: femur 0.54, tibia 0.17, tarsus 0.25, claw 0.14; female: femur 0.56, tibia 0.19, tarsus 0.23, claw 0.15; hind leg: male: femur 0.39, tibia 0.29, tarsus I 0.32, tarsus II 0.13, claw 0.08; female: femur 0.42, tibia 0.33, tarsus I 0.32, tarsus II 0.13, claw 0.08. Palmar bristles: about 13 in lower row and ca. 11 in upper row.

Male. Fore femur (Fig. 35) with a pair of pegs in proximal third, one peg dorsally at distal third and two pegs dorsodistally; tibia without dorsoapical peg; pala with three comparatively short dorsal hairs, 10–12 short bristles in upper row, distal bristle of upper row much stouter and longer than other upper bristles, and 14 to 16 longer bristles in lower row. Claw simple, elongate (Fig. 36). Dorsum of abdomen: prestrigilar lobe (Fig. 46) with a pointed apex, strigil small and narrow (Fig. 53), comb with about 75 teeth, free lobe of left part of tergite VIII (Fig. 69) caudally truncate. Left paramere

(Fig. 83) with a wide shaft, apex with short longitudinal grooves; right paramere (Fig. 82) with a medium-sized shaft and a slightly expanded apex, basal lobe strongly developed, stridulatory ridges not observed. Mediocaudal lobe of sternite VII (Fig. 61) with four bristles.

Female. Fore femur with the same general arrangement of pegs and setae as in male. Seminal capsule of spermatheca urn-shaped (Fig. 93).

Comparative notes. The hemelytral pattern is diagnostic among the Melanesian *Micronecta* fauna. *Micronecta liewi* is similar to *M. melanopardala melanopardala* Nieser & Chen, 2003 described from the Philippines by having a similar transverse band midway along the hemelytra, but it differs from *M. melanopardala melanopardala* by lacking a dark patch on the clavi as in *M. melanopardala.* In general, *M. liewi* has more distinct dark markings than in *M. melanopardala adiaphana* Nieser & Chen, 2003. Furthermore, in both subspecies of *M. melanopardala*, the shafts of the right parameres are more slender than *M. liewi*, and the apex of the right paramere of *M. melanopardala* is not expanded.

The strongly developed distal bristle of the upper row on the male pala (Figs 35, 36) gives impression of an additional claw as in the subgenus *Unguinecta* Nieser, Chen & Yang, 2005 from southern continental Asia. However, the four bristles on mediocaudal lobe of sternite VII of the male, the shape of the parameres, and the shape of the free lobe on the left part of tergite VIII of the male will all allow placement in the subgenus *Micronecta*.

Etymology. This species is named in honor of Dr. Thor Seng Liew (NBC Naturalis and Sabah University, Malaysia), for his outstanding contributions to the study of the biodiversity of Sabah, and his invaluable help with our work on water bugs in Borneo.

Habitat. The type series was collected in a small, virtually stagnant bay on the downstream side of Kibambangan waterfall (Fig. 98).

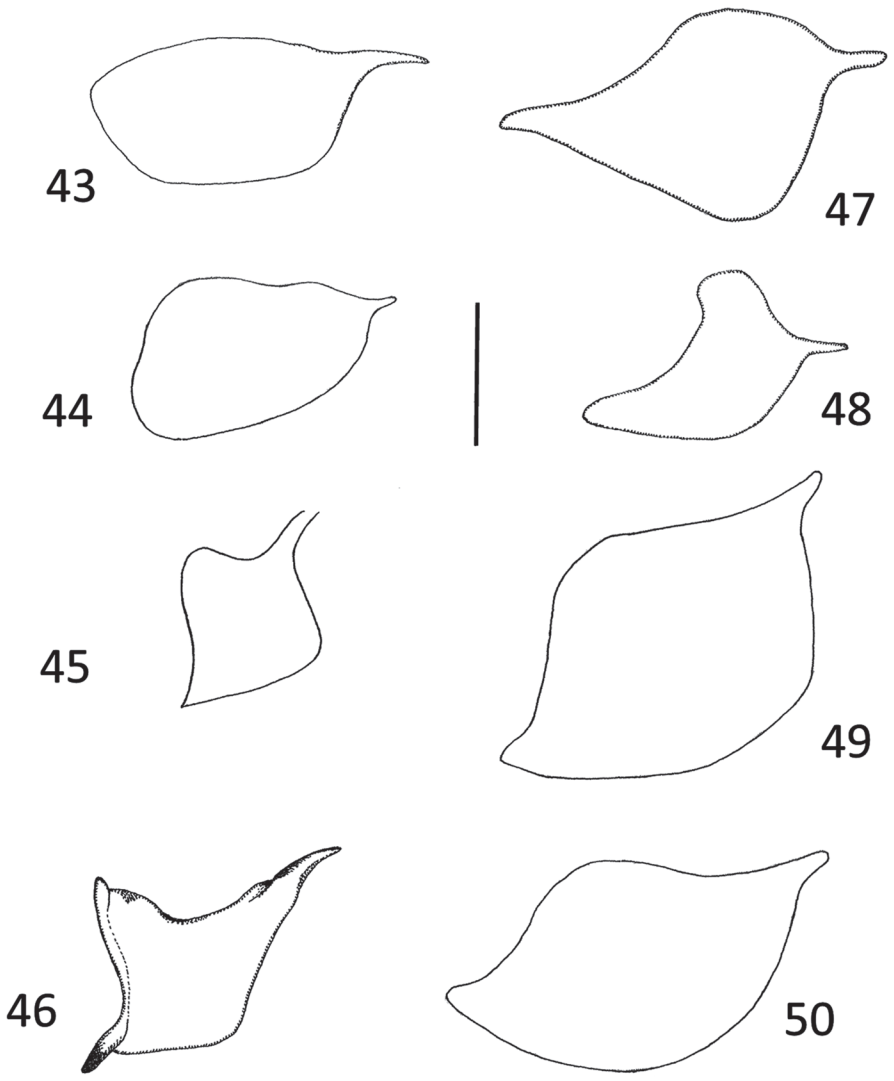
Distribution. Malaysia: Sabah (this paper).

Micronecta (*Micronecta*) *lakimi* sp. n.

http://zoobank.org/F8B54026-F285-47D7-954F-8D522FDA38EB
Figs 18, 27, 37, 38, 47, 54, 62, 70, 80, 81, 94, 99

Type material examined. Holotype: male (body length 1.00 mm, in RMNH), MALAYSIA: Sabah, Kota Belud Dist., Crocker Range, Mahua Substation, Mahua waterfall (fig. 99), 05°47.59'N, 116°24.08'E, 1215 m. a.s.l., 21.IX. 2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1281. Paratypes: same data as holotype, 7 males, 25 females; MALAYSIA: Sabah, Kota Belud Dist., Crocker Range Park, Sungai Mahua near entrance of Mahua Substation, 05°47.53'N, 116°24.19'E, 1053 m. a.s.l., 22.ix.2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1283, 10 males, 3 females. (Paratypes in RMNH, NCTN, NMPC, ZCSM).

Description. Macropterous form (Fig. 18). Generally a medium-sized (body length 2.1–2.2), rather dark grayish-brown species, without obvious markings.



Figures 43–50. *Micronecta* spp., prestrigilar flap on abdominal segment V, male; in dorsal view: **43** *M. decorata* Lundblad, 1933 **44** *M. ludibunda* Breddin, 1905 **45** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **46** *M. liewi* sp. n., paratype **47** *M. lakimi* sp. n., paratype **48** *M. skutalis* Nieser & Chen, 1999 **49** *M. kymatista* Nieser & Chen, 1999 **50** *M. quadristrigata* Breddin, 1905. Scale bar: 0.1 mm

Dimensions. Length: male 2.07–2.22, female 2.11–2.13; width: male 0.92–1.00 female 1.01–1.04; diatone: male 0.74–0.76, female 0.75–0.77; width of pronotum: male 0.83–0.88, female 0.84–0.88; ocular index: male 1.57–1.89, female 1.76–2.05. Body length slightly over twice maximal width (male 2.16/0.97, female 2.12/1.02). Head in dorsal view short, its median length less than half the median length of pronotum (male 0.14/0.33, female 0.15/0.36). Head narrower than pronotum, synth-

lipsis 1.5–1.7 times as wide as the posterior margin of an eye (male 0.35/0.23, female 0.37/0.22).

Colour. Vertex yellowish, with a small dark brown point at middle of posterior margin (raised for air intake), eyes grayish. Pronotum unicolorous, medium-brown except for a narrow yellow transverse band along posterior margin. Scutellum reddish brown. Hemelytra medium brown, clavus with a reddish stripe along the scutellar margin, pruinose area at base of embolar groove black, apical third of corium light brown, laterally with a reddish tinge. Frons medium brown, rostrum with a dark median gray marking. Thoracic and abdominal venter dull dark grayish to blackish. Legs pale yellow, anterior femur with a brownish stripe and intermediate tarsus I with a small black spot distally.

Pronotum well developed, dorsally convex with lateral margins distinctly truncate (Fig. 18), about 2.5 times as wide as long (W/L male 0.85/0.34, female 0.87/0.36). Hemelytra (Fig. 27) smooth, beset with small, distinct spinules, most notably on corium. Spines laterally on abdominal segments: V with three short and one long spine; VI with two short and two long spines; VII with two short and two long spines; VIII with four or five short spines and two long hair–like bristles.

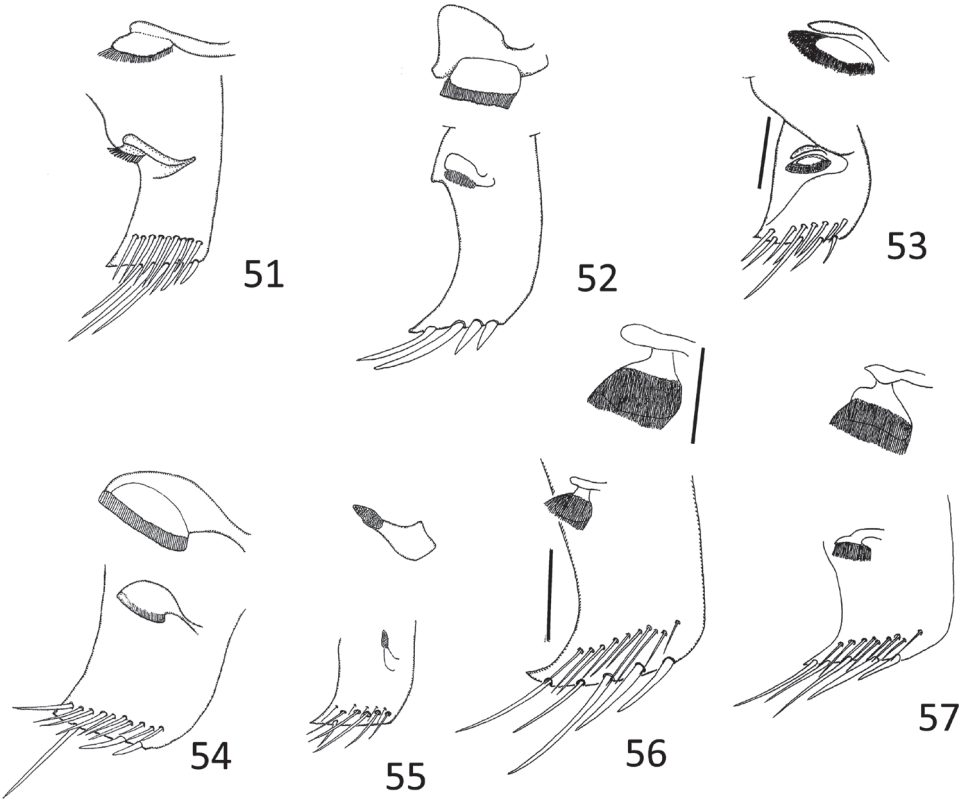
Legs. Length of leg segments: fore leg: male: femur 0.27, tibia 0.13, pala 0.14; female: femur 0.26, tibiotarsus 0.26; middle leg: male: femur 0.66, tibia 0.20, tarsus 0.37, claw 0.21; female: femur 0.65, tibia 0.23, tarsus 0.36, claw 0.21; hind leg: male: femur 0.49, tibia 0.35, tarsus I 0.38, tarsus II 0.16, claw 0.10; female: femur 0.46, tibia 0.38, tarsus I 0.38, tarsus II 0.16, claw 0.10. Palm of pala with about 14 bristles in upper row and about 17 in lower row.

Male. Fore femur (Fig. 37) with a pair of pegs on proximal third and one peg dorsodistally; tibia without dorsoapical peg; pala with three comparatively short dorsal hairs. Claw simple, dilated distally (Fig. 38). Dorsum of abdomen: prestrigilar flap (Fig. 47) with a elongate, weakly acute apex; strigil comparatively large, comb (Fig. 54) narrow, with about 75 teeth; free lobe of left part of tergite VIII (Fig. 70) with a somewhat sinuate apex with about 30 bristles. Left paramere (Fig. 81) with a wide, roughly parallel-sided shaft, apex abruptly narrowed; right paramere (Fig. 80) with a medium-sized shaft and an expanded apex with a short finger–like projection; basal lobe well developed, with 25 stridulatory ridges. Mediocaudal lobe of sternite VII (Fig. 62) with four bristles.

Female. General arrangement of bristles on fore femur is the same as in male. The seminal capsule of spermatheca mushroom–shaped (Fig. 94).

Comparative notes. The right paramere is apically somewhat similar to that of *M. ornitheia* Nieser et al., 2005 from Yunnan, China. However, the shaft of the right paramere of *M. orniteia* is narrower, the left paramere is apically truncate; and it is a smaller species; body length of *M. orniteia* is 1.7–1.9, body length of *M. lakimi* is 2.1–2.2.

Etymology. The species is named after Dr. Maklarin Lakim for his great service organizing the joint expedition to Sabah Parks in 2012, and his various activities in support of biodiversity exploration in Sabah Parks.



Figures 51–57. *Micronecta* spp., right part of tergite VI with strigil (scale 0.05 mm), males, in dorsal view: **51** *M. decorata* Lundblad, 1933 **52** *M. ludibunda* Breddin, 1905 **53** *M. liewi* sp. n., paratype **54** *M. lakimi* sp. n., paratype **55** *M. skutalis* Nieser & Chen, 1999 **56** *M. kymatista* Nieser & Chen, 1999 **57** *M. quadristrigata* Breddin, 1905. Scale bars: 0.1 mm

Habitat. The type series was collected downstream of Mahua waterfall, at the edge of the stream with a slow current (Fig. 99).

Distribution. Malaysia: Sabah (this paper).

Micronecta (*Micronecta*) *skutalis* Nieser & Chen, 1999

Figs 19, 28, 39, 40, 48, 55, 63, 71, 84, 85, 95

Micronecta skutalis Nieser & Chen, 1999: 86–87 (original description).

Type material examined. Holotype macropterous male (RMNH), MALAYSIA: Sabah, 60 km W of Lahad Datu, Danum Valley Field Centre at junction of Sungai Segama and Sungai Palum Tambun, bridge of Segama, 4°58'N, 117°48'E, 750m a.s.l.,

edge of untouched lowland rainforest, 14 march 1987, at light, 18.20–22.30h leg. Van Tol & Huisman. Paratypes, same data as holotype 12male 11 females (RMNH).

Additional material examined. MALAYSIA: Borneo: Sabah: 60 km West of Lahad Datu: Danum Valley Field Centre, at junction of Sungai Segama and Sungai Palum Tambun, 4°58'N, 117°48'E, 150 m a.s.l., 14.iii.1987, 18.20–22.30 hr., edge of untouched evergreen lowland forest, leg. J. van Tol & Huisman, 5 males, 14 females. (RMNH, 2 males, 2 females NCTN); 75 km West of Lahad Datu, confl. S. Sabran, S. Danum, S/N, 4°57'N, 117°41'E, 200 m, 23.x.1987, leg. J. Huisman & R. de Jong, 1 male, 2 females; 10 km SE of Ranau, Kg. Nalapak, Sungai Kananapun, 5°58'N, 116°47'E, 7.ii.1987, leg. J. Huisman, 2 females (RMNH). All macropterous, collected at light.

Redescription (based on dry specimens mounted on carton). Macropterous form (Fig. 19). A small (length 1.5–1.7 mm), light to medium-brown species; hemelytra smooth, with a variable number of small pegs scattered over their surface.

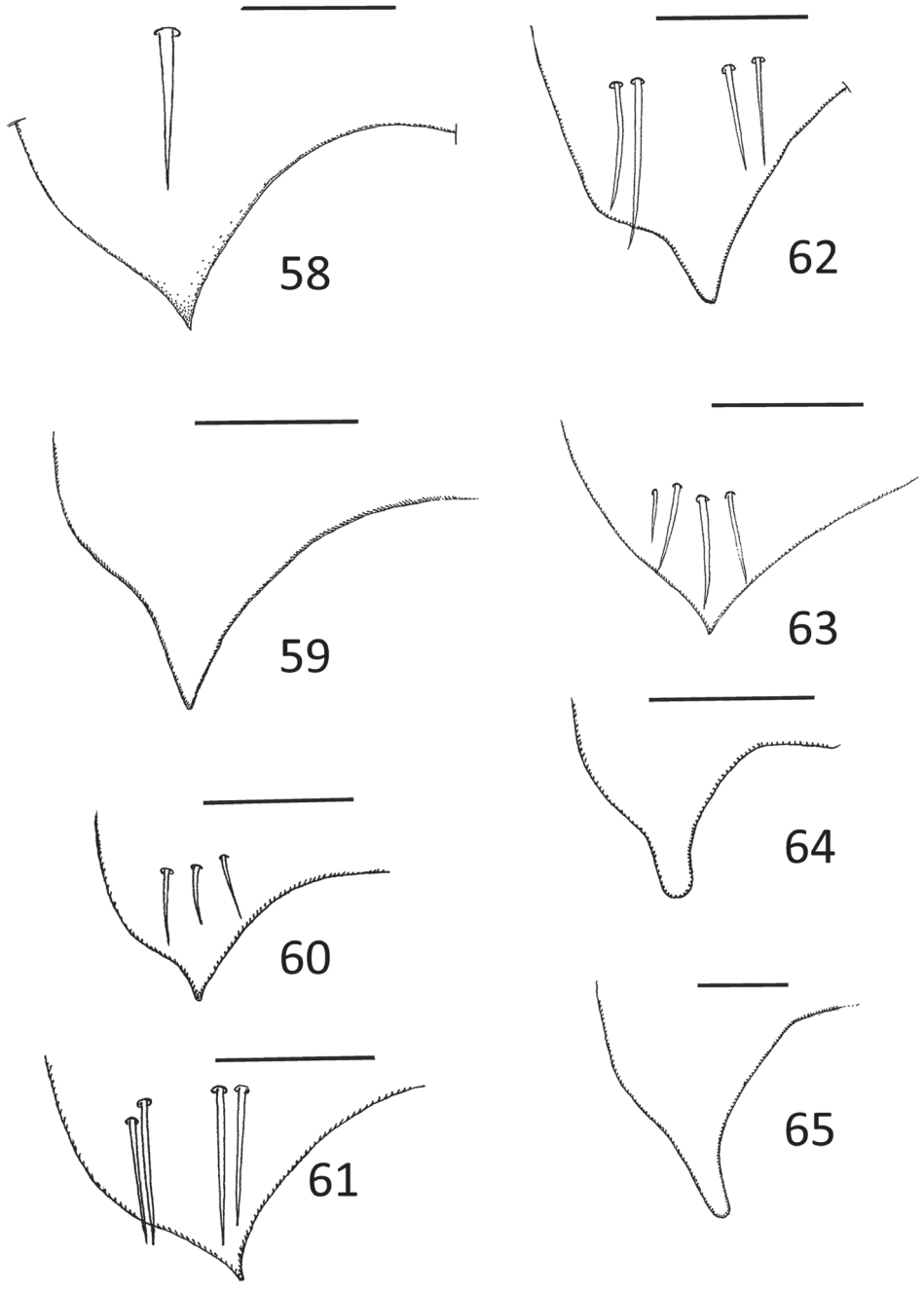
Dimensions. Length, male 1.52–1.57, female 1.53–1.70; width, male 0.63–0.70, female 0.62–0.70; diatone, male 0.53–0.56, female 0.51–0.55; width of pronotum, male 0.56–0.61, female 0.57–0.59; ocular index, male 1.44–1.61, female 1.57–1.86. Body length 2.3–2.6 the maximal width. Pronotum slightly wider than head, synthlipsis wider than the posterior width of an eye (S/E 0.23/0.17).

Colour. Frons and vertex sordid yellow, eyes grayish. Pronotum and hemelytra sordid yellow to light brown; hemelytra with an often indistinct, transverse medium to dark brown band at middle (Fig. 28), left membrane medium to dark brown. Venter: thorax sordid yellow, laterally infuscate; abdomen grayish brown, medially variably lighter. Legs pale yellow.

Pronotum dorsally convex, 2–2.5 times as wide as long (W/L 2.1–2.7, Fig. 19). Hemelytra beset with spinules arranged in longitudinal rows and along membranal suture. Spines laterally on abdominal segments: V with two short and one long stout spine; VI with two short and one or two longer spines; VII with two short and two long stout spines; VIII with five short spines and two long hair-like bristles.

Legs. Length of leg segments: fore leg: male: femur 0.31, tibia 0.15, pala 0.21; female: femur 0.32, tibiotarsus 0.32; middle leg: male: femur 0.53, tibia 0.19, tarsus 0.26, claw 0.19; female: femur 0.50, tibia 0.18, tarsus 0.26, claw 0.15; hind leg: male: femur 0.32, tibia 0.28, tarsus I 0.25, tarsus II 0.12, claw 0.09; female: femur 0.33, tibia 0.29, tarsus I 0.27, tarsus II 0.12, claw 0.09. Palmar bristles 15–19 in upper row, about 14–17 in lower row.

Male. Fore femur (Fig. 39) with a pair of pegs on proximal third, and two or three small pegs distally; tibia with two to three small spines near dorsal margin; pala (Fig. 40) with three long dorsal hairs. Claw simple, clavate. Dorsum of abdomen: prestrigilar flap (Fig. 48) with a short apex; strigil (Fig. 55) small, sub-oval, one comb with about 60 teeth; free lobe of left part of tergite VIII (Fig. 71) more or less parallel-sided, softly curved, with a rounded apex and about10 apical bristles. Mediocaudal lobe of sternite VII (Fig. 63) short, acute, with four larger bristles. Left paramere (Fig. 85) parallel-



Figures 58–65. *Micronecta* spp., mediocaudal process of sternite VII, in ventral view: **58** *M. decorata* Lundblad, 1933 **59** *M. ludibunda* Breddin, 1905 **60** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **61** *M. liewi* sp. n., paratype **62** *M. lakimi* sp. n., paratype **63** *M. skutalis* Nieser & Chen, 1999 **64** *M. kymatista* Nieser & Chen, 1999 **65** *M. quadristrigata* Breddin, 1905. Scale bars: 0.1 mm (**58–64**); 0.05 mm (**65**).

sided, apically rounded, with an indentation at the base of the shaft; right paramere in lateral view (Fig. 84) apically dilated, basal lobe with about eight stridulatory ridges.

Female. Fore femur with the same general arrangement of pegs and setae as in male. Seminal capsule of spermatheca ovate (Fig. 95).

Comparative notes. Its small size separates this species from other Bornean species of *Micronecta*, except for *M. lumutensis* (see that species).

Habitat. The specimens all have been collected at light near a stream.

Distribution. Malaysia: Sabah (Nieser and Chen 1999).

Subgenus *Sigmonecta* Wróblewski, 1962

Type species. *Micronecta quadristrigata* Breddin, 1905, by monotypy.

Diagnosis. Medium-sized to larger *Micronecta*, body length 2.2–3.2 mm. Males with process of abdominal sternite VII elongate, tongue-like, with a rounded tip (Figs 64, 65), and without larger bristles; strigil present; free lobe of tergite VIII sigmoid (Fig. 72); and left paramere with a sickle-shaped apex (Figs 87, 89).

Remarks. Wróblewski (1962: 176) erected *Sigmonecta* as a new subgenus for *Micronecta quadristrigata* Breddin, 1905, without describing the subgenus. His comments were as follows: "I have already stressed in an earlier paper (Wróblewski 1960a) the isolated systematic position of *M. quadristrigata* Bred. Now I propose to place it in a separate, so far monotypic subgenus *Sigmonecta* subg. n., named so on account of the sigmoid outline of the eighth abdominal tergite in the males."

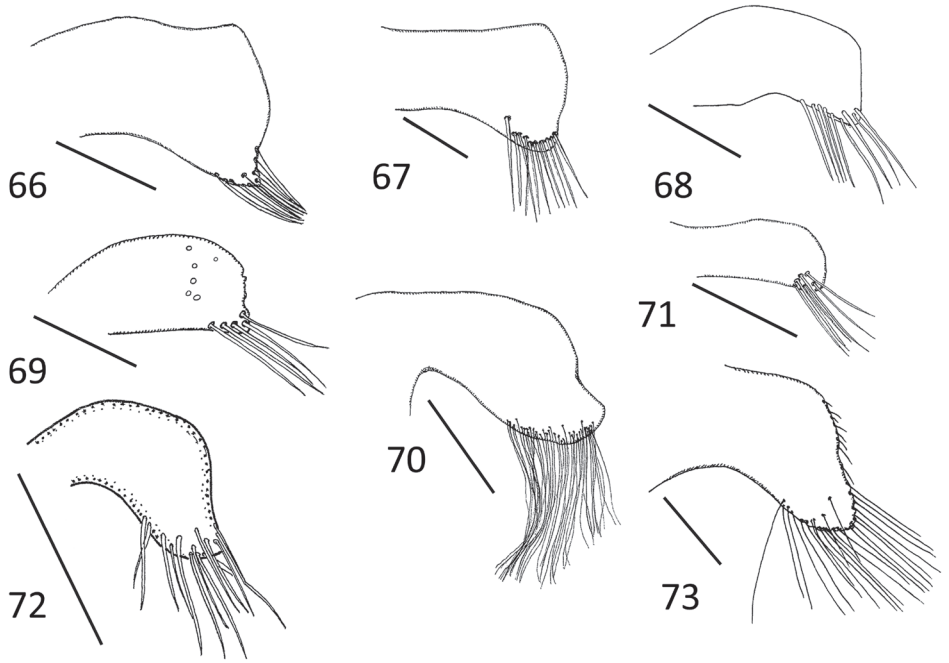
Micronecta (*Sigmonecta*) *kymatista* Nieser & Chen, 1999

Figs 8, 20, 41, 49, 56, 64, 72, 86, 87, 96

Micronecta kymatista Nieser & Chen, 1999: 82–83 (original description).

Type material examined. Holotype macropterous male (RMNH), **INDONESIA: Sulawesi Utara**, Dumoga Bone N.P. Malibagu Road 10 km H, ca. 250m a.s.l., 2 sept.1985, secondary growth, at light, leg. J. Huijbregts, HH437. Paratypes, same data as holotype, 14 males, 16 females (RMNH).

Additional Material examined. INDONESIA: Sulawesi Utara: Dumoga Bone N.P., Malibagu Road, 10 km N, ca. 250 m a.s.l., 2.ix.1985, second growth, at light, leg. J. Huijbregts, 1 female. **Sulawesi Tenggara:** Wawonggole, Sungai Anggoro, 20.ii.1989, sluggish stream in open woodland, leg. N. Nieser, N8801, 1 female; **Sulawesi Tenggara:** Desa Kagunyala, pond overgrown by *Azolla* and *Lemna*, 21.ii.1989, leg. N. Nieser, N8906, 1 male; **Sulawesi Tenggara:** Pulau Buton, mangrove swamp along road Bau-bau to Lawele, 9.iii.1989, leg. N. Nieser, 2 males (all macropterous paratypes, in NCTN).



Figures 66–73. *Micronecta* spp., free lobe at right side of tergite VIII, in dorsal view: **66** *M. decorata* Lundblad, 1933 **67** *M. ludibunda* Breddin, 1905 **68** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **69** *M. liewi* sp. n., paratype **70** *M. lakimi* sp. n., paratype **71** *M. skutalis* Nieser & Chen, 1999 **72** *M. kymatista* Nieser & Chen, 1999 (scale 0.2 mm) **73** *M. quadristrigata* Breddin, 1905. Scale bars: 0.01 mm (**66–71, 73**); 0.2 mm (**72**).

Redescription. Macropterous form. Generally a quite large (body length 2.8–3.1), light to medium-brown species; corium with four longitudinal, brownish stripes, very often interrupted.

Dimensions. Length: male 2.8–2.9, female 2.9–3.1; width: male 1.25–1.32, female 1.28–1.39; diatone: male 1.01–1.03, female 1.04–1.11; width of pronotum: male 0.98–1.01, female 1.02–1.08; ocular index: male 1.25–1.32, female 1.17–1.30. Body length 2.15 times maximal width (male 2.46/1.10, female 2.79/1.22). Head slightly wider than pronotum (male 1.02/1.00, female 1.08/1.05), synthlipsis 1.2 times as wide as the posterior margin of an eye.

Colour. Frons and vertex sordid yellow, eyes grayish. Pronotum light to medium brown, disk unmarked, posterior margin with a distinct yellowish stripe. Hemelytra sordid yellow to light brown, clavus with a darker medium-brown stripe along the suture between clavus and corium suture, corium typically with four fragmented longitudinal medium-brown stripes (Fig. 20), embolium yellowish with three or four indistinct brownish spots; right membrane poorly delimited from the corium, with the same colour and texture but without darker stripes; left membrane more distinctly separated from corium, hyaline to somewhat smoky and more membranous than the corium. Venter, abdomen, thorax, and legs pale yellow.

Pronotum well developed, dorsally convex with lateral margins straight or more or less truncate (Fig. 20), about three times as wide as long (W/L male 1.00/0.34, female 1.05/0.36). Hemelytra smooth, beset with numerous small but distinct spinules. Spines laterally on abdominal segments: V with two short and one longer stout spine; VI with two or three short and one long spine; VII with two or three short and one long stout spine; VIII with five or six short and one longer, stout spine and two long hair-like bristles.

Legs. Length of leg segments: fore leg: male: femur 0.26, tibia 0.14, pala 0.14; female: femur 0.26, tibiotarsus 0.26; middle leg: male: femur 0.70, tibia 0.23, tarsus 0.30, claw 0.25; female: femur 0.76, tibia 0.23, tarsus 0.33, claw 0.26; hind leg: male: femur 0.46, tibia 0.36, tarsus I 0.40, tarsus II 0.13, claw 0.08; female: femur 0.48, tibia 0.37, tarsus I 0.44, tarsus II 0.16, claw 0.08. Palmar bristles: 15 in upper and lower row.

Male. Fore femur with a pair of pegs on proximal third, and a pair of small pegs distally; tibia with a dorsoapical peg. Pala (Fig. 41) with three long dorsal hairs, the apical bristles in lower row distinctly thicker than the bristles of lower row. Claw broadly clavate, gradually dilated from base to apex, without ventral notch. Dorsum of abdomen: prestrigilar lobe (Fig. 49) difficult to observe, strigil (Fig. 56) with one, relatively broad comb with about 50 elongate teeth. Median lobe of sternite VII (Fig. 64) apically narrow with a rounded apex, without obvious longer bristles. Free lobe of left part of tergite VIII (Fig. 72) sigmoid with about 12 apical bristles. Medial margin of right lobe of tergite VIII with 28–35 bristles caudally, placed in a double to triple row on caudal half (Fig. 8). Left paramere (Fig. 87) with a comparatively narrow shaft and a sickle-shaped apex; right paramere, in lateral view (Fig. 86), with an evenly curved, more or less parallel-sided, apically tapering shaft, basal lobe with about 40 stridulatory ridges on the pars stridens.

Female. Fore leg with the same general arrangement of pegs and setae as in male. Seminal capsule of spermatheca elongate-clavate (Fig. 96).

Comparative notes. This species is similar to *M. quadristrigata*, which is smaller on average and has fewer bristles on the caudal half of inner margin of right part of tergite VIII in males (see key and Figs 8–9).

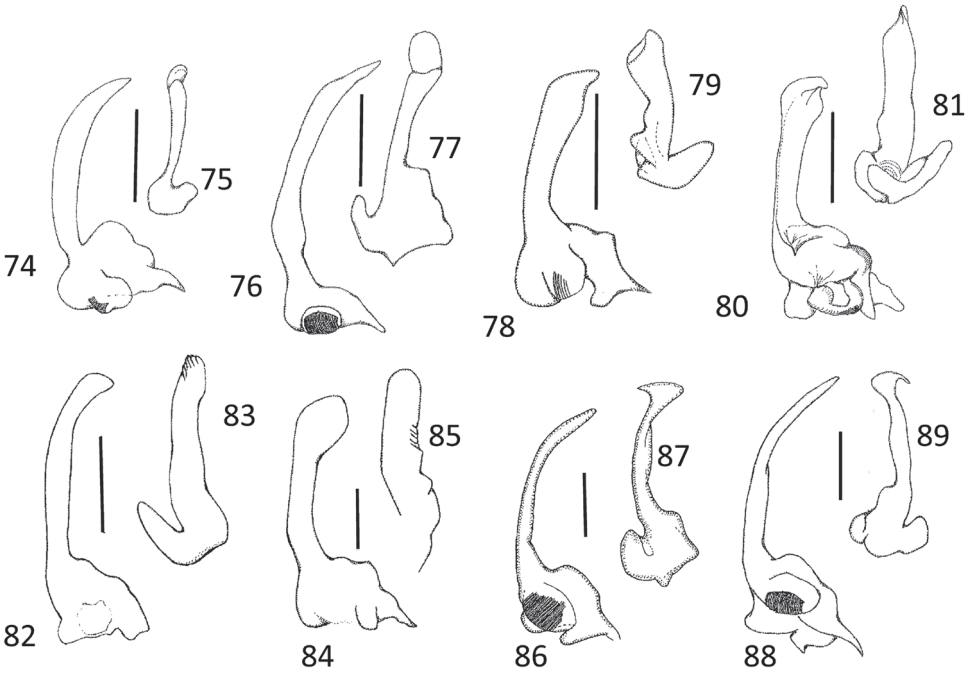
Habitat. This species has been found in ponds and sluggish streams mostly in less disturbed areas.

Distribution. Indonesia: Sulawesi and Borneo (Kalimantan Timur) (Nieser and Chen 1999).

Micronecta (*Sigmonecta*) *quadristrigata* Breddin, 1905, new record for Borneo

Figs 5, 9, 21, 23, 29, 42, 50, 57, 65, 73, 88, 89, 97

Micronecta quadristrigata Breddin, 1905a: 57 (original description).
Micronecta quadristrigata: Breddin 1905b: 156–157 (extensive description).
Micronecta quadristrigata: Lundblad 1933: 87–191 (redescription).
Micronecta quadristrigata: Wróblewski 1960: 301–304 (additional distributional and morphological notes).



Figures 74–89. *Micronecta* spp., parameres: **74, 76, 78, 80, 82, 84, 86, 88**: right parameres in external view; **75, 77, 79, 81, 83, 85, 87, 89**: left parameres. **74–75** *M. decorata* Lundblad, 1933 **76–77** *M. ludibunda* Breddin, 1905 **78–79** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **80–81** *M. lakimi* sp. n., paratype **82–83** *M. liewi* sp. n., paratype **84–85** *M. skutalis* Nieser & Chen, 1999 **86–87** *M. kymatista* Nieser & Chen, 1999 **88–89** *M. quadristrigata* Breddin, 1905. Scale bars: 0.1 mm.

Micronecta (*Sigmonecta*) *quadristrigata*: Wróblewski 1962: 176 (introducing subgenus).
Micronecta quadristrigata: Wróblewski 1968: 776 (checklist).
Micronecta quadristrigata: Wróblewski 1972: 29–133 (redefinition of species).
Micronecta (*Sigmonecta*) *quadristrigata*: Jansson 1995: 34 (catalogue).
Micronecta quadristrigata Cassis & Goss, 1995: 69 (distribution in Australia)
Micronecta quadristrigata: Nieser and Chen 1999: 80 [recorded from Indonesia (Sulawesi) and Philippines (Mindanao)].
Micronecta quadristrigata: Chen et al. 2005: 420 (checklist).
Micronecta quadristrigata: Tinerella 2008: 39–145 [distribution in New Guinea Island, record from Indonesia (Moluccas)].
Micronecta (*Sigmonecta*) *quadristrigata*: Linnavuori et al 2011: 77–178 (record from United Arab Emirates).
Micronecta quadristrigata: Tinerella 2013: 102 (redescription, additional records in Australia).
For a discussion on the status of *M. minthe* Distant, 1911, which is considered by some authors as a subspecies or synonym of *M. quadristrigata*, see Jansson (1995) and Wróblewski (1972a).

Type material examined. Syntype, INDONESIA: "Kotype; Djokjokarta (= Yogyakarta), **Java**, K. Kraepelin; leg. 18.III.1904, ded. 8.VI.1904; Breddin determ.; Lundblad revid. 1934", 1f (ZMUH); syntype, INDONESIA: "Kotype; Buitenzorg (= Bogor)", 1m 1f (ZMUH).

Additional material examined. MALAYSIA: Sabah: Kota Belud Dist., Mt. Kinabalu, pond at Kampong Kiau, 06°01.48'N, 116°29.14'E, 1003 m a.s.l., 15.ix.2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1273, 9 males, 15 females; Sabah, Kota Belud Dist., Mt. Kinabalu, Kota Belud, Head Quarter of Kinabalu Park, tributary of Sungai Kadamaian, 06°02.09'N, 116°29.39'E, 1410 m. a.s.l., 16.ix.2012, leg. P. Chen, N. Nieser & J. Lapidin, CN1275, 2 males; all macropterous (NCTN).

Redescription. Macropterous form. Generally a medium-sized to quite large (body length reported 2.2–3.2, most specimens 2.5–3.0), yellowish to light-brown species, with four variable, indistinct, longitudinal, brown stripes on corium.

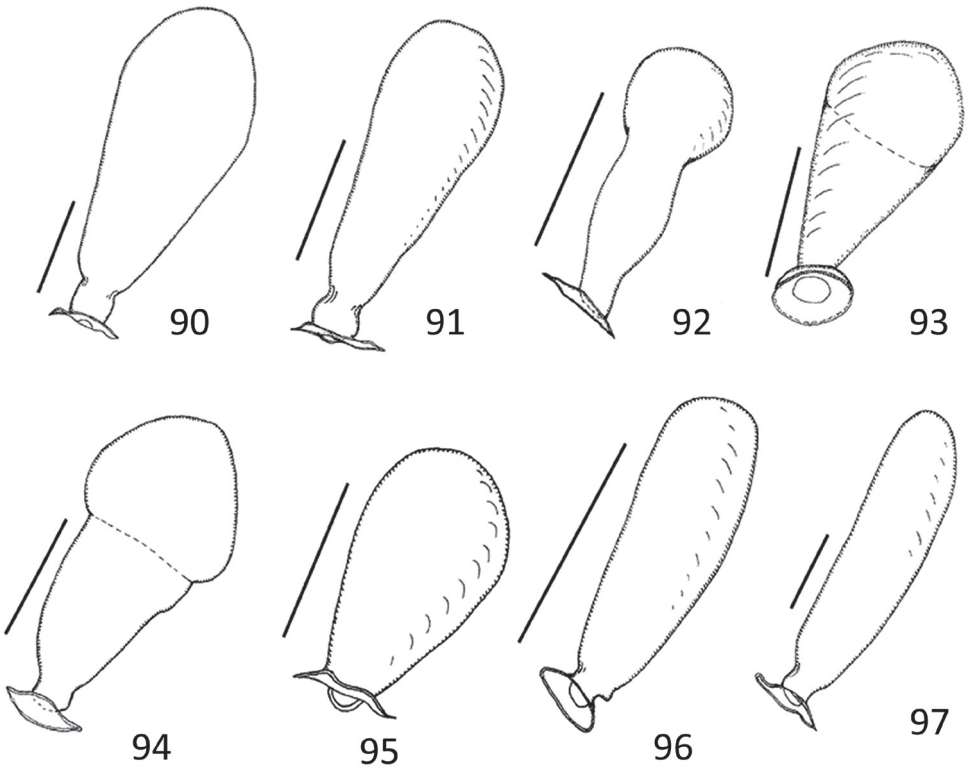
Dimensions. Length: male 2.2–2.9, female 2.5–3.2; width: male 1.07–1.15, female 1.12–1.37; diatone: male 0.83–1.12, female 0.87–1.18; width of pronotum: male 0.82–1.11, female 0.86–1.17; ocular index: male 1.20–1.55, female 1.17–2.16. Body length two and a quarter times maximal width (male 2.46/1.10, female 2.79/1.22). Head slightly wider than pronotum (male 0.89/0.88, female 0.99/0.98), synthlipsis 1.4–1.5 times as wide as the posterior margin of an eye.

Colour. Frons and vertex sordid yellow, eyes grayish. Pronotum light brown, virtually unmarked in most specimens, in some specimens, with two indistinct, usually interrupted transverse stripes, posterior margin with a poorly defined yellowish stripe. Hemelytra sordid yellow to light brown, clavus with a darker medium-brown stripe along the claval suture, and a smaller medium-brown streak near the inner angle; corium typically with four interrupted, longitudinal, medium- brown stripes (Figs 21, 23, 29), embolium with four black spots; right membrane poorly delimited from the corium, with the same colour and texture but without darker stripes; left membrane more distinctly separated from corium, hyaline and more membranous than the corium. Venter, thorax, and legs pale yellow, abdomen yellowish to light brown.

Pronotum well developed, dorsally convex with lateral margins straight or more or less truncate (Fig. 21), slightly over 2.5 times as wide as long (W/L male 0.88/0.34, female 0.98/0.37). Hemelytra smooth, beset with numerous small but distinct spinules. Spines laterally on abdominal segments: V with three short and one longer stout spine; VI with two short and two long spines; VII four short and one long stout spine; VIII with six short to longer, stout spines and one long hair-like bristle.

Leg. Length of leg segments: fore leg: male: femur 0.38, tibia 0.16, pala 0.16; female: femur 0.38, tibiotarsus 0.36; middle leg: male: femur 0.89, tibia 0.26, tarsus 0.38, claw 0.34, female; femur 0.98, tibia 0.28, tarsus 0.41, claw 0.3; hind leg: male: femur 0.58, tibia 0.42, tarsus I 0.42, tarsus II 0.19, claw 0.10; female: femur 0.62, tibia 0.46, tarsus I 0.46, tarsus II 0.21, claw 0.12. Palmar bristles: 14 to 15 in upper row, 11 to 12 in lower row.

Male. Fore femur (Fig. 42) with a pair of pegs in proximal third, two (in some specimens only one) small pegs about midway dorsally and a small peg dorsodistally; tibia with a larger peg subventrally on apical third and two small dorsoapical pegs; pala with



Figures 90–97. *Micronecta* spp., seminal capsule of spermatheca, in dorsal view: **90** *M. decorata* Lundblad, 1933 **91** *M. ludibunda* Breddin, 1905 **92** *M. lumutensis* Chen, Nieser & Lansbury, 2008 **93** *M. liewi* sp. n., paratype **94** *M. lakimi* sp. n., paratype **95** *M. skutalis* Nieser & Chen, 1999 **96** *M. kymatista* Nieser & Chen, 1999 **97** *M. quadristrigata* Breddin, 1905. Scale bars: 0.1 mm (**90–96**); 0.05 mm (**97**).

four long dorsal hairs, distal bristle of lower row much stouter and longer than other lower bristles. Claw plump, clavate. Dorsum of abdomen: prestrigilar lobe with a short, broadly rounded apex, strigil (Fig. 57) sub-oval, comb with about 25 long teeth, free lobe of left part of tergite VIII (Fig. 73) sigmoid-shaped. Left paramere (Fig. 89) with a wide shaft, apex sickle-shaped; right paramere in lateral view (Fig. 88) with an evenly curved shaft, basal lobe strongly developed with about 50 stridulatory ridges; in dorsolateral view, the shaft is somewhat sinuous. Mediocaudal lobe of sternite VII (Fig. 65), long, with apical part elongate and obtusely rounded apically, without larger bristles.

Female. Fore femur with the same general arrangement of pegs and setae as in male. The seminal capsule of spermatheca elongate-clavate (Fig. 97).

Notes. *M. quadristrigata* might have an even broader range of size variation. Wróblewski (1960a) reported that females had a length up to 3.3 mm from Hong Kong, and Leong (1966) measured females with a length up to 3.4 mm from the Malay Peninsula. However, we have never seen specimens with a length over 3.1 mm.

Comparative notes. See discussion under *M. kymatista*.

Figure 98. Waterfall in Sungai Kibabangan (above) at Substation Inobong, the Sabah Parks, Sabah Malaysia; downstream of the waterfall (below), where the specimens of *Micronecta liewi* sp. n. were collected.

Habitat. Various stagnant and slowly flowing waters, especially in agricultural fields, including rice fields.

Distribution. Widely spread through South and Southeast Asia to Hong Kong and Taiwan, and through Indonesia to the Philippines, New Guinea, and N. Australia; United Arab Emirates (Linnavuori et al 2011), Iran (Wróblewski 1960), India (Hutchinson 1940),

Figure 99. Waterfall Mahua (above) in Mahua Sub-station, The Sabah Parks, Sabah, Malaysia; downstream of the waterfall (below), one of the sites where *M. lakimi* was collected.

Sri Lanka (Wróblewski 1964), Thailand (Wróblewski 1972), Vietnam (Wróblewski 1962), southern China, including Taiwan (Wróblewski 1968, 1972), West Malaysia (Leong 1966), Indonesia (Breddin 1905a, Lundblad 1933, Nieser and Chen 1999, Tinerella 2013), Philippines (Polhemus and Reisen 1976, Nieser and Chen 1999), New Guinea (Tinerella 2008), and Australia (Cassis and Gross 1995, Tinerella 2013).

Discussion of faunistic components in Borneo

All Bornean species of Micronectidae belong to the dominant genus *Micronecta* Kirkaldy, 1897, which contains about 130 described species. The present knowledge of the Bornean fauna (and the Malesian fauna as a whole) of Micronectidae is still insufficient to discuss its proper biogeographical affinities. Judging from the literature, lowland species, such as *M. decorata*, *M. ludibunda*, *M. quadristrigata*, tend to be more widespread than species from hilly areas, such as *M. lakimi*, *M. liewi*, and *M. lumutensis*. This conclusion might be partly artificial because most taxa of Micronectidae are collected at light. They easily escape from the casual collector in the field due to their small size. The shallow stagnant waters in lowland ponds and marginal bays of streams are less stable than the stagnant waters in hills or mountains, such as a pond at the foot of a waterfall. Moreover, lowland species were found several times in very high densities, whereas species from hilly areas were always found in moderate to low densities. We hypothesize that lowland species more often colonize new habitats and therefore fly more often.

Of the eight species of *Micronecta* known from Borneo, three are so far endemic to the island: *M. lakimi*, *M. liewi*, and *M. lumutensis*. Their localities are all in mountainous areas. It is unclear which species are closely related to *M. lakimi* and *M. liewi*. But *Micronecta lumutensis* apparently is closely related to *M. skutalis*, which was described from Sabah and also has been found on Palawan in the Philippines (Nieser and Chen 2003). Another species related to *M. lumutensis* and *M. skutalis* is *M. abra* Nieser & Chen, 2003 described from Palawan. These three species apparently constitute a species-group by having small body size, and each of them has limited distributional range around Borneo.

Micronecta quadristrigata is a widespread species. In the west, it reaches the United Arab Emirates and southern Iran (Linnavuori et al. 2011, Wróblewski 1960). The area around the Gulf of Persia and the Gulf of Oman is considered to belong to the Palaearctic Region (Aukema and Rieger 1995), but for water bugs it has a strong Oriental element as well as some species of African origin (Linnavuori et al. 2011). Besides the water bugs, the water beetles also show Oriental elements in Arabian Peninsula. Hájek and Wewalka (2009) stated: "Our study further reiterates that the Arabian Peninsula is a typical transition area between the neighboring major zoogeographical regions". We agree with their observations based on the recent studies by different authors of insect fauna in the Arabian Peninsula, which has emphasized an interesting point from a zoogeographical point of view.

Eastward, *M. quadristrigata* reaches New Guinea and northern Australia (Tinerella 2013), indicating that this species probably originated in the Oriental origin. The open and shallow man-made waters, such as rice fields, provide conditions that have allowed micronectid to spread westward and eastward.

Micronecta kymatista is closely related to *M. quadristrigata*, but according to the locality information from Sulawesi and Borneo, it seems to prefer habitats somewhat less influenced by human activities. This might also explain the wide distribution of the

other two lowland species *M. decorata* and *M. ludibunda*. It is clear that *M. decorata* is an Oriental element ranging from northern Thailand to Borneo, Java and Sumatra (Lundblad 1933). *Micronecta ludibunda* is also widespread, ranging from India and Sri Lanka to New Guinea and the Solomon Islands (Tinerella 2008). As its closest relative, *M. albifrons* (Motschulsky, 1863), known from India and Sri Lanka (Wróblewski 1968), is also considered a species of Oriental origin which has spread eastward. The four "lowland species" occurring in Borneo belong to the common Oriental elements.

Choi (1996) has pointed out that "the sedimentary basin of Mt. Kinabalu was sinuated between three crustal or tectonic plates - The South China Sea Plate to the north, the Sulu Sea Plate to the east, and the main Eurasian Plate to the west". The uplifting of the Crocker-Trus Madi area began 40 million years ago with its collision with these other plates. The movement slowed down about 10 million years ago, although Mt. Kinabalu is said to be still pushing up at a rate of 5 mm per year, the Crocker-Trus Madi area has been pushed up into mountain ranges. According to radiometric age determinations, Mt. Kinabalu is somewhat younger, with the cooling of its magma taking place 10–4 million years ago. *Micronecta lakimi* and *M. liewi* are both from the Crocker Range and not closely related to the other species of *Micronecta* collected so far in Borneo. It is, therefore, possible that the origin of these two newly described species coincided with the rising of the Crocker Range.

In view of the endemism of various organisms in Mt. Kinabalu (Wong and Phillipps 1996), these two newly described species might also be endemic to this area. However, this point of view needs to be proved by further explorations in Sabah and Borneo, notably the confirmation whether *M. lakimi* and *M. liewi* are endemic in the area of Crocker Range.

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RESEARCH ARTICLE

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Molecular phylogeny of the genus *Dicronocephalus* (Coleoptera, Scarabaeidae, Cetoniinae) based on mtCOI and 16S rRNA genes

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Abstract

The seven species belonging to the genus *Dicronocephalus* are a very interesting group with a unique appearance and distinct sexual dimorphism. Only one species among them, *D. adamsi*, has been known in the Korean fauna. This species is recognized as having a wide distribution from Tibet to Korean Peninsula and is currently represented by two subspecies that have separated geographical ranges. The phylogenetic relationships of *D. adamsi* were still unclear. The phylogeny of *Dicronocephalus* is reconstructed with a phylogenetic study of five species including four subspecies based on a molecular approach using mitochondrial COI and 16S rRNA genes. Our results are compared with the results obtained by previous authors based on morphological characters. They show that the tested taxa are divided into two major clades. Clade A consists of two species (*D. adamsi* + *D. yui*) and Clade B includes the others (*D. dabryi* + *D. uenoi* + *D. wallichii*). This result generally supports Kurosawa's proposal except that *D. dabryi* and *D. uenoi* are newly recognized as members of a monophyletic group. We propose that *D. adamsi drumonti* is a junior subjective synonym of *D. adamsi adamsi*. These results show that three members of the *D. wallichii* group should be treated as species rather than subspecies. However, further research including analyses of different genetic markers is needed to reconfirm our results.

* These authors contributed equally to this work.

Keywords
Dicronocephalus, phylogenetic relationships, *D. adamsi*, taxonomy, Scarabaeidae, new synonymy, Korea

Introduction

Genus *Dicronocephalus* Hope, 1831 is a group of medium- to large-sized beetles with a unique appearance among Cetoniinae representatives. The members of the genus show distinct sexual dimorphism such as antler-like clypeal horns and prolonged tarsomeres in males (Šípek et al. 2008). This genus is composed of seven species including nine subspecies: *D. adamsi adamsi* Pascoe, 1863; *D. adamsi drumonti* Legrand, 2005; *D. dabryi* (Lucas, 1872); *D. shimomurai* Kurosawa, 1986; *D. uenoi uenoi* Kurosawa, 1968; *D. uenoi katoi* Kurosawa, 1968; *D. bieti* Pouillaude, 1914; *D. wallichii wallichii* Hope, 1831; *D. wallichii bourgoini* Pouillaude, 1914; *D. wallichii bowringi* Pascoe, 1863; *D. yui yui* Kurosawa, 1968; and *D. yui cheni* Kurosawa, 1986 (Legrand 2005, Krajcik 2014). Geographically, the genus is widely distributed from the Himalayan foothills of Nepal to Vladivostok in Russia and to Korea, but the distribution of most species and subspecies is rather limited. In particular, *D. shimomurai*, *D. uenoi uenoi*, *D. uenoi katoi*, *D. wallichii bourgoini*, *D. yui yui*, and *D. yui cheni* are endemic to the small island of Taiwan. One species, *D. dabryi,* is only known in West China and Myanmar. The remaining species and subspecies are widely distributed in Asia occurring throughout the Manchuria and Indo-China (Kurosawa 1986, Šípek et al. 2008, Young 2012, Krajcik 2014).

Kurosawa (1986) proposed dividing this genus into three groups on the basis of the morphological characters: 1) the *adamsi* species-group (*D. adamsi*, *D. shimomurai*, and *D. yui*); 2) the *wallichii* species-group (*D. w. wallichii*, *D. w. bourgoini*, *D. w. bowringi*, and *D. dabryi*); and 3) the *D. uenoi* species-group (*D. uenoi*). However, he did not explain the phylogenetic relationships between these species.

Among the seven species of *Dicronocephalus*, only *D. adamsi* is found in the Korean fauna. This species was described from Korea, but it has been known to have a wide range across Korea, China, Tibet, and Vietnam. The range of this species is divided by a wide geographical gap between Liaoning and Shanxi provinces of China (Young 2012). Legrand (2005) divided *D. adamsi* into two subspecies based on this distribution pattern and morphological differences. He described populations occurring in west China as *D. adamsi drumonti*. This classification was accepted by Krajcik (2014), but not by Young (2012).

The subspecies of *D. wallichii* (*D. w. wallichii*, *D. w. bourgoini*, and *D. w. bowringi*) were originally described as valid species (Hope 1831, Pascoe 1863, Pouillaude 1914). While some authors have treated these taxa as subspecies (Paulian 1960, Mikšić 1971, 1977, Krajčík 1998, Sakai and Nagai 1998, Šípek et al. 2008, Young 2012, Krajcik 2014), some others have treated them as species (Kurosawa 1968, Devecis 2008). The controversy over whether they should be dealt with at the species or sub-species level has continued without in-depth analysis.

During a review of the genus *Dicronocephalus*, several issues were encountered, such as validation of species or subspecies rank of taxa composing *D. adamsi* and *D. wallichi* (sensu lato) and the lack of phylogenetic analysis of the genus. To resolve these questions, phylogenetic analysis was performed for the genus using *cytochrome c oxidase subunit I* (COI) and *16S ribosomal RNA* (16S rRNA) mitochondrial gene sequences as well as examination of their morphological diagnostic characters.

Materials and methods

Specimen sampling and examination

Fifty specimens of *Dicronocephalus* belonging to five species and seven subspecies from four countries were obtained (Fig. 1, Table 1), but we were unable to obtain specimens of the remaining two species, *D. bieti* and *D. shimomurai*. For examining male genitalia, these were extracted from the abdomens and cleaned by heating with 10% KOH solution in a WiseTherm®HB-48P heating block at 60 °C for 1~2 hours. Male genitalia were preserved in microvials with glycerine after examination. Photographs of external morphology and genitalia were taken with a Canon EOS 10D camera and stacked with a combineZM program (Hadley 2006). Based on previous studies (Pascoe 1863, Pouillaude 1914, Kurosawa 1968, 1986, Young 2012), diagnostic characters were obtained to provide precise criteria for species identification. In this study, the most recent taxonomic scheme by Krajcik (2014) was followed, especially for subspecies treatment of *D. wallichii*. All examined specimens are stored in the Department of Agricultural Biology, National Academy of Agricultural Biology (NAAS), Jeonju, Korea.

DNA extraction, amplification and sequencing

Genomic DNA (gDNA) was extracted from middle legs removed from dried specimens of all species and accomplished using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Polymerase Chain Reaction (PCR) was performed in order to amplify the cytochrome *c* oxidase subunit I gene (COI) and *16S ribosomal RNA* gene (16S rRNA) using Accupower PCR PreMix (Bioneer, Daejeon, Korea). The universal primer set LCO1490/HCO2198 (Folmer et al. 1994) for amplifying the DNA barcoding region (658bp) of COI sequences was not successful for all samples; this may be caused by the degraded quality of gDNA (Goldstein and Desalle 2003, Hajibabaei et al. 2006; Wandeler et al. 2007). We applied the PCR methodology for retrieving COI sequences from old specimens given in Han et al. (2014) and designed new primer pairs: LCO-Ceto232F (5'–GCHTTYCCYCGAATAAATAAYATA–3') corresponding to HCO2198 and HCO-Ceto367R

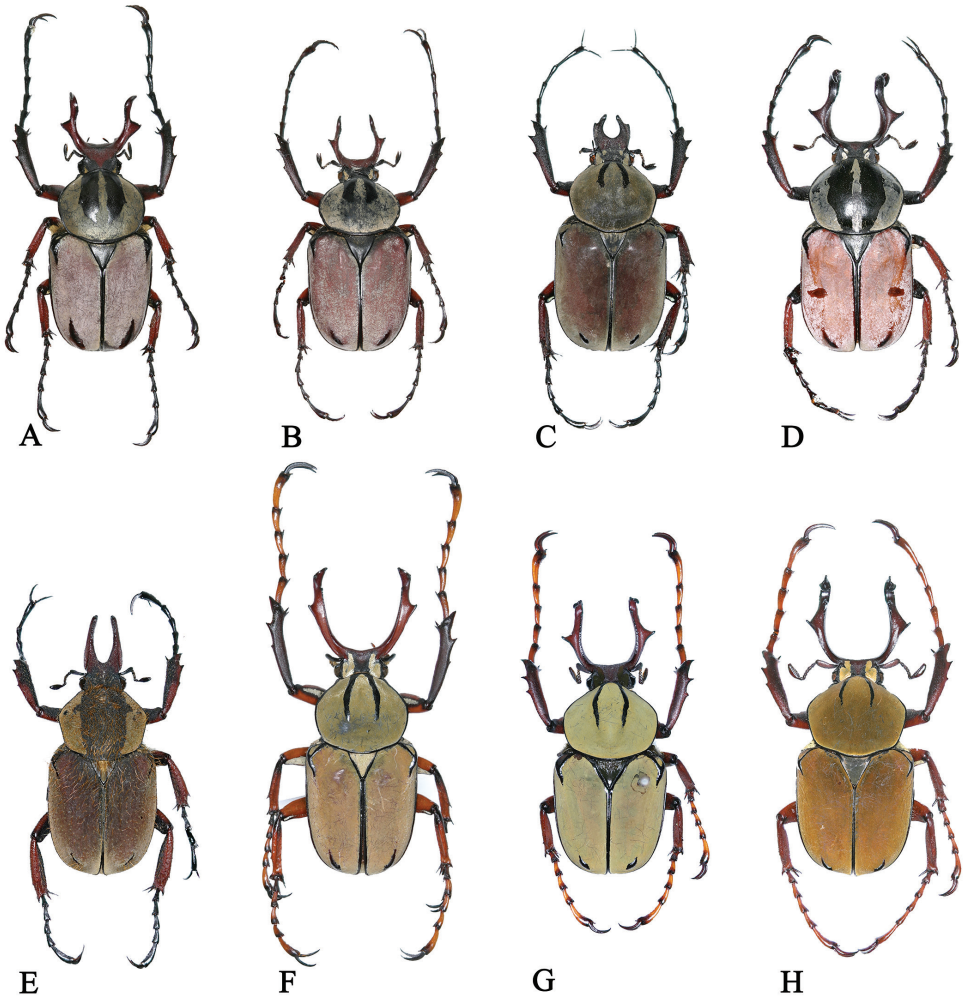

Figure 1. The male habitus of species and subspecies of *Dicoronocephalus*. **A** *D. adamsi adamsi* **B** *D. a. drumonti* **C** *D. yui yui* **D** *D. dabryi* **E** *D. uenoi katoi* **F** *D. wallichii bowringi* **G** *D. w. wallichii* **H** *D. w. bourgoini*.

(5'–ACDGTYCADCCNGTTCCTGCNCC–3') corresponding to LCO1490. 16S rRNA was targeted in a 600 bp region with two primers, 16SB/16SA, that successfully amplified in Lucanidae and Elateridae (Hosoya et al. 2001, Hosoya and Araya 2005, Han et al. 2009, 2010). PCR amplification conditions were as follows: for COI, initial denaturation at 94 °C for 5 min, then 45 cycles at 94 °C for 30 s, 46 °C for 25 s, and 72 °C for 45 s followed by a final extension at 72 °C for 3 min, and for 16S rRNA, initial denaturation at 94 °C for 5 min, then 40 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 45 s followed by a final extension at 72 °C for 5min. The amplicons were purified using a QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) after the product yield was monitored by 0.7% agarose gel electrophoresis.

Table 1. Collection and voucher information for specimens.

Sample no.	Species	Locality	Data collected	Sex	Voucher no.	Sequencing	
						GBAn of COI	GBAn of 16S
1	*Dicronocephalus adamsi adamsi*	Muju, JB, South Korea	6. VI. 2012	F	7258	KM390855	KM390809
2	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	19. V. 2009	M	7300	KM390856	KM390810
3	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	19. V. 2009	M	7301	KM390857	KM390811
4	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	19. V. 2009	M	7302	KM390858	KM390812
5	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	19. V. 2009	F	7303	KM390859	KM390813
6	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	25. V. 2013	M	7696	KM390860	KM390814
7	*Dicronocephalus adamsi adamsi*	Sangdaewon-dong, Jungwon-gu, Seongnam, GG, South Korea	25. V. 2013	M	7697	KM390861	KM390815
8	*Dicronocephalus adamsi adamsi*	Tongrim, North Korea	VII. 1995	M	7683	KM390862	–
9	*Dicronocephalus adamsi adamsi*	North Korea	IV. 2002	M	7684	KM390863	KM390816
10	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7264	KM390864	KM390817
11	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7265	KM390865	KM390818
12	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7267	KM390866	KM390819
13	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7268	KM390867	KM390820
14	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7269	KM390868	KM390821
15	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7270	KM390869	KM390822
16	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7272	KM390870	KM390823
17	*Dicronocephalus adamsi adamsi*	Mt. Wu Long, Dandong, Liaoning, China	15. VII. 2009	M	7273	KM390871	KM390824
18	*Dicronocephalus adamsi drumonti*	Sichuan, China	VI. 2008	M	7677	KM390872	KM390825
19	*Dicronocephalus adamsi drumonti*	Sichuan, China	VI. 2008	F	7678	KM390873	KM390826
20	*Dicronocephalus adamsi drumonti*	Sichuan, China	VI. 2008	F	7679	KM390874	–
21	*Dicronocephalus adamsi drumonti*	Sichuan, China	VI. 2008	F	7680	KM390875	KM390827
22	*Dicronocephalus adamsi drumonti*	Mt. Foding, Guizhou, China	–	F	7688	KM390876	KM390828
23	*Dicronocephalus adamsi drumonti*	Tibet, China	–	M	7685	KM390877	–
24	*Dicronocephalus adamsi drumonti*	Tibet, China	–	M	7686	KM390878	KM390829
25	*Dicronocephalus adamsi drumonti*	Tibet, China	–	F	7687	KM390879	–
26	*Dicronocephalus adamsi drumonti*	Tibet, China	VIII. 2005	F	7689	KM390880	KM390830
27	*Dicronocephalus yui yui*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	F	7290	KM390881	KM390831

Sample no.	Species	Locality	Data collected	Sex	Voucher no.	Sequencing	
						GBAn of COI	GBAn of 16S
28	*Dicronocephalus yui yui*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	F	7291	KM390882	KM390832
29	*Dicronocephalus yui yui*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	F	7292	KM390883	KM390833
30	*Dicronocephalus dabryi*	Hanyan, Sichuan, China	16–17. VI. 2007	M	7278	KM390884	KM390834
31	*Dicronocephalus dabryi*	Hanyan, Sichuan, China	16–17. VI. 2007	M	7279	KM390885	KM390835
32	*Dicronocephalus dabryi*	H-1601m, Env. Xichang city, S. Sichuan, China	12. VI. 2009	M	7375	KM390886	KM390836
33	*Dicronocephalus dabryi*	H-1601m, Env. Xichang city, S. Sichuan, China	12. VI. 2009	F	7376	KM390887	KM390837
34	*Dicronocephalus dabryi*	China	2005	M	7690	KM390888	KM390838
35	*Dicronocephalus uenoi katoi*	Chiayi, Taiwan	VIII. 2011	M	7285	KM390889	KM390839
36	*Dicronocephalus uenoi katoi*	Chiayi, Taiwan	VIII. 2011	M	7286	KM390890	KM390840
37	*Dicronocephalus uenoi katoi*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	M	7287	KM390891	KM390841
38	*Dicronocephalus uenoi katoi*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	M	7288	KM390892	KM390842
39	*Dicronocephalus uenoi katoi*	A- Li-Shan, Chiayi county, Taiwan	IV. 2012	M	7289	KM390893	KM390843
40	*Dicronocephalus wallichii bowringi*	Mt. Lianyuan, Hunan, China	VII. 2006	M	7692	KM390894	KM390844
41	*Dicronocephalus wallichii bowringi*	Mt. Lianyuan, Hunan, China	VII. 2006	F	7693	KM390895	KM390845
42	*Dicronocephalus wallichii bowringi*	Mt. Guangwu, Sichuan, China	–	M	7694	KM390896	KM390846
43	*Dicronocephalus wallichii bowringi*	Mt. Guangwu, Sichuan, China	–	F	7695	KM390897	KM390847
44	*Dicronocephalus wallichii wallichii*	Taeng, Mae, Mai, Ching, N. Thailand	VII. 2010	M	7274	KM390898	KM390848
45	*Dicronocephalus wallichii wallichii*	Taeng, Mae, Mai, Ching, N. Thailand	IV. 2008	M	7275	KM390899	KM390849
46	*Dicronocephalus wallichii bourgoini*	Beitou, Taipei, Taiwan	V. 2008	F	7277	KM390900	KM390850
47	*Dicronocephalus wallichii bourgoini*	Beitou, Taipei, Taiwan	V. 2008	M	7280	KM390901	KM390851
48	*Dicronocephalus wallichii bourgoini*	Beitou, Taipei, Taiwan	V. 2008	M	7281	KM390902	KM390852

Sample no.	Species	Locality	Data collected	Sex	Voucher no.	Sequencing	
						GBAn of COI	GBAn of 16S
49	*Dicronocephalus wallichii bourgoini*	Beitou, Taipei, Taiwan	V. 2008	F	7282	198 bp	KM390853
50	*Dicronocephalus wallichii bourgoini*	Beitou, Taipei, Taiwan	V. 2008	F	7283	KM390903	KM390854
51	*Protaetia brevitarsis**	Korea	–	–	–	KC775706	KC775706

* denotes outgroup taxa data extracted from GenBank. GBAn is denoted the GenBank accession number.

DNA sequencing was performed using an automated DNA sequencer (ABI 3730xl 96-capillary DNA analyzer; Applied Biosystems, Foster City, CA) with the same primers used for PCR. All sequences (excepting a 198 bp fragment of COI in no. 7282) are available from GenBank under accession numbers KM390855–KM390903 for COI and KM390809–KM390854 for 16S rRNA (Table 1).

Phylogenetic analysis

For the phylogenetic analyses, three data sets were used, a 658 bp fragment of COI, 520 bp fragment of 16S rRNA sequences, and the concatenated COI and 16S rRNA sequences. The data sets were aligned using ClustalW in MEGA 5.2 (Tamura et al. 2011), and genetic distances were calculated using Kimura's two-parameter test (Kimura 1980). The phylogenetic analyses were constructed using maximum likelihood (ML), Bayesian inference methods (BI), and maximum parsimony (MP).

ML analysis was performed with GARLI 2.0 (Zwickl 2011), and the analysis was initiated at a random start tree using GTR+I+G model parameters selected by MrModelTest (Nylander 2004), with a 10,000 generation search algorithm and 1,000 bootstrap replications. The frequencies with which to log the best score ("logevery") and to save the best tree to file ("saveevery") were set to 10,000 and 10,000 respectively, and the number of generations without topology improvement required for termination ("genthreshfortopoterm") was set to 5,000. At the end of the analysis, there was no improvement in the tree topology by a log likelihood of 0.01 or better. The bootstrap values were calculated using the SumTrees program of the DendroPy package (Sukumaran and Holder 2010).

BI analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run with one cold and three heated chains (temperature set to 0.2) for 5,000,000 generations and tree sampling every 100 generations. The posterior probabilities were then obtained and a majority-rule consensus tree was generated from the remaining trees after discarding the first 25% of samples.

MP analysis was performed with TNT 1.1 (Goloboff et al. 2008). The analyses, followed by tree bisection reconnection (TBR) branch swapping, used default options that performed 100 random additional sequences and saved up to ten trees per replication. To obtain the strict consensus tree, symmetric resampling (Goloboff et al. 2003) with a 33% change probability and jack-knifing with a 36% removal probability were implemented using a traditional search with 1,000 replications. Each set of results was summarized in terms of absolute frequency, and the group support values were analyzed. For bootstrap value (BP) in ML and MP, and posterior probability value (PP) in BI, supporting values of <70% as "weak", 70–79% as "moderate", 80–89% as "strong", and ≥ 90% as "very strong" support were used.

Results

Nucleotide information for COI and 16S rRNA

The data set of COI, with no evidence of indel (insertion/deletion) events, had 144 (21.9%) variable sites (Vs). Of these, 140 (21.3%) were parsimoniously informative sites (PIs). The data set of 16S rRNA, with indel events at three sites, consisted of 43 (8.3%) Vs, of which 41 (7.9%) were PIs. There was about 2.6 times more variability and the level of PIs was about 2.7 times greater in COI than in that in 16S rRNA.

Phylogenetic analyses of COI

Phylogenetic inferences based on three analyses (ML, BI, and MP) reconstructed the same topologies for COI (Fig. 2; for BI, ML and MP tree data not shown, see Suppl. material 1 for sequences), and there was separation into two major clades (A and B) with very strong supporting values (100%), except for ML. Eight ingroup taxa representatives including subspecies were clearly clustered into seven monophyletic groups corresponding to nominal species; the two subspecies of *D. adamsi* formed one cluster. Their terminal nodes were well supported, but the values of ML and BI were very low in *D. yui yui* (<50% in ML and 53% in BI) and *D. wallichii bowringi* (<50% in ML and 56% in BI).

The intra-specific distances of COI were rather low, ranging from 0–2.3%. The inter-specific divergences were highly variable, ranging from 2.7%–16.7%. The distances between the ingroup and outgroup taxa ranged from 16.1%–20.1% (Table 2).

Clade A is composed of *D. adamsi adamsi*, *D. a. drumonti*, and *D. yui yui* with strong bootstrap support (>72%). The two subspecies of *D. adamsi* did not separate into two distinct subgroups. The genetic divergences between the two subspecies were relatively low (0–1.7%); moreover, *D. a. drumonti* shared haplotypes with *D. a. adamsi* from Korea and China. *D. yui yui* was sister to *D. adamsi* with distinct inter-specific divergences (5.6%–7.3%).

Clade B is composed of *D. dabryi*, *D. uenoi katoi*, and three subspecies of *D. wallichii* with strong bootstrap supports by ML and BI, but relatively low support (56%–62%) by MP. Among the members of Clade B, *D. dabryi* and *D. uenoi katoi* formed a monophyletic group with very strong supporting values in all analyses and with distinct inter-specific divergences (5.6%–8.9%). The intra-specific divergences of these two species (0–1.5% in *D. dabryi*, 0.2%–2.3% in *D. u. katoi*) were explicitly lower than their inter-specific values. The three subspecies of *D. wallichii* were clustered as a monophyletic group and clearly subdivided. *D. w. bowringi* diverged early from an ancestor, and then *D. w. wallichii* and *D. w. bourgoini* underwent subsequent separation with strong bootstrap supports by ML (83%) and BI (99%); however, despite low divergences within each subspecies ranging from 0.3%–0.8%, the

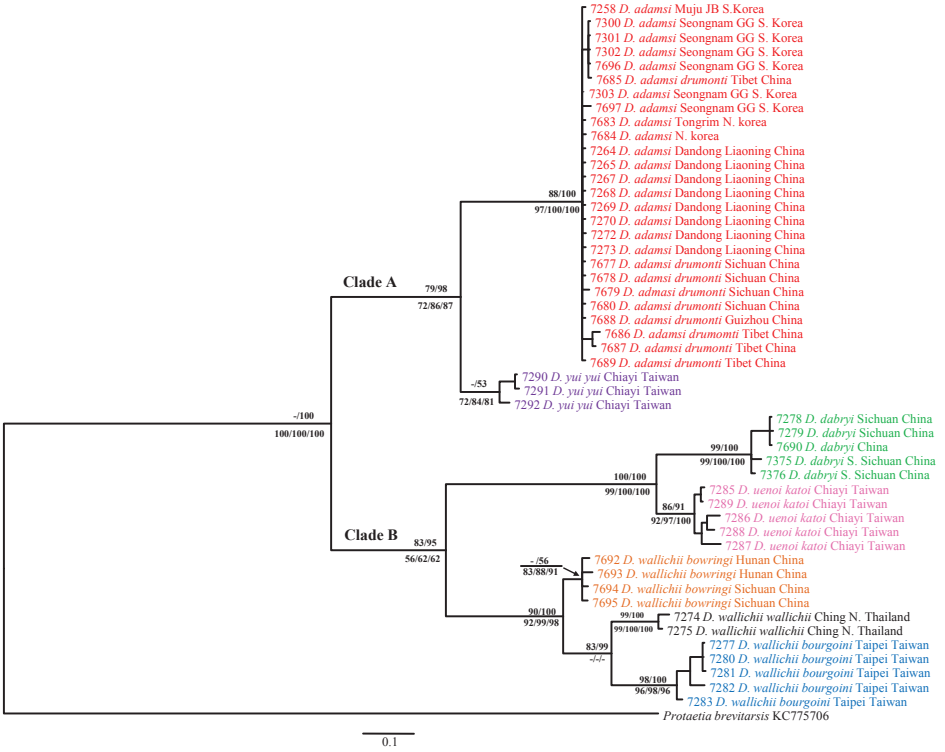

Figure 2. Phylogenetic relationships among *Dicronocephalus* species reconstructed with Bayesian inference using COI sequences. Numbers above branches indicate ML bootstrap values and Bayesian posterior probabilities. Numbers below branches are bootstrap, symmetric resampling, and jacknife support from parsimony searches, respectively. Scale bar represents 10% nucleotide mutation rate.

genetic divergences between these subspecies were unexpectedly variable ranging from 2.7%–8.1%. Genetic divergences were larger between *D. w. bowringi* and both *D. w. wallichii* (4.3%–5.0%) and *D. w. bourgoini* (4.8%–8.1%), than those between *D. w. wallichii* and *D. w. bourgoini* (2.7%–5.7%).

Phylogenetic analyses of 16S rRNA

ML, BI, and MP analyses of 16S rRNA resulted in considerably similar topologies to those of COI (Fig. 3 for BI, ML and MP tree data now shown, see Suppl. material 2 for sequences), but a polytomy was found in *D. yui yui* and paraphyly in *D. w. bowringi* with respect to *D. w. wallichii*.

The intra-specific pairwise distances of 16S rRNA were relatively low, ranging from 0–0.4%. The inter-specific divergences ranged from 0.8%–6.3%. The distances between the ingroup and outgroup taxa ranged from 9.7%–11.8% (Table 3). The

Table 2. Pairwise distance of COI within and between *Dicronocephalus* spp.

	No. of samples	Within species	Between subspecies & species						
			D. a. adamsi* + *D. a. drumonti	***D. yui yui***	***D. dabryi***	***D. uenoi katoi***	***D. w. bowringi***	***D. w. wallichii***	***D. w. bourgoini***
D. adamsi adamsi + *D. adamsi drumonti*	26	0.006 (0–0.017)							
D. yui yui	3	0.011 (0.002–0.017)	0.062 (0.056–0.073)						
D. dabryi	5	0.008 (0–0.015)	0.150 (0.130–0.162)	0.140 (0.130–0.149)					
D. uenoi katoi	5	0.013 (0.002–0.023)	0.150 (0.131–0.167)	0.135 (0.128–0.150)	0.069 (0.056–0.089)				
D. wallichii bowringi	4	0.006 (0.003–0.008)	0.120 (0.104–0.131)	0.117 (0.105–0.127)	0.139 (0.130–0.152)	0.117 (0.105–0.134)			
D. w. wallichii	2	0.006 (0.006–0.006)	0.133 (0.126–0.141)	0.123 (0.121–0.124)	0.132 (0.125–0.137)	0.135 (0.125–0.144)	0.048 (0.043–0.050)		
D. w. bourgoini	5	0.003 (0–0.006)	0.123 (0.109–0.134)	0.122 (0.120–0.124)	0.146 (0.131–0.163)	0.128 (0.104–0.147)	0.060 (0.048–0.081)	0.047 (0.027–0.057)	
*Protaetia brevitarsis**	1	–	0.175 (0.168–0.179)	0.168 (0.164–0.170)	0.196 (0.192–0.201)	0.191 (0.188–0.196)	0.179 (0.166–0.188)	0.198 (0.197–0.199)	0.176 (0.161–0.189)

Numbers are indicated as mean (minimum-maximum) of the pairwise distance. *denotes outgroup taxon

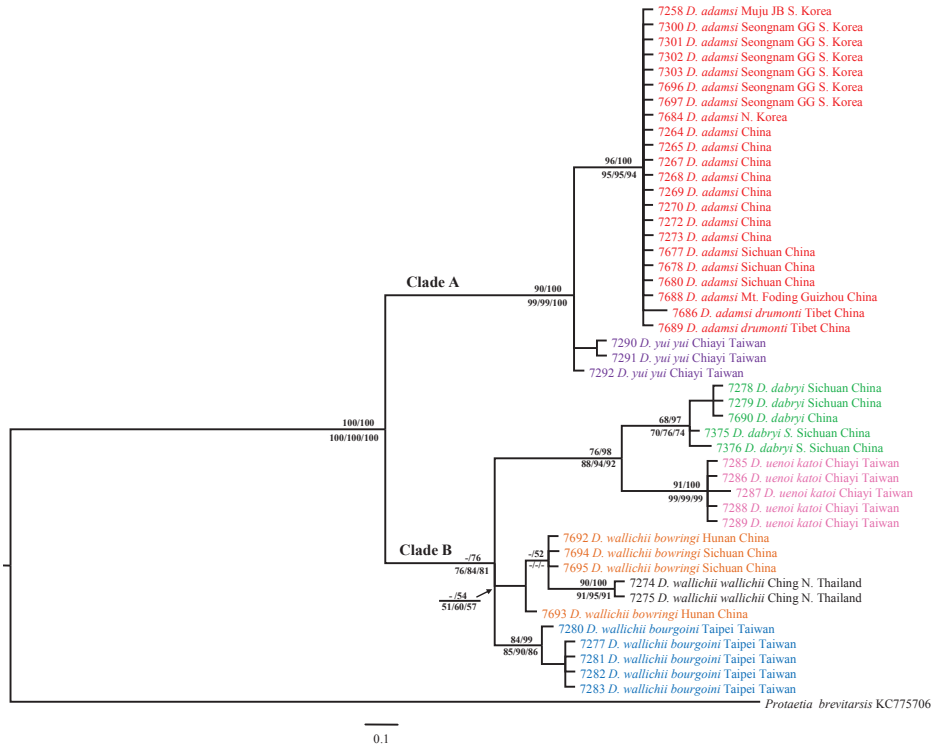

Figure 3. Phylogenetic relationships among *Dicronocephalus* species reconstructed with Bayesian inference using 16S rRNA sequences. Numbers above branches indicate ML bootstrap values and Bayesian posterior probabilities. Numbers below branches are bootstrap, symmetric resampling, and jacknife support from parsimony searches, respectively. Scale bar represents 10% nucleotide mutation rate.

lowest inter-specific divergence range (0.8%–1.2%) was revealed between *D. adamsi* and *D. yui yui*, and this is rather similar to the divergence ranges of the *D. wallichii* subspecies (0.8%–1.6%).

Dicronocephalus adamsi was clustered as a sister to *D. yui yui* in Clade A with strong bootstrap support (>90%), while the remaining taxa were clustered into Clade B with relatively low supporting values (>76%) in BI and MP. The monophyly of *D. adamsi*, *D. uenoi katoi*, *D. w. wallichii*, and *D. w. bourgoini* was well supported by bootstrap analyses (>84%). In contrast, in all analyses a polytomy was found in *D. yui yui* and ML and BI showed paraphyly of *D. w. bowringi*. We showed that these phenomena were caused by few parsimony-informative nucleotide variations in conserved regions. A comparison of each of those sequences, showed that *D. y. yui* has different substitutions at 326 nucleotide position. Two samples (7290 and 7291) have "C", while one sample (7292) has "T". On the other hand, *D. w. bowringi* has a substitution occurred in 196 nucleotide position. The 7693 sample has "G", while the other samples (7692, 7694, and 7695) and two samples (7274 and 7275) of *D. wallichii* have "A" at this site (Suppl. material 2).

Table 3. Pairwise distance of 16S ribosomal RNA within and between *Dicronocephalus* spp.

	No. of samples	Within species	Between subspecies & species						
			D. a. adamsi* + *D. a. drumonti	***D. yui yui***	***D. dabryi***	***D. uenoi katoi***	***D. w. bowringi***	***D. w. wallichii***	***D. w. bourgoini***
D. a. adamsi + *D. a. drumonti*	22	0.000 (0.000–0.002)							
D. yui yui	3	0.001 (0.000–0.002)	0.009 (0.008–0.012)						
D. dabryi	5	0.002 (0.000–0.004)	0.057 (0.054–0.060)	0.050 (0.046–0.052)					
D. uenoi katoi	5	0.001 (0.000–0.002)	0.059 (0.058–0.063)	0.052 (0.050–0.054)	0.020 (0.018–0.022)				
D. wallichii bowringi	4	0.001 (0.000–0.003)	0.046 (0.042–0.055)	0.039 (0.034–0.049)	0.035 (0.028–0.047)	0.036 (0.032–0.047)			
D. w. wallichii	2	0.000 (0.000–0.000)	0.050 (0.050–0.050)	0.043 (0.042–0.044)	0.030 (0.030–0.032)	0.034 (0.034–0.036)	0.009 (0.008–0.011)		
D. w. bourgoini	5	0.001 (0.000–0.002)	0.048 (0.048–0.048)	0.041 (0.040–0.042)	0.032 (0.028–0.034)	0.034 (0.032–0.036)	0.012 (0.008–0.016)	0.015 (0.014–0.016)	
*Protaetia brevitarsis**	1	–	0.104 (0.104–0.106)	0.102 (0.101–0.104)	0.103 (0.101–0.104)	0.104 (0.104–0.104)	0.103 (0.097–0.118)	0.099 (0.099–0.099)	0.101 (0.099–0.102)

Numbers are indicated as mean (minimum-maximum) of the pairwise distance. *denotes outgroup taxon

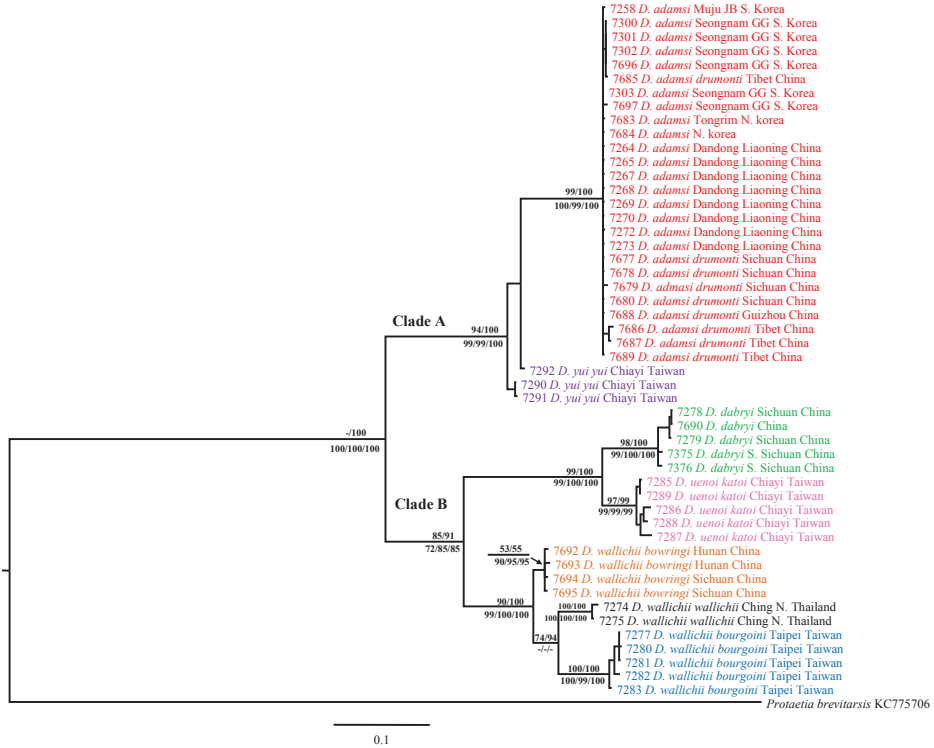

Figure 4. Phylogenetic relationships among *Dicronocephalus* species reconstructed with Bayesian inference using COI and 16S rRNA sequences. Numbers above branches indicate ML bootstrap values and Bayesian posterior probabilities. Numbers below branches are bootstrap, symmetric resampling, and jacknife support from parsimony searches, respectively. Scale bar represents 10% nucleotide mutation rate.

Phylogenetic analyses of COI and 16S rRNA

In the combined data set of COI and 16S rRNA, phylogenetic reconstructions produced topologies congruent with the COI analyses. The nodal supporting values were improved compared with the analyses based on each gene (Fig. 4, see Suppl. material 3 for sequences). Monophyly of the seven taxa including subspecies was strongly supported by bootstrap values >90%, except for low support of 53% and 55% in ML and BI, respectively, for the terminal node of *D. w. bowringi*. *D. w. wallichii* was grouped as a sister to *D. w. bourgoini* based on the results of the COI analyses with a high value in BI (94%) and moderate value in ML (74%), but not in MP (Fig. 4).

Re-examination of morphological diagnostic characters

The 19 diagnostic characters used to classify species or subspecies were re-examined in order to determine whether they are suitable for identification (Table 4). Of these characters,

Table 4. Diagnostic characters of ***Dicronocephalus***.

Character		states	Reference
Body	1. Color in male (Fig. 1)	0) grayish brown	Kurosawa (1968)
		1) dark brown	
		2) yellowish brown	
		3) dark yellowish brown	
		4) green-yellowish brown with pale purple on elytra	
	2. Color in female	0) dark blackish body without marking	Kurosawa (1986)
		1) not dark blackish body	
	3. Pronotal and elytral colors (Fig. 1)	0) pronotum and elytra different	Pouillaude (1914)
		1) pronotum and elytra similar	
	4. Dorsal surface	0) pilose with brownish semirecumbent hairs	Pouillaude (1914) Kurosawa (1968)
		1) almost hairless	
		2) sparsely pilose with hair	
Head	5. Development of antlers	0) a pair of antlers in male very short, undeveloped, approximate to each other anteriorly	Kurosawa (1968)
		1) antlers in male long and well developed, curving upwards apically and broadly separated from each other	
	6. Inferior dentation of antlers	0) clearly projected upward	Kurosawa (1968)
		1) weakly prominent	
		2) absent	
	7. Shape of anterior edge of clypeus (Fig. 5)	0) simple without angular projection	Pouillaude (1914)
		1) with an angular projection	
	8. Circular indentation of clypeus	0) with a strong or weak circular indentation on the edge	Pouillaude (1914)
		1) without circular indentation on the edge	
Pronotum	9. Pronotal bands	0) reaching posterior border	Pouillaude (1914) Young (2012)
		1) not reaching posterior border	
	10. Central carinae	0) carinae defined	Pascoe (1866)
		1) carinae nearly indistinct	
	11. Extending of carinae	0) extending beyond the middle	Kurosawa (1968)
		1) never extending beyond the middle	
		2) no carina	
	12. The widest portion	0) widest near the middle	Kurosawa (1968)
		1) widest in front of the middle	
Elytra	13. Surface	0) with two black dots	Young (2012)
		1) without black dot	
	14. Shoulder (Fig. 6)	0) with triangular umbone	Pascoe (1866)
		1) without triangular umbone	
	15. Apicosutural angle (Fig. 7)	0) rounded	Pouillaude (1914)
		1) projected	
Metasternum	16. Metasternal process	0) obtuse, rather rounded	Kurosawa (1968) Young (2012)
		1) rectangular or acute, moderately produced	
		2) triangularly and sharply produced	

Character		states	Reference
Abdomen	17. Abdominal sternites in male	0) covered with yellowish grey powder	Pouillaude (1914)
		1) normal, not covered with yellowish grey powder	
Legs	18. Color of tarsi	0) clear reddish brown (=testaceous)	Pascoe (1866) Pouillaude (1914) Young (2012)
		1) black or very dark brown	
	19. Length of tarsi	0) anterior tarsi of the male about as long as posterior ones	Kurosawa (1968)
		1) anterior tarsi distinctly longer than the others	

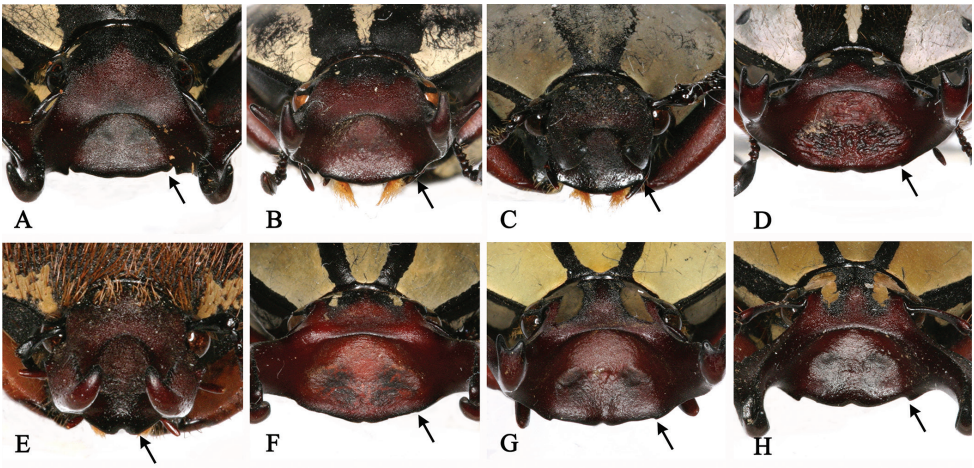

Figure 5. Anterior edge of clypeus of *Dicronocephalus*. **A** *D. adamsi adamsi* **B** *D. a. drumonti* **C** *D. yui yui* **D** *D. dabryi* **E** *D. uenoi katoi* **F** *D. wallichii bowringi* **G** *D. w. wallichii* **H** *D. w. bourgoini*.

mentioned in previous studies, 13 are clearly suitable for species or subspecies identification; however, we recognized six characters that are ambiguous and not applicable (Table 5). For example, Pouillaude (1914) mentioned three diagnostic characters as follows: 1) *D. dabryi* has a different color of the pronotum and the elytra compared with *D. wallichii* subspecies (Fig. 1); 2) *D. w. wallichii* can be separated from the others (*D. adamsi*, *D. w. bowringi*, *D. w. bourgoini*, *D. dabryi*, and *D. beiti*) by having no angular projection at the base of the anterior edge of the clypeus (Fig. 5); and 3) *D. w. bourgoini* can be distinguished from the others by the projected apicosutural angle of the elytra (Fig. 6). However, none of these characters has proven to be suitable for species identification. We observed that the color of the pronotum and the elytra of *D. dabryi* was the same with grayish powder in freshly collected specimens, but it has faded gradually in old specimens (Fig. 1D). Also the anterior edge of the clypeus of *D. w. wallichii* (Fig. 5G) was sinuate in the middle, similar to that of *D. w. bourgoini* (Fig. 5H), and did not match the description by Pouillaude. We therefore consider that these characters might have been mistakenly described and illustrated by Pouillaude (1914). In addition, the projection of the apicosutural angle of the elytra of *D. w. bourgoini* was not distinct and could not separate this taxon from the other

Table 5. Data matrix for *Dicronochephalus* species in this study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
D. adamsi adamsi	**0**	0	**1**	**1**	**1**	?	1	0	1	1	**2**	**0**	**1**	1 (rarely 0)	0	**1**	0	1	**1**
D. adamsi drumonti	**0**	**0**	**1**	**1**	**1**	?	**0**	**0**	**1**	**1**	**2**	**0**	**1**	**1 (rarely 0)**	**0**	**1**	**0**	**1**	**1**
D. yui yui	**1**	0	**1**	1	0	**2**	**0**	**0**	**1**	**1**	**1**	0 (or 1)	**1**	**1 (rerely 0)**	**?**	1	**0**	**1**	1
D. dabryi	**0**	1	0 (or 1)	1	**1**	?	1	1	0	**1**	**2**	**1**	0	**1 (rarely 0)**	0	0	1	1	**1**
D. uenoi katoi	1	**1**	**1**	0	0	**2**	**1**	**0**	**0**	**1**	**2**	**1**	**1**	**1**	**?**	0	**1**	**1**	0
D. w. bowringi	**3**	1	1	**1**	**1**	0 (or 1)	0 (or 1)	1	1	0	0	**0**	**1**	0	0	**1**	1	0	**1**
D. w. wallichii	**2**	1	1	**1**	**1**	**0**	0 (or 1)	1	1	0	0	**0**	**1**	**0**	0	2	1	0	**1**
D. w. bourgoini	4	1	1	1	1	1	1	1	1	**0**	1	1 (rarely 0)	**1**	**0**	1	1	1	0	1
Results of examination	C	C	U	C	C	U	U	C	C	C	C	U	C	U	U	C	C	C	C

Boldic numbers indicate additionally examined diagnostic characters at each species in this study.

Parentheses denote the characteristic represeted by our examination.

Question marks indicate the ambiguous character state to be difficult determination in our examination.

'C' is clear and 'U' is unclear characters resulted in this study.

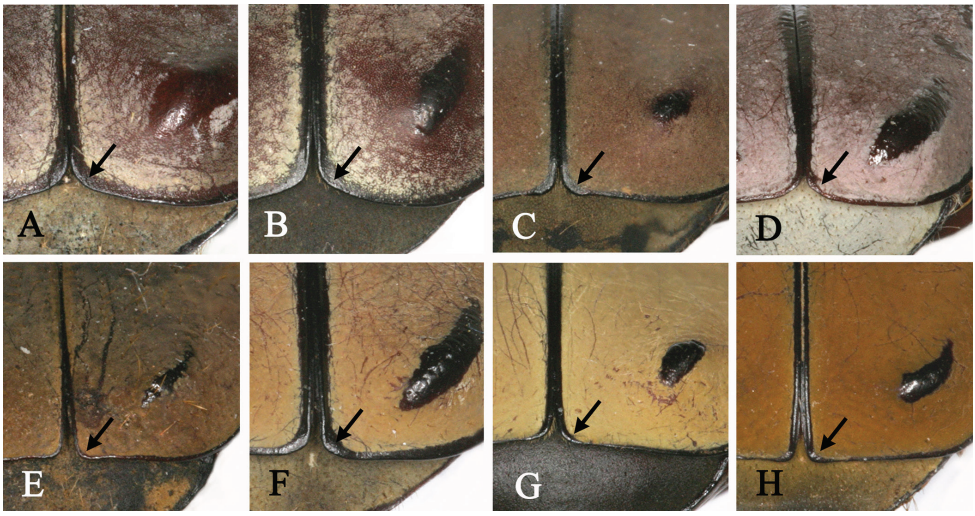

Figure 6. Apicosutural angle of *Dicronocephalus*. **A** *D. adamsi adamsi* **B** *D. a. drumonti* **C** *D. yui yui* **D** *D. dabryi* **E** *D. uenoi katoi* **F** *D. wallichii bowringi* **G** *D.w. wallichii* **H** *D. w. bourgoini*.

species and subspecies (Fig. 6H). We consider that using another character such as "the posterior margin of the elytra is round or truncated" may more diagnostic than the former character as shown in Fig. 6. Pascoe (1863) used the triangular umbone on the shoulder of the elytra (Fig. 7) to distinguish *D. a. adamsi* from *D.w. bowringi*. But, we consider that the presence of a triangular umbone is as an unsuitable character. We found this state also in some specimens of *D. adamsi*, although the size of the triangular umbone was small and variable in each specimen. Kurosawa (1986) used the widest portion of the pronotum as a distinguishing character state, but this was variable in all specimens of *D. w. bourgoini* and not distinct enough to be used in species and subspecies identification.

Legrand (2005) used six diagnostic characters to distinguish between the two subspecies, *D. a. adamsi* and *D. a. drumonti*. Among them, we found four characters, namely body size, general body shape, longitudinal bands on the pronotum, and the shape of the triangular umbone of the elytra, to be ambiguous. He also illustrated the metasternal process and the parameres and explained in the key to subspecies that the ridge of the metasternal process does not reach the plate, and the process is weakly raised and more rounded anteriorly in *D. a. drumonti*. Also, the parameres of *D. a. drumonti* are shorter and with more acute lateral angles than of *D. a. adamsi*. However, we found that these characters were variable in the specimens from the two geographically isolated populations (Fig. 8). For example, the shape of the lateral angles of the parameres of Tibetan *D. a. drumonti* (Fig. 8C, D) is similar to that of a *D. a. adamsi* from South Korea (Fig 8K, L), and another specimen of *D. a. drumonti* from Sichuan, China (Fig. 8G, H) resembles a *D. a. adamsi* from Dandong, China (Fig. 8S, T). We did not find any significant diagnostic characters to separate the two subspecies and therefore the new synonymy is here proposed (*Dicronocephalus adamsi drumonti* Legrand, 2005 = *Dicronocephalus adamsi adamsi* Pascoe, 1863, syn. nov).

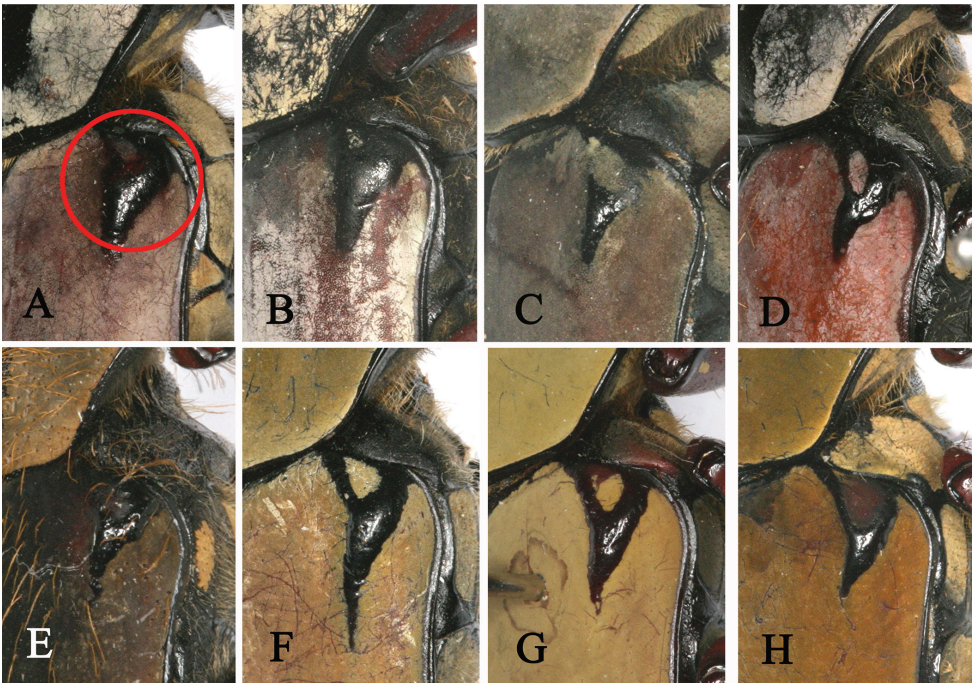

Figure 7. Umbone (in the circle) of shoulder of *Dicronocephalus*. **A** *D. adamsi adamsi* **B** *D. a. drumonti* **C** *D. yui yui* **D** *D. dabryi* **E** *D. uenoi katoi* **F** *D. wallichii bowringi* **G** *D. w. wallichii* **H** *D. w. bourgoini*.

Discussion

From the results inferred from ML, BI, and MP methods using COI and 16S rRNA genes, the genus *Dicronocephalus* includes two major lineages, one with *D. adamsi* and *D. yui yui* and another with *D. dabryi*, *D. uenoi katoi*, *D. w. bowringi*, *D. w. wallichii*, and *D. w. bourgoini* (Figs 1–3). The specimens of eight taxa including subspecies clustered into seven groups and their monophyly was strongly supported in all analyses. However, *D. w. bowringi* was found to be paraphyletic and the monophyly of *D. yui yui* was not confirmed in the 16S rRNA based analyses. In the same analyses we also failed to identify the monophyly of *D. yui yui* (Fig. 3). Paraphyly or polytomy of the two species was the result of a few pasimony-informative nucleotide substitutions. This has a significant effect on phylogenetic reconstructions when the genetic divergences within and between species are low.

In all topologies, *D. adamsi* is sister to *D. yui yui*; the same was suggested by Kurosawa (1986). He grouped *D. adamsi*, *D. shimomurai*, and *D. yui* as the *adamsi* species-group and mentioned that the female dark blackish body without markings might be the main characteristic of this group. The abdomen covered with whitish powder is also a trait that is only shared by *D. adamsi* and *D. yui* among the examined species (Pouillude 1914, Kurosawa 1986).

In contrast with the molecular data of the *adamsi* species-group, our results for the other congeners do not support the view of Kurosawa (1986). *D. uenoi katoi* is treated

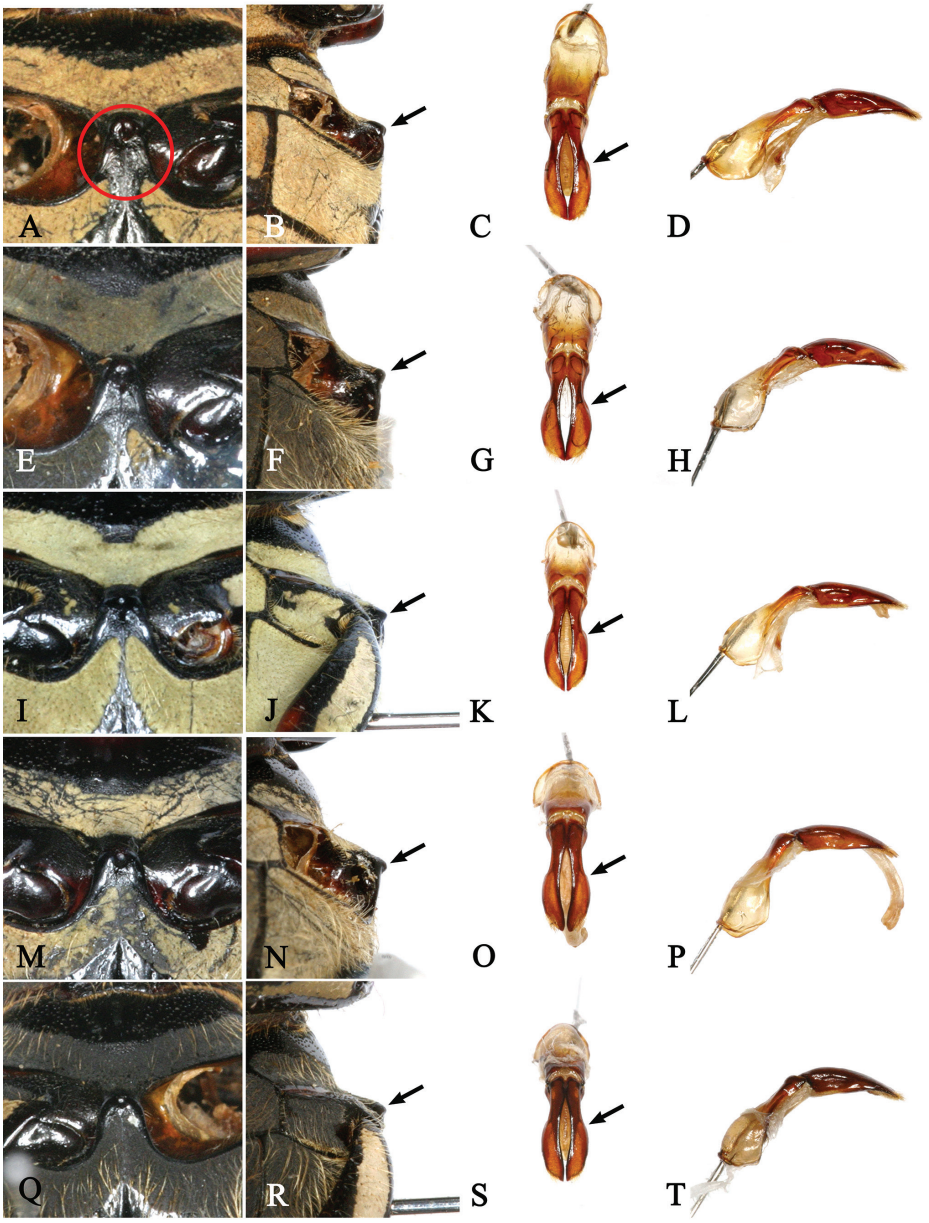

Figure 8. Metasternal process (in the circle) and aedeagi of *Dicronocephalus adamsi drumonti* and *D. a. adamsi*. **A, B, C, D** *D. a. drumonti* (Tibet) **E, F, G, H** *D. a. drumonti* (Sichuan) **I, J, K, L** *D. a. adamsi* (South Korea) **M, N, O, P** *D. a. adamsi* (North Korea) **Q, R, S, T** *D. a. adamsi* (Dandong, China).

as a separate group in his paper, but it appears a sister taxon of *D. dabryi* in our study, although the general appearance of *D. uenoi katoi* is rather similar to that of *D. yui yui*. Especially, these two species share two characters: the pronotal bands reaching the

posterior border and the obtuse metasternal process. Pouillaude (1914) also noted that *D. dabryi* has tawny erect hair on the pronotum and elytra. We could observe that the pronotum and elytra are sparsely pilose and the hairs are much denser and longer on the ventral side compared with the other congeners. Furthermore, in the male genitalia, the parameres of the two species are similar and much shorter than those of other species. In this study, the pilose body, which is represented as a unique character of *D. uenoi katoi* by Kurosawa (1986), is considered as autapomorphy, which may have been rapidly acquired during allopatric speciation in Taiwan because *D. uenoi katoi* was isolated from a continental ancestor. This interpretation disagrees with Kurosawa's presumption that *D. uenoi katoi* is the most primitive in this genus.

Regarding the status of the subspecies of *D. adamsi*, Legrand (2005) recognized discontinued distribution and morphological differences between two geographically separated populations; however, we consider almost all of the diagnostic characters as being unsuitable for distinguishing these two subspecies. Furthermore, the molecular data indicates that the two subspecies form a monophyletic group with low genetic divergences (0–1.7%) and individuals of the both subspecies share haplotypes. Therefore, our results provide strong evidence that *D. a. drumonti* should be synonymized with *D. a. adamsi*.

The three subspecies of *D. wallichii* were originally described as separate species (Hope 1831, Pascoe 1863, Pouillaude 1914). Subsequently their status was lowered to subspecific (Paulian 1960, Mikšić 1971, 1977, Krajcik 1998, Sakai and Nagai 1998, Šípek et al. 2008, Young 2012, Krajcik 2014). However, Kurosawa (1968) disagreed with Paulian (1960) as he considered that there were significant morphological differences between them such as the characteristics of the antlers, the clypeus, the marginal carinae of the pronotum, and the metasternal process. Devecis (2008) also proposed that the taxa be restored as species based on the morphological differences such as color of the dorsal setation, shape of the antlers, and length of the pronotal bands. Results of our molecular analyses showed that the three subspecies of *D. wallichii* form a monophyletic group with high supporting values and large genetic distances. The average pairwise distances (4.7%–6.0%) of COI between *D. wallichii bowringi* + *D. wallichii wallichii* and *D. wallichii bowringi* + *D. wallichii bourgoini*. *D. wallichii wallichii* + *D. wallichii bourgoini* were slightly lower than the average inter-specific distances of *D. adamsi* + *D. yui yui* (6.2%) and *D. dabryi* + *D. uenoi katoi* (6.9%) (Table 2). Also, in 16S rRNA analysis, the pairwise distances between the three subspecies of *D. wallichii* were similar to (0.8%–1.6%) the distance between *D. adamsi* and *D. yui yui* (0.8%–1.2%) (Table 3). Our phylogenetic analyses explicitly explain their evolutionary history. *D. w. bowringi* is the most primitive among this group and *D. w. wallichii* might be separated by parapatric speciation in the continental region. Also, *D. w. bourgoini* might have undergone allopatric speciation after colonizing the volcanic island of Taiwan. Our results support specific rather than subspecific rank of the three members of *D. wallichii*. We revealed them as being in a monophyletic cluster (Mishler and Theriot 2000, Wiens and Penkrot 2002) with each other separated by distinct genetic gaps in the COI and COI+*16S* analyses, although not in the 16S rRNA analy-

sis. Also, our study showed two distinguishable morphological characters, namely the color of the dorsal body side in males and the shape of the metasternal process (Table 5). However, this evidence is not strong enough to propose specific rank for each of them. A recent study showed that the high genetic divergence of COI alone cannot be a reason for species separation in *Cetonia aurata aurata* (Ahrens et al. 2013). There is a need for additional analyses with representative sample sizes and the use of multiple genetic loci to reconfirm our results.

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Supplementary material 1

COI **sequences dataset of *Dicronocephalus* species in this study.**
Authors: Ga-Eun Lee, Taeman Han, Jongchel Jeong, Seong-Hyun Kim, In Gyun Park, Haechul Park
Data type: (DNA sequences)
Explanation note: This COI data includes 50 individual sequences of the examined *Dicronocephalus* species and subspecies in this study
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Supplementary material 2

16S rRNA **sequences data set of *Dicronocephalus* species in this study.**
Authors: Ga-Eun Lee, Taeman Han, Jongchel Jeong, Seong-Hyun Kim, In Gyun Park, Haechul Park
Data type: (DNA sequences)
Explanation note: This 16S rRNA data includes 46 individual sequences of the examined *Dicronocephalus* species in this study.
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Supplementary material 3

The combined dataset of COI **and** 16S rRNA **of *Dicronocephalus* species in this study.**
Authors: Ga-Eun Lee, Taeman Han, Jongchel Jeong, Seong-Hyun Kim, In Gyun Park, Haechul Park
Data type: (DNA sequences)
Explanation note: There is the concatenated sequences of COI and 16S rRNA genes correspondence with each sample.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

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Revised and annotated checklist of aquatic and semi-aquatic Heteroptera of Hungary with comments on biodiversity patterns

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Abstract

A basic knowledge of regional faunas is necessary to follow the changes in macroinvertebrate communities caused by environmental influences and climatic trends in the future. We collected all the available data on water bugs in Hungary using an inventory method, a UTM grid based database was built, and Jackknife richness estimates and species accumulation curves were calculated. Fauna compositions were compared among Central-European states. As a result, an updated and annotated checklist for Hungary is provided, containing 58 species in 21 genera and 12 families. A total 66.8% of the total UTM 10 × 10 km squares in Hungary possess faunistic data for water bugs. The species number in grid cells numbered from 0 to 42, and their diversity patterns showed heterogeneity. The estimated species number of 58 is equal to the actual number of species known from the country. The asymptotic shape of the cumulative species curve predicts that additional sampling efforts will not increase the number of species currently known from Hungary. These results suggest that the number of species in the country was estimated correctly and that the species accumulation curve levels off at an asymptotic value. Thus a considerable increase in species richness is not expected in the future. Even with the species composition changing the chance of species turn-over does exist. Overall, 36.7% of the European water bug species were found in Hungary. The differences in faunal composition between Hungary and its surrounding countries were caused by the rare or unique species, whereas 33 species are common in the faunas of the eight countries. Species richness

does show a correlation with latitude, and similar species compositions were observed in the countries along the same latitude. The species list and the UTM-based database are now up-to-date for Hungary, and it will provide a basis for future studies of distributional and biodiversity patterns, biogeography, relative abundance and frequency of occurrences important in community ecology, or the determination of conservation status.

Keywords
Water bugs, estimated species richness, new species records, *Notonecta reuteri reuteri*

Introduction

Aquatic and semi-aquatic Heteroptera (water bugs) are important components of aquatic ecosystems for several reasons. Water bugs act both as consumers of algae and leaf litter at lower trophic levels and as prey for fish and other organisms at higher trophic levels (McCafferty 1981, Hutchinson 1993). Water bugs can be found on the macrophyte stands, of the benthic region, beneath open water or on the surface. However, both the surface dwellers and the truly aquatic forms occupy a particular niche within an ecosystem (Savage 1989). Moreover, several species are considered as flagship or umbrella species for ecosystem protection (Whiteman and Sites 2008). In addition to their ecological role, some species even have high economic importance as top predators or food sources for protected or endangered animals (or even humans), the significance of which has probably been underestimated (Papáček 2001).

There are conflicting opinions in the literature as to whether aquatic bugs are good indicators of the ecological status. However, communities of aquatic Heteroptera *per se* have generally been studied less frequently than the assemblages of aquatic macroinvertebrates as a whole (Turić et al. 2011). Aquatic bugs – except for nymphs and *Aphelocheirus aestivalis* – are air-breathers, thus, they exist under a wide range of water quality conditions, including waters poor in oxygen. On the other hand, the distribution of some taxa is correlated with several biotic and abiotic factors (e.g., Macan 1938, 1954, Savage 1982, Tully et al. 1991, Savage 1994, Sládeček and Sládečková 1994, Hufnagel et al. 1999, Jardine et al. 2005, Nosek et al. 2007). Consequently, some aquatic bugs show great sensitivity to environmental stressors, whereas some other species are more resilient to environmental changes which, on the whole makes them doubtful indicators of water quality. This ecological difference may be related to their geographic distribution. Due to their high dispersal ability, some species with a wide ecological tolerance to environmental constraints can be found in almost every freshwater habitat across the Holarctic Region. Besides these cosmopolitan taxa, there are species which occur exclusively in specific habitats (Macan 1954, Savage 1994).

The aquatic and semi-aquatic Heteroptera are composed of two monophyletic infraorders (Gerromorpha, Nepomorpha), which together encompass 92% of the aquatic and semi-aquatic species, with the remaining species belonging to the more or less water dependent Leptopodomorpha (Polhemus and Polhemus 2008). In the Palaearctic Region, there are more than 100 Gerromorpha and 200 Nepomorpha species.

The first major catalogue of species in the Palaearctic Region was published by Aukema and Rieger (1995), who presented all the synonyms and distribution information based on the original descriptions. This work was later supplemented and up-dated by Aukema et al. (2013). In Hungary, active taxonomical and faunistical studies have been conducted since 1870. The first Heteroptera checklist, encompassing both terrestrial and aquatic species, was published by Horváth (1918), and since then, Hungarian experts have published almost 100 publications containing faunistic data on aquatic bugs. The large amount of relevant new information was summarised in a new checklist by Kondorosy (1999). Since 1999, attention focused mainly on the autecology of aquatic and semi-aquatic Heteroptera and has remained relevant since the EU Water Framework Directive (WFD) was adopted (European Commission 2000). The WFD, undoubtedly, represents a milestone in the research of the aquatic and semi-aquatic Heteroptera fauna of Hungary, and the member states of the European Union.

The implementation of the WFD required intensive faunistical and ecological surveys across Hungary. The first country-wide survey of aquatic and semi-aquatic Heteroptera was carried out in 2005 under the framework of the ECOSURV project (Kiss et al. 2006a,b). The increasing intensity of faunistic research is clearly illustrated by the fact that more papers were published during the last 15 years (N = 103, 1999-2014) than in the previous decades (N = 95, prior to 1999). Many localities that had been poorly studied before were sampled and five heteropteran species new to the Hungarian fauna have been detected since 1999. Consequently, this large amount of new data warrants a comprehensive faunistical overview of this group.

The main goals of the present paper are (1) to provide a revised and annotated checklist of the aquatic and semi-aquatic Heteroptera fauna of Hungary, (2) to assess the UTM-based distributional patterns during three distinct intervals of research to show the biodiversity trends in Hungary over more than 100 years, (3) to describe the current state-of-the-art of water bug studies in Hungary, and (4) to compare the number of species with those of the neighbouring countries. Finally, by synthesizing this information, key areas for future research are identified.

Material and methods

Geographic and hydrological background

Hungary is located in the Carpathian Basin, the largest intramontane basin in Europe (Gábris and Nádor 2007). Most of the country lies below 200 m a.s.l.; the highest point in the country is Kékes (1 014 m) and the lowest spot is located near Szeged in the south (77.6 m). Based on the ecoregion classification schemes of rivers and lakes (EEA 2004, Illies 1978), Hungary belongs to the Pannonian Ecoregion. This alluvial basin is formed by the Danube River and its main tributaries, the Tisza and Dráva Rivers. The hydrology of Hungary is primarily determined by these large potamal rivers. The most characteristic water body types are the small lowland streams, oxbows, swamps, and

soda pans formed by fluvial erosion and deflation (Borics et al. 2014). Besides these types of waters, large, shallow lakes (e.g., Lake Balaton, Lake Velence and Fertő) provide unique habitats for aquatic and semi-aquatic Heteroptera in Hungary.

Database, statistical analyses

As a first step, a database was constructed that contained information on the taxa occurring in Hungary and their known locations. During the building of the database, two main sources were considered: published papers, and data from the regular surveillance monitoring operated by the National Environmental Authorities since 2005. As a result, 22 587 records from 198 papers published between 1878 and 2014 are included in the database. Records were only included when the specimen was identified to species and when the locality of occurrence was clearly indicated. For mapping the distribution patterns, all records were arranged into 10 × 10 km UTM grids. Non-verifiable records were omitted from the database. To reveal the trends in the growth of knowledge regarding water bugs, the database was divided into three time periods: the first part included all records before 1918, the second part included all records before 1999, and the third part contained all data before 2014, respectively. Each sub-database was then considered as a matrix with UTM grids in columns and species in rows. Each species has presence-absence data in cells appertaining only to those UTM grid cells, in which aquatic and semi-aquatic Heteroptera data occurred during the given period. Based on these sub-databases, species accumulation curves and richness estimates were calculated with PAST 3.02 (Hammer et al. 2001). Jackknife 1 was used as a non-parametric estimator, because it is useful for evaluating the expected richness for incidence data (Melo 2004, Gotelli and Colwell 2010).

The composition of water bug assemblages of the neighbouring countries were compared by using non-metric multidimensional scaling (NMDS). The dissimilarity of assemblages based on presence-absence data was quantified by the Jaccard index (Legendre and Legendre 1998). The correlation between the number of species and the number of UTM grids was also calculated with PAST 3.02.

Compiling the checklist

The names of the species were updated according to Aukema and Rieger (1995) and Aukema et al. (2013). A detailed taxonomic classification is listed in the current checklist, with the author of each taxonomy level given. New records were identified by the authors Kiss et al. (2009), Soós et al. (2009), and Soós et al. (2010). All of the changes between the second and the latest checklist (Kondorosy 1999) were noted, and finally we produced an updated checklist of Hungarian aquatic and semi-aquatic Heteroptera. Following Nieser (2002), we considered the subfamily Micronectinae to have family rank as Micronectidae.

Results

Based on the results of data mining and the Hungarian surveillance monitoring, 58 water bug species representing 21 genera and 12 families are currently known from Hungary (Table 1). The occurence of the species in Hungary is now documented for 37 species of Nepomorpha (Nepidae – 2, Micronectidae – 5, Corixidae – 19, Naucoridae – 1, Aphelocheiridae – 1, Notonectidae – 7, Pleidae – 1) and 21 species of Gerromorpha (Mesoveliidae – 2, Hebridae – 2, Hydrometridae – 2, Veliidae – 6, Gerridae – 9). No representatives of the families Belostomatidae and Ochteridae were found.

Although the first checklist listed only 31 species (Horváth 1918), this number increased by 23 species and none disappeared during the time to the second checklist (Kondorosy 1999). From the second checklist to date, the species list has been expanded by five species. Four of these have already been published; *Notonecta maculata* and *Notonecta meridionalis* by Soós et al. (2009), *Anisops sardeus sardeus* by Soós et al. (2010), and *Sigara hellensii* by Kiss et al. (2009), whereas the fifth species, *Notonecta reuteri reuteri* is here recorded for the first from Hungary (see below).

Figure 1 represents the species accumulation curves of aquatic and semi-aquatic Heteroptera during the three distinct intervals. The species richness estimators suggest that a large number of species living in the country were not collected before 1918. The estimated number of species was 41, whereas the observed number was only 32. The monotonic increase of the curve confirms that the estimated richness was considerably higher at that time than the observed one. The curve based on data before 1999 showed only a slightly higher estimated taxa richness in Hungary (54) than the observed number of species (52). Based on the most recent (current) checklist, the estimated richness curve flattens off soon after the number of UTM grids increases to 100. The estimated number of species is 58, which is equal to the observed one.

There are 1061 UTM grid cells in Hungary, 709 of which contain aquatic and semi-aquatic Heteroptera records (66.8% of the total) (Figure 2). The species number in any given grid cell ranged from 0 to 42. The most diverse UTM grid cell was BT70 with 42 species (part of Lake Balaton). Eight grid cells had an outstandingly high number of species ($n > 30$). Twenty to 30 species occurred in 71 grid cells (10% of the cells in which aquatic and semi-aquatic Heteroptera were found), 10 to 20 species occurred in 204 grid cells (29%), and less than 10 species occurred in 426 grid cells (60%). Finally, there were 352 UTM grid cells without records.

The number of species occurring in Hungary (58) corresponds to 36.7% of the water bug fauna of Europe. The number of species was higher in Hungary than in Slovakia (55), Serbia (54), and Slovenia (49); almost the same as in Croatia (59); and slightly lower than in Austria (62), Ukraine (68) and Romania (72) (Table 2, Suppl. material 1). The scatter plot of the NMDS (Figure 3) showed that Hungary had almost the same species list as Slovakia, whereas the other countries surrounding them had slightly different water bug faunas.

Table 1. Updated checklist of aquatic and semi-aquatic Heteroptera (Heteroptera: Nepomorpha, Gerromorpha) occurred in Hungary, with the year of the first published occurrence and the author(s).

Taxa	Year of first published occurrence, and author
Nepomorpha	
Nepidae	
Nepa cinerea Linnaeus, 1758	1918 Horváth
Ranatra (*Ranatra*) *linearis* (Linnaeus, 1758)	1918 Horváth
Micronectidae	
Micronecta (*Dichaetonecta*) *pusilla* (Horváth, 1895)	1918 Horváth
Micronecta (*Dichaetonecta*) *scholtzi* (Fieber, 1860)	1918 Horváth
Micronecta (*Micronecta*) *griseola* Horváth, 1899	1916 Horváth
Micronecta (*Micronecta*) *minutissima* (Linnaeus, 1758)	1962 Wróblewski
Micronecta (*Micronecta*) *poweri poweri* (Douglas & Scott, 1869)	1960 Wróblewski
Corixidae	
Cymatia coleoptrata (Fabricius, 1777)	1885 Horváth
Cymatia rogenhoferi (Fieber, 1864)	1885 Horváth
Callicorixa praeusta praeusta (Fieber, 1848)	1959 Soós
Corixa affinis Leach, 1817	1918 Horváth
Corixa panzeri Fieber, 1848	1959 Soós
Corixa punctata (Illiger, 1807)	1918 Horváth
Hesperocorixa linnaei (Fieber, 1848)	1918 Horváth
Hesperocorixa sahlbergi (Fieber, 1848)	1918 Horváth
Paracorixa concinna concinna (Fieber, 1848)	1885 Horváth
Sigara (*Microsigara*) *hellensii* (C.R. Sahlberg, 1819)	2009 Kiss
Sigara (*Pseudovermicorixa*) *nigrolineata nigrolineata* (Fieber, 1848)	1918 Horváth
Sigara (*Retrocorixa*) *limitata limitata* (Fieber, 1848)	1918 Horváth
Sigara (*Retrocorixa*) *semistriata* (Fieber, 1848)	1918 Horváth
Sigara (*Sigara*) *assimilis* (Fieber, 1848)	1959 Soós
Sigara (*Sigara*) *striata* (Linnaeus, 1758)	1918 Horváth
Sigara distincta (Fieber, 1848)	1918 Horváth
Sigara (*Subsigara*) *falleni* (Fieber, 1848)	1918 Horváth
Sigara (*Subsigara*) *fossarum* (Leach, 1817)	1990 Bakonyi
Sigara (*Vermicorixa*) *lateralis* (Leach, 1818)	1918 Horváth
Naucoridae	
Ilyocoris cimicoides cimicoides (Linnaeus, 1758)	1918 Horváth
Aphelocheiridae	
Aphelocheirus (*Aphelocheirus*) *aestivalis* (Fabricius, 1794)	1918 Horváth
Notonectidae	
Anisops sardeus sardeus Herrich-Schaeffer, 1849	2010 Soós
Notonecta (*Notonecta*) *glauca glauca* Linnaeus, 1758	1918 Horváth
Notonecta (*Notonecta*) *lutea* Müller, 1776	1918 Horváth
Notonecta (*Notonecta*) *maculata* Fabricius, 1794	2009 Soós
Notonecta (*Notonecta*) *meridionalis* Poisson, 1926	2009 Soós
Notonecta (*Notonecta*) *viridis* Delcourt, 1909	1931 Horváth
Notonecta (*Notonecta*) *obliqua* Thunberg, 1787	1938 Visnya
Notonecta (*Notonecta*) *reuteri reuteri* Hungerford, 1928	recent paper

Taxa	Year of first published occurrence, and author
Pleidae	
Plea minutissima minutissima Leach, 1817	1918 Horváth
Gerromorpha	
Mesoveliidae	
Mesovelia furcata Mulsant et Rey, 1852	1915 Horváth
Mesovelia thermalis Horváth, 1915	1999 Kiss
Hydrometridae	
Hydrometra gracilenta Horváth, 1899	1899 Horváth
Hydrometra stagnorum (Linnaeus, 1758)	1878 Horváth
Hebridae	
Hebrus (*Hebrus*) *pusillus pusillus* (Fallén, 1807)	1878 Horváth
Hebrus (*Hebrusella*) *ruficeps* Thomson, 1871	1918 Horváth
Veliidae	
Microvelia (*Microvelia*) *buenoi* Drake, 1920	1988 Vásárhelyi and Bakonyi
Microvelia (*Microvelia*) *reticulata* (Burmeister, 1835)	1916 Horváth
Microvelia (*Picaultia*) *pygmaea* (Dufour, 1833)	1916 Horváth
Velia (*Plesiovelia*) *caprai caprai* Tamanini, 1947	1923 Horváth
Velia (*Plesiovelia*) *affinis filippii* Tamanini, 1947	1938 Visnya
Velia (*Plesiovelia*) *saulii* Tamanini, 1947	1969 Benedek
Gerridae	
Aquarius najas (De Geer, 1773)	1918 Horváth
Aquarius paludum paludum Fabricius, 1794	1918 Horváth
Gerris (*Gerris*) *argentatus* Schummel, 1832	1878 Horváth
Gerris (*Gerris*) *lacustris* (Linnaeus, 1758)	1878 Horváth
Gerris (*Gerris*) *odontogaster* (Zetterstedt, 1828)	1918 Horváth
Gerris (*Gerris*) *thoracicus* Schummel, 1832	1918 Horváth
Gerris (*Gerris*) *gibbifer* Schummel, 1832	1918 Horváth
Gerris (*Gerriselloides*) *asper* (Fieber, 1860)	1918 Horváth
Limnoporus rufoscutellatus (Latreille, 1807)	1918 Horváth

Table 2. Number of species of aquatic and semi-aquatic Heteroptera from Hungary and neighbouring countries compared to the 158 species in Europe. Data on the number of established species in specific countries taken from different papers.

Countries	Gerromorpha	Nepomorpha	Total number of species	% of the total number of species in Europe
Slovenia	20	29	49	31.0
Slovakia	20	35	55	34.2
Serbia	23	31	54	34.2
Hungary	21	37	58	36.7
Croatia	22	37	59	37.3
Austria	22	40	62	39.2
Ukraine	24	44	68	43.0
Romania	28	43	72	45.6

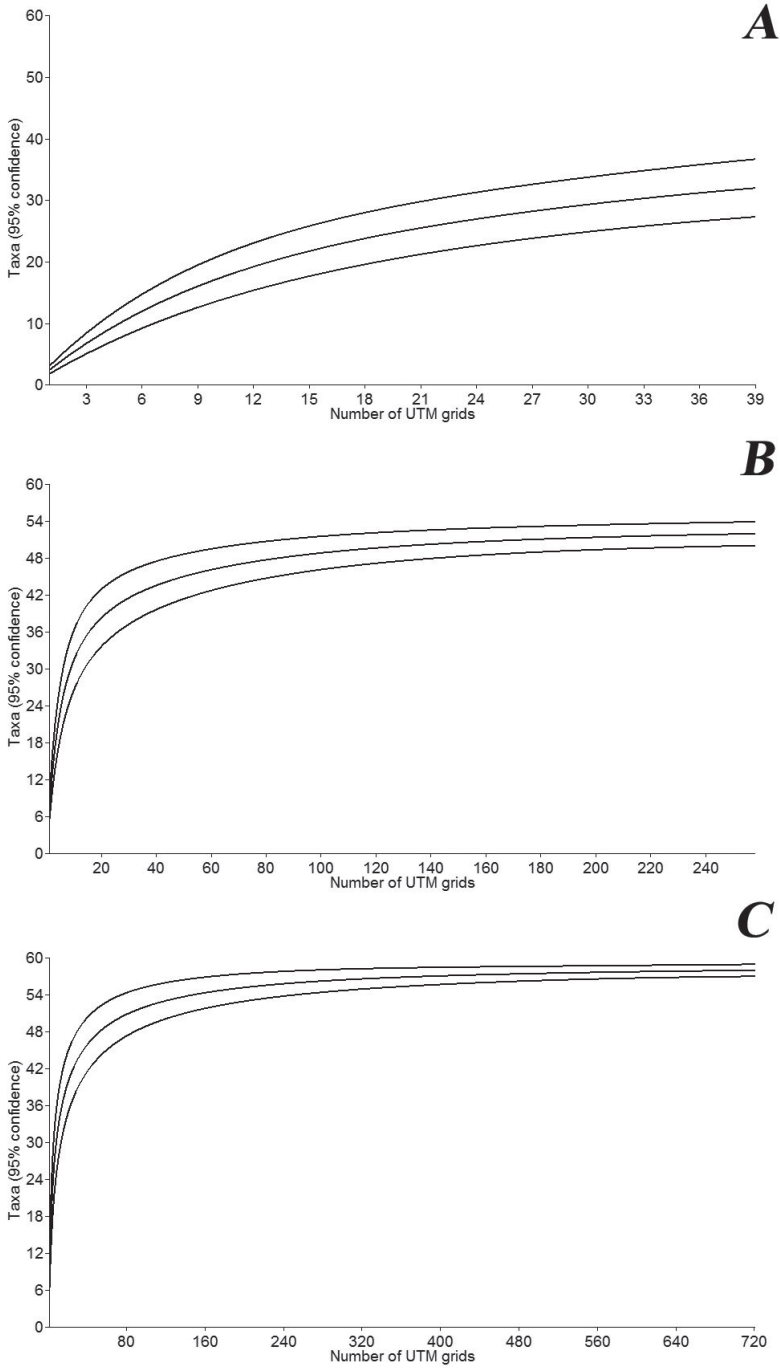

Figure 1. Observed and estimated species richness based on the checklist of given periods. Cumulative species curves produced by PAST 3.02 software package. **A** based on data before the first checklist (published in 1918) **B** based on data before the second checklist (published in 1999) **C** based on the whole database (present work).

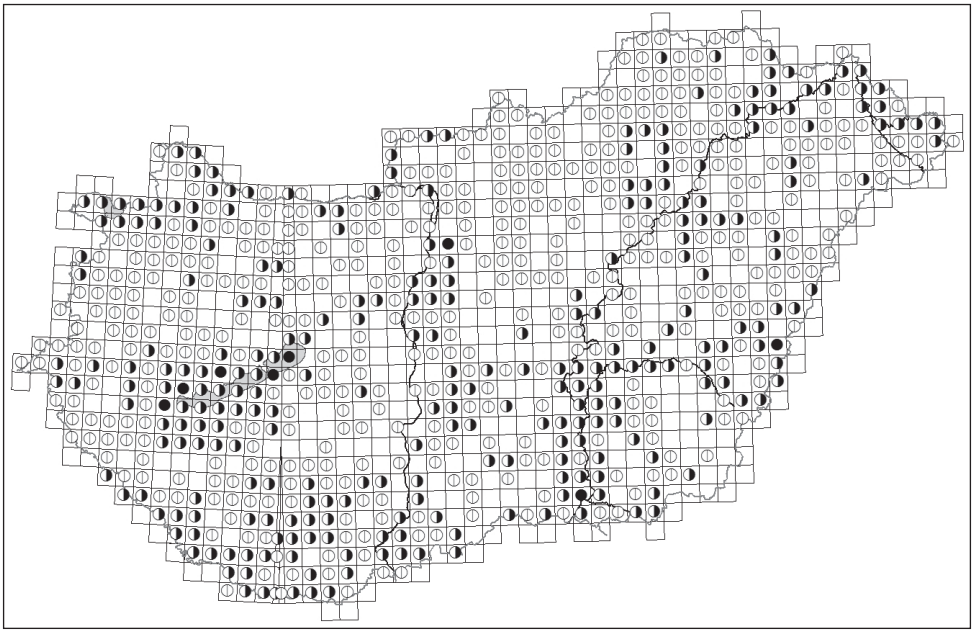

Figure 2. Aggregate records of aquatic and semi-aquatic Heteroptera (Heteroptera: Nepomorpha, Gerromorpha) in Hungary depicted on UTM grids map. Empty circles refer to UTM grids with a lower number of species ($N < 10$), half full circles refer to UTM grids with an average number of species ($10 < N < 30$), and full circles refer to the most diverse UTM grids ($N > 30$).

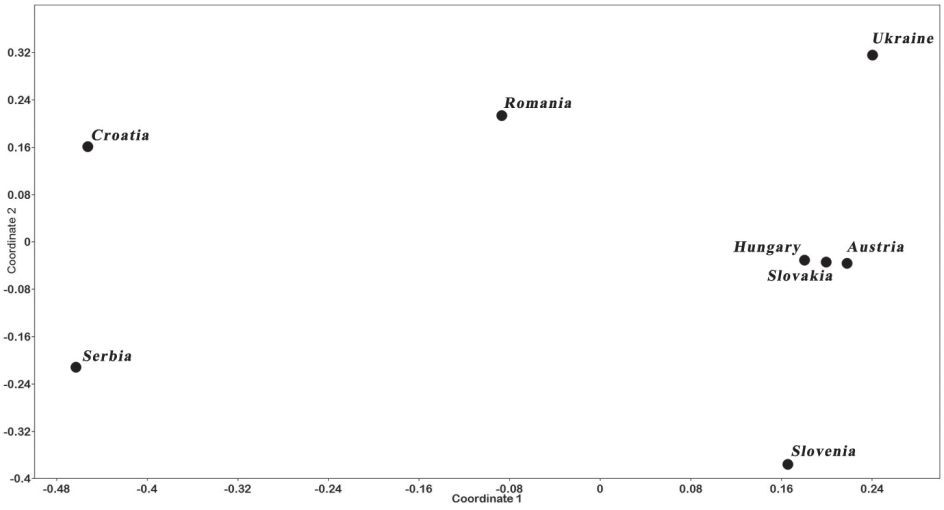

Figure 3. Ordination of the neighbouring countries based on presence-absence data of aquatic and semi-aquatic Heteroptera species (with Jaccard similarity index, Final stress = 0.1998).

First record of *Notonecta reuteri reuteri*

Material examined. *Notonecta reuteri reuteri* Hungerford, 1928: Érd, 1934, 3 females, Pudleiner lgt., P. Boda & P. Kment det. (coll. Hungarian Natural History Museum, Budapest).

Former publications mentioned *N. reuteri reuteri* as a species expected to occur in the Hungarian fauna (Soós et al. 2009, Soós 1963) because it was found in the neighbouring countries. However, it is a tyrphobiont species usually inhabiting higher altitudes in Central Europe (Štys 1960, Wróblewski 1980), i.e., habitats generally absent in Hungary. Recently, 3 females were discovered in the unidentified material of the Hungarian Natural History Museum and were definitively identified as *N. reuteri reuteri*. *Notonecta lutea* and *N. reuteri reuteri* both have the same yellowish scutellum and body shape, but the species are distinguished from each other by the male and female genitalia as well as by the shape of the last abdominal sternum of the female (Štys 1960) . There are no recent records of this species from Hungary; it has not been found since 1934, but there is a chance it will be rediscovered in the future. Including *N. reuteri reuteri*, there are now eight species of Notonectidae recorded from Hungary (Soós et al. 2009).

Discussion

Increased sampling effort contributes to a better knowledge of regional faunas (Dennis et al. 1999, Stander 1998, Rocchini et al. 2011). The number of estimated species in the first period (until 1918) is only a rough estimate due to the small sample size (Figure 1A). It is striking that the small sample size provides a relatively high number of species (Colwell and Coddington 1994). The reason for this lies in how studies were conducted in the beginning of the 20th century. During that period, researchers primarily surveyed the most interesting, particular and diverse habitats. These purposeful and directional studies resulted in the collection of 31 species in a short period of time. More frequent and broadly based studies then yielded higher estimated taxon numbers until 1999. Based on the shape of the species accumulation curve estimated from the entire database until 2013, it appears likely that an increase in sampling efforts will not result in an increase in the number of species currently known from Hungary. Surprisingly, the constantly changing number of studies and the alternating sampling intensity throughout the decades had no traceable influence on the chances of the appearance of a new species. The average rate of species discovery has remained the same, at around 2.85 species per 10 years (23 species in 81 years between 1918 and 1999, and 4 species in 14 years between 2000 and 2014). The constant rate of discovery has no scientific explanation, and can only be considered as a statistical coincidence without any ecological background.

Is the Hungarian aquatic and semiaquatic bug fauna, currently at 58 species, completely known? Our results suggest that the number of species in the country is es-

timated correctly and that the species accumulation curve levels off at an asymptotic value, a considerable increase in species richness is not expected in the future. It is clear that species composition may change and that the opportunity of species turnover exists. Turnover of species, or finding additional species new to Hungary, depends on the current characteristics of water bodies and on the biological attributes controlling the dispersal and persistence of their potential colonists (Case and Cody 1987). In former publications, 24 species were considered as expected species on Hungarian fauna (Soós 1963, Benedek 1969). Six of these species are now confirmed members of the fauna, and the others might appear in the future. What a species needs and what the environment supplies is species-specific, but due to the fact that the borders of several eco-regions meet in the Carpathian Basin, Mediterranean, and Eurosiberian species occur along with Holarctic and Palaearctic species (Josifov 1986). Because of this biogeographic setting, the chance for the appearance of additional species is difficult to predict accurately.

Among these expected species, some alien species show a recent range expansion northwards in Europe (Van de Meutte et al. 2010, Boda et al. 2012, Guareschi et al. 2013, Barbora and Marek 2014, Reduciendo Klementová and Svitok 2014). Several new records and regular findings of *Anisops sardeus sardeus* were published from all across Europe during the last five years (Berchi 2011, Khatukhov et al. 2011, Kment and Beran 2011, Cianferoni and Pinna 2012, Cianferoni and Terzani 2013, Reduciendo Klementová and Svitok 2014) and from Hungary (Soós et al. 2010). In addition, Hungary is a potential area of invasion of another alien bug *Trichocorixa verticalis verticalis* (Fieber, 1851) (Corixidae). The possibility of the future occurrence of this taxon is high for several reasons. First, this species lives in brackish and saline waters in both juvenile and adult phases, salinity tolerance is one of the key factors for its expanding range (Van De Meutter et al. 2010), and the Carpathian Basin is extremely rich in soda pans. Second, climate change is generally expected to result in increased salinization of water bodies. Finally, the resting eggs of this species are able to survive in extreme environments (Tones 1977, Kelts 1979). These facts together can facilitate the appearance of this species and the survival of the pioneer individuals in Hungary (Guareschi et al. 2013).

The national biodiversity monitoring system of Hungary is operated at approximately 1200 samplings stations from 558 UTM grid cells and thus provides a broad spatial coverage. With the addition of UTM grid cells where further studies were carried out with various purposes and which provided valid data (198 papers altogether), the spatial coverage has now reached two thirds of the area of Hungary. The most diverse grid cells may have particular significance for biodiversity conservation as hotspots of species richness. However, the eight grids with an outstandingly high number of species ($N > 30$) can also result from unusually high sampling effort. Five from the eight cells belong to Lake Balaton and its tributaries, one of the most frequently studied shallow lakes in Europe (BT70: Horváth 1931, Bakonyi and Vásárhelyi 1988, Bíró and Hufnagel 1998, 2001, Bíró 2003, Sipkay et al. 2005, Vásárhelyi and Bakonyi 2005, 2012; XM67: Soós 1959, Kondorosy et al. 1996, 2011, Bíró and Hufnagel 1998, Kiss

et al. 2008, Móra et al. 2008; XM78: Horváth 1931, Soós 1959, Wróblewski 1960, Bíró and Hufnagel 1998, Kiss et al. 2008, Kondorosy 2011; XM99: Soós 1959, Bíró and Hufnagel 1998, Rozner 2004, Móra et al. 2007, 2011, Szekeres and Csányi 2010; and YM29: Horváth 1931, Soós 1959, Wróblewski 1960, Bíró and Hufnagel 1998, Móra et al. 2007, Kiss et al. 2008, Soós et al. 2009). Grid cells with similarly high richness also occur near Szeged, at the site of a periodic and long-term study (DS32: Vellay 1899, Czógler 1937, Csongor 1956, Soós 1959, Csabai et al. 2010); near Budapest, at the site of a continuous but medium-term (1991–1996) ecological study (CT66: Hufnagel 1994, 1998); and Kis-Sárrét Nature Conservation area (SE, Hungary), at the site of an intensive but short-term study with several sampling times per year (ET40: unpublished personal data). These considerations suggest that these regions are not necessarily hotspots of species richness, they rather reflect a disproportionately high sampling effort in these grid cells. On the other hand, the UTM grid cells with no records show a random and patchy pattern. Surveys in these UTM grids provide some chance for the appearance of species new to the country.

A comparison of species composition with that of neighbouring countries is difficult because of the high variation in latitude, area, climate, altitude, and the number and types of watercourses. In Hungary, all but one catchment area originates in the surrounding mountain ranges (the Alps to the west, Carpathians to the north and east, and Dinarids to the south) and thus extends beyond the country borders. As a result, drift phenomena from upstream reaches can be more frequent and important than one might think. No species occurs exclusively in Hungary, which could be explained by these geographical features, the fact that the country borders are not aligned with any geographical feature and that aquatic bugs have good dispersal abilities. Dispersal studies indicate that 32% of the fauna can be found in the air as common species (Csabai et al. 2012, Boda and Csabai 2013, Boda et al. 2014). On the other hand, the species/area relationship suggests that the number of species in an area correlates strongly and positively with the size of that area. In the last decade, specialists in neighbouring countries made a considerable effort to explore the aquatic and semi-aquatic Heteroptera fauna (AUSTRIA: Rabitsch 2008a,b; CROATIA: Kment and Beran 2011, Turić et al. 2011; ROMANIA: Berchi 2011, 2013, Berchi et al. 2011, 2012, Ilie and Olosutean 2012; SERBIA: Živić et al. 2007, Šeat 2011, 2013, Protić 2011, Protić and Živić 2012; SLOVAKIA: Klementová et al. 2012, Kment et al. 2013, Reduciendo Klementová and Svitok 2014; SLOVENIA: Gogala 2003, 2009; UKRAINE (including Crimea): Putshkov and Putshkov 1996, Grandova and Prokin 2012, Grandova 2013, 2014). Consequently, the aquatic and semi-aquatic Heteroptera fauna of these countries is adequately known, except for Ukraine, the large area of which sets a natural limit to the number of surveys. In our case, there is a strong positive correlation between the number of species and the area of the countries ($r = 0.695$, $n = 8$, $p < 0.05$). Moreover, the correlation coefficient is even higher and significant ($r = 0.905$, $n = 7$, $p < 0.05$) with Ukraine excluded from the analysis because of its under-studied status.

The plot of the NMDS and the geographical map has shown the same organizing principles. Hungary and Slovakia together are roughly at the same latitude with Austria and two other countries with similar geographical/environmental conditions (Romania, Ukraine), whereas countries reaching into the Mediterranean Region are

located further south (Croatia, Serbia, Slovenia). The differences in faunal composition seen in the plot should be due to the rare or unique species, and 33 species are common in the faunas of the eight countries (Suppl. material 1). It is well known that latitude has a major influence on species diversity (Fischer 1960) with species richness increasing from high latitudes toward the tropics (Rosenzweig 1995). The latitudinal pattern of aquatic bugs is currently unknown, and has been rarely studied for the whole macroinvertebrate community. Our data suggests that there is no evidence for such a latitudinal diversity gradient at our spatial scale. However, our data confirm that latitude *per se* cannot be a determinant of species richness; diversity only correlates with a number of potentially causal environmental factors (Gaston 2000). Even if species richness does not show correlation with latitude, similar species compositions were observed in the countries positioned along the same latitude. We found three main groups based on species number and fauna composition: (1) slightly lower number of species, but unique fauna composition, e.g., Slovenia, Serbia and Croatia; (2) average number of species, with highly overlapping fauna composition, e.g., Hungary, Slovakia and Austria; (3) higher number of species with many species in common with countries in group 2 along with some extra species occurring in larger and more heterogeneous countries (Romania, Ukraine).

We conclude that the species list and the UTM-based database are now up-to-date for Hungary. These will provide a basis for future studies of distributional and biodiversity patterns, biogeography, relative abundances and frequency of occurrences important in community ecology, or the determination of conservation status.

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Supplementary material 1

Checklist of aquatic and semi-aquatic Heteroptera (Heteroptera: Nepomorpha, Gerromorpha) occurred in Hungary, and the neighbouring countries

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Systematics of the *Rhinella margaritifera* complex (Anura, Bufonidae) from western Ecuador and Panama with insights in the biogeography of *Rhinella alata*

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Abstract

The *Rhinella margaritifera* species group consists of 17 species of toads distributed in tropical and subtropical South America and eastern Central America. The identity of some of its species is poorly understood and there are numerous undescribed cryptic species. Among them, the status of *Rhinella margaritifera* is one of the most problematic. Its range includes lowland rainforests separated by the Andes, the Chocoan rainforest to the west and the Amazonian rainforest to the east. This distribution is puzzling because the Andes are an old and formidable barrier to gene flow and therefore should generate vicariant speciation between disjunct lowland populations. Herein we clarify the taxonomy of populations of the *R. margaritifera* complex from Central America and the Chocó region of South America. The morphological and genetic variation of *R. margaritifera* was examined from 39 populations from Chocó, 24 from the upper Amazon region of Ecuador, and 37 from Panama, including the holotype of the Panamanian *R. alata*. Phylogenetic analyses were performed based on mitochondrial genes 12S rRNA, 16S rRNA, and cytochrome c oxidase I (COI) and the nuclear gene Tyrosinase (Tyr). The genetic and morphological data show that Panamanian and Chocoan populations are conspecific. In the phylogeny, populations from Chocó and Panama form a well-supported clade. The morphology of the holotype of *R. alata* falls within the variation range of Panamanian and Chocoan populations. Based on all this evidence, we assign the populations from western Ecuador and Panama to *R. alata* and demonstrate that the unusual distribution pattern of "*R. margaritifera*" on both sides of the Andes was an artifact of incorrectly defined species boundaries.

Keywords
Andes, Biogeography, Chocó, Morphology, Panama, Phylogeny, *Rhinella alata*

Introduction

Rhinella is a genus of bufonid frogs distributed from southern Texas, through southern Sonora (Mexico), south tropical Mexico, Central America, and South America. There are 87 recognized species of *Rhinella* (Frost, 2014) among which 17 belong to the *R. margaritifera* species group (Lavilla et al. 2013, Moravec et al. 2014). Thirteen of these species are distributed throughout the Amazon Basin, the Guyanas and Central America, while *R. hoogmoedi* Caramaschi & Pombal, 2006 occurs in the Brazilian Atlantic Forest, *R. scitula* (Caramaschi & Niemeyer, 2003) and *R. ocellata* (Günther, 1858) in the Brazilian Cerrado, and *R. paraguayensis* Ávila, Pansonato & Strüssmann, 2010 in the Brazilian Pantanal (Caramaschi and Niemeyer 2003, Caramaschi and Pombal 2006, Lima et al. 2007, Fouquet et al. 2007a, Ávila et al. 2010, Frost 2014). They inhabit the forest floor and their cryptic coloration mimics the forest leaflitter. Morphologically they have been characterized by the presence of hypertrophied supra and postorbital crests, especially in females. Putative synapomorphies for the group are the expansion of the posterior ramus of the pterygoid and nasals that articulate laterally with the preorbital process of the maxilla (Pramuk 2006).

The *R. margaritifera* species group (formerly *Bufo typhonius* or *Bufo margaritifer* group) has one of the most complex histories in the systematics of Neotropical anurans (Hoogmoed 1986, 1989, 1990, Hass et al. 1995, Fouquet et al. 2007b). The boundaries among its species member are poorly understood as a result of a highly variable intraspecific morphology and scant morphological differentiation between some species. In addition, some of the type material is unavailable or poorly preserved and several species descriptions lack details. Despite recent progress in the systematics of the group (i.e. Vélez-Rodriguez 2004, Pramuk 2006, Fouquet et al. 2007b, 2012b, Ávila et al. 2010, Lavilla et al. 2013, Moravec et al. 2014) a number of cryptic species still need to be identified, specially among Amazonian populations (Hoogmoed 1990, Hass et al. 1995, Vélez-Rodríguez 2004, Pramuk 2006, Fouquet et al. 2007b, Lavilla et al. 2013, Moravec et al. 2014).

Two species of the *R. margaritifera* group have been reported west of the Andes (Chocó region, humid forests west of the Andes in Colombia and Ecuador) and in eastern Panama: *R. alata* and *R. margaritifera*. *R. alata* was described by Thominot (1884) as *Bufo alatus*, based on an adult male collected at Obispo, Isthmus of Panama. Boulenger (1885) considered it a junior synonym of "*B. typhonius*", and Hoogmoed (1986, 1989) suggested that it was, possibly, a synonym of *B. acutirostris* (Spix, 1824). La Marca (1997) reported populations of *R. alata* from northern Venezuela. Gorzula and Señaris (1999) suggested that *R. margaritifera* only occurs in southern Venezuela and *R. alata* north of the Orinoco. However, Barrio-Amorós (1999 "1998", 2004) disagreed with both reports and considered that *R. alata* was not distributed in Venezuela.

Rhinella margaritifera was described by Laurenti in 1768. It occurs in eastern Panama (Frost 2014), the Chocoan lowlands of western Ecuador and western Colombia (e.g. Anderson 1945, Miyata 1982, Ruiz-Carranza et al. 1996, Ortega-Andrade et al. 2010, Ortiz et al. 2013, Ron et al. 2014), Amazonia and vicinities in Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela (Lavilla et al. 2013). A genetic study by Fouquet et al. (2007b), using two mitochondrial genes (12S and 16S) and the two nuclear genes (Tyrosinase and 18S), showed that *R. margaritifera* was paraphyletic and contained up to 11 cryptic species. Populations from the Chocó region have been widely referred as *R. margaritifera* although Solis et al. (2010) remarked that populations from the Ecuadorian Chocó might belong to a separate species. Unfortunately, they did not provide further details.

The distribution of *R. margaritifera* in the humid lowlands west and east of the Andes is intriguing because, particularly for amphibians, the Andes represent a formidable barrier to gene flow (e.g. Santos et al. 2009). Despite similar environmental conditions, only four amphibian species are shared between the lowland rainforests of the Amazon basin and the Chocó: *R. margaritifera*, *R. marina*, *Hypsiboas boans* and *Trachycephalus typhonius*. Moreover, there is genetic and morphological evidence suggesting that populations on each side of the Andes of *R. marina* and *Trachycephalus typhonius* represent separate species (Slade and Moritz 1998, Ron and Read 2011). Thus, the distribution of *R. margaritifera* is suggestive of either an unusual biogeographic history or the existence of cryptic species.

Herein, genetic and morphological information were integrated to clarify the taxonomy of the populations of *R. margaritifera* from Panama and the Chocoan region. Populations from the western and eastern Andean slopes were compared to test the role of the Andes as a dispersal barrier in shaping the evolution of the *R. margaritifera* species complex.

Methods

Population sampling

Populations from Panama, the Ecuadorian Chocó, and the Amazon basin were sampled (Figs 1 and 2). Specimens examined morphologically are listed in Appendix 1; specimens analyzed genetically are listed in Table 1.

Morphometric analyses were based on 120 adult specimens of *R. margaritifera* from Panama (14 specimens from 10 populations), Ecuadorian Chocó (74 specimens, 37 populations), and the Ecuadorian Amazon (32 specimens, 18 populations). Qualitative morphological characters were examined in the same specimens and 28 additional individuals from 27 Panamanian populations (Figs 1 and 2; Appendix 1).

Genetic analyses were based on newly generated sequences of *R. margaritifera* from 32 individuals and 19 populations: *R. margaritifera* from the Ecuadorian Chocó (12 individuals, 7 populations); *R. margaritifera* from Panama (3 individuals, 2 popula-

tions) and *R. margaritifera* from the Amazon basin (17 individuals, 10 populations), and six sequences for the outgroups (see Table 1). Sequences of eight *R. dapsilis* were generated, including all available homologous sequences for the *R. margaritifera* species group from GenBank (http://www.ncbi.nlm.nih.gov/genbank; Table 1). *R. marina*, *R. chavin*, *R. nesiotes* and *R. festae* were included as outgroups. The morphometric and genetic analyses were based on the same individuals, when possible. Several specimens used in the morphological analyses lacked tissues and were not included in the genetic analyses. However, their identification was unambiguous based on geographic distribution and morphological characters.

Examined specimens are deposited at the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ, Quito, Ecuador), the American Museum of Natural History (AMNH, New York, USA), Círculo Herpetológico de Panama (CH, Panama, Panama), Centro de Ornitología y Biodiversidad (CORBIDI, Lima, Perú) and Museo de Vertebrados de la Universidad de Panama (MVUP). We also examined photographs of the holotypes of *R. alata* from Musée National d'Historie Naturelle (MNHN, Paris, France). Tissues were obtained from the QCAZ and CH collections. Tissues (liver or thigh muscle) were stored in 95% ethanol.

Morphological analyses

Morphological terminology and abbreviations follow Vélez-Rodriguez (2004) and Narvaes and Rodrigues (2009). Sexual maturity was determined by the presence of nuptial pads in adult males and convoluted oviducts or mature eggs in gravid females. Specimens from the QCAZ collection were euthanized with the anesthetic spray Roxicaine, fixed in 10% formalin, and preserved in 70% ethanol.

The goal of the morphological analyses was to compare three geographic regions: (1) Chocó (2) Panama, and (3) upper Amazon basin. Because the phylogeny showed that Panama and Chocó populations are conspecific, we also compared Chocó + Panama vs. upper Amazon. Morphometric analyses were based on adult and well-preserved specimens (Simmons 2002). We measured the following variables: (1) SVL (snout-vent length, from the tip of snout to the mid-vent); (2) TL (tibia length, from the outer edge of flexed knee to the heel); (3) FL (femur length, from the mid-venter to the outer edge of flexed knee); (4) HL (head length, from the posterior margin of tympanum to the tip of snout); (5) HW (head width, between knobs at angles of jaws, if present); (6) STCH (supratympanic crest height, the distance between the angle of the jaw and the highest point of the ridge above of the tympanum); (7) SOCH (supraorbital crest height, the distance between the angle of jaw and the highest point of the ridge at the mid-orbit); (8) NSD (nostril-snout distance, from the nostril to the tip of the snout); (9) IND (inter-nostril distance, distance between nostrils); (10) TD (tympanum diameter, from the posterior to the anterior edge of the tympanum); (11) FT (foot length, from the posterior edge of the metatarsal tubercle to the tip of the toe IV). Measurements were taken with digital calipers (to the nearest 0.01 mm). Two qualitative mor-

phological characters were also analyzed: (1) vertebral apophyses (present/absent) and (2) bony knob at angle of jaws (present/absent).

Principal Components Analysis (PCA) and Discriminant Function Analysis (DFA) were used to assess morphometric differentiation between Chocó, upper Amazon, and Panama. To remove the effect of body size (SVL), the MANOVA and PCA were applied to the residuals from the linear regressions between the measured variables and SVL, for males and females separately. For the PCA, only components with eigenvalues > 1 were retained. All measurements were first subjected to the Shapiro-Wilk normality to test for normal distribution (Shapiro and Wilk 1965). Data not normally distributed were log-transformed. Levene's test was used to determine if variables were homoscedastic (Levene 1960). Number of analyzed specimens were (1) Chocó: 43 males and 31 females, (2) Panama: 6 males and 8 females, (3) upper Amazon basin: 16 males and 16 females. All analyses were performed using JMP® 9.0.1 (SAS Institute 2010).

DNA extraction, amplification, and sequencing

Total DNA was extracted from muscle or liver tissue preserved in 95% ethanol or tissue storage buffer using standard guanidine thiocyanate protocol (M. Fujita, unpublished) with modifications. Polymerase Chain Reaction (PCR) was used to amplify the mitochondrial genes 12S rRNA, 16S rRNA, cytochrome c oxidase I (COI) and nuclear gene Tyrosinase (Tyr). PCR amplifications were carried out under standard protocols. Using standard primers developed by Bossuyt and Milinkovitch (2000), Goebel et al. (1999), Pauly et al. (2004), and Meyer et al. (2005). Amplicons were sequenced by Macrogen Inc., Seoul, Korea.

Phylogenetic analyses and genetic distances

Preliminary sequence alignment was done with Geneious Pro 5.4.6 (Drummond et al. 2011). The sequence matrix was imported to Mesquite 2.75 (Maddison and Maddison 2011) and the ambiguously aligned regions were adjusted manually to produce a parsimonious alignment. Phylogenetic trees were obtained using Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and Maximum Likelihood (ML) in Garli 2.0 (Zwickl 2006). The best-fit models of sequence evolution were selected under the Akaike information criterion (AIC) and the best partitioning scheme for the combined nucleotide data set and the models of character evolution for the BI and ML were estimated with PartitionFinder 1.0.1 (Lanfear et al. 2012). We ran three analyses: (1) the complete multi-locus data set, (2) only mitochondrial genes, (3) only the nuclear gene.

The Bayesian search consisted of two parallel runs each with 130×10^6 generations with four Markov chains. The convergence of the runs was assessed with Tracer 1.5 (Rambaut and Drummond 2007) evaluating the effective sample sizes and stopping

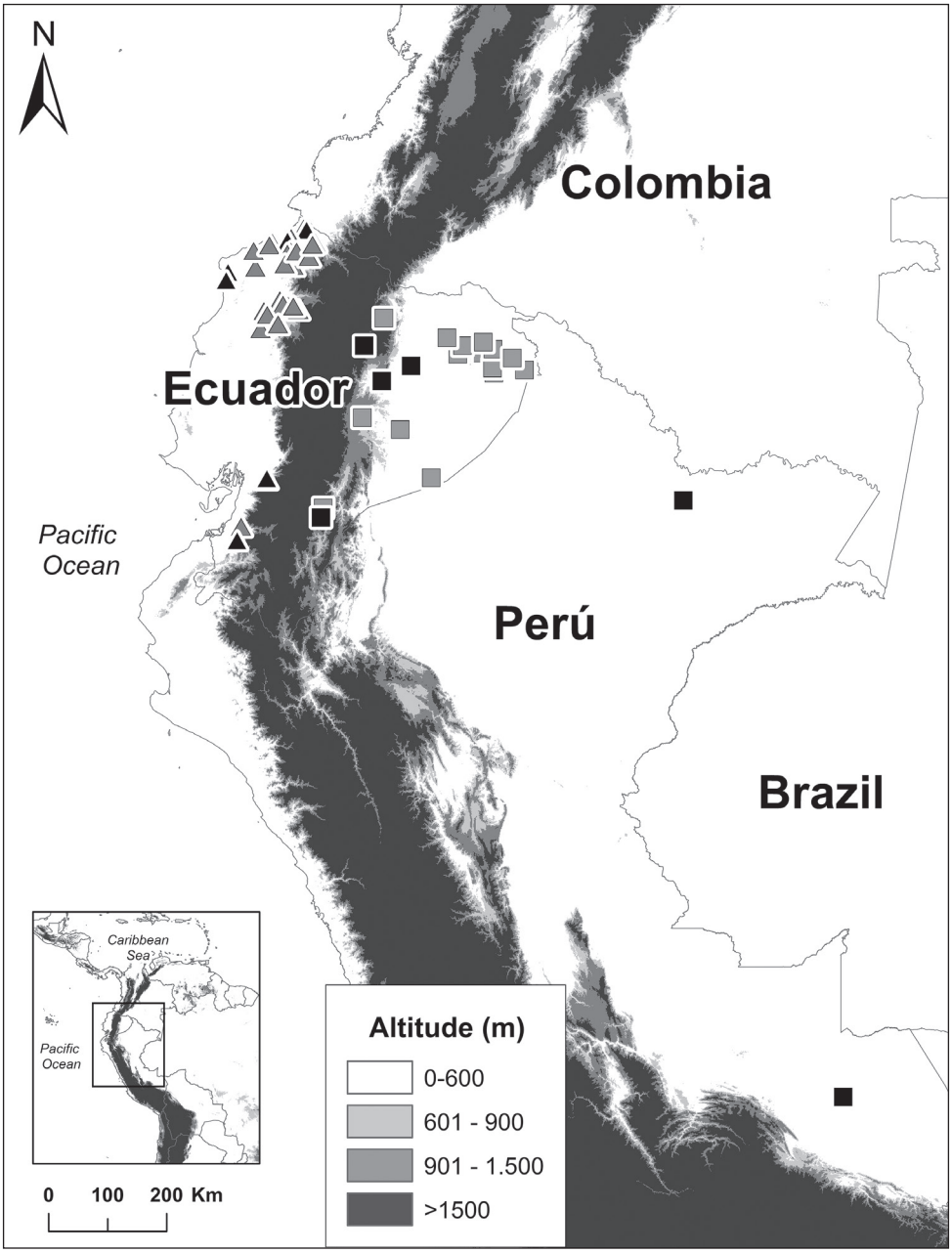

Figure 1. Localities of the *Rhinella margaritifera* group from Chocó (triangles) and Amazon (squares). Gray for specimens analyzed morphologically, black for specimens analyzed both genetically and morphologically. Specimens (listed in Appendix 1 and Table 1) are deposited at the Museo de Zoología of Pontificia Universidad Católica del Ecuador (QCAZ), Centro de Ornitología y Biodiversidad (CORBIDI), and National Museum of Natural History (USNM).

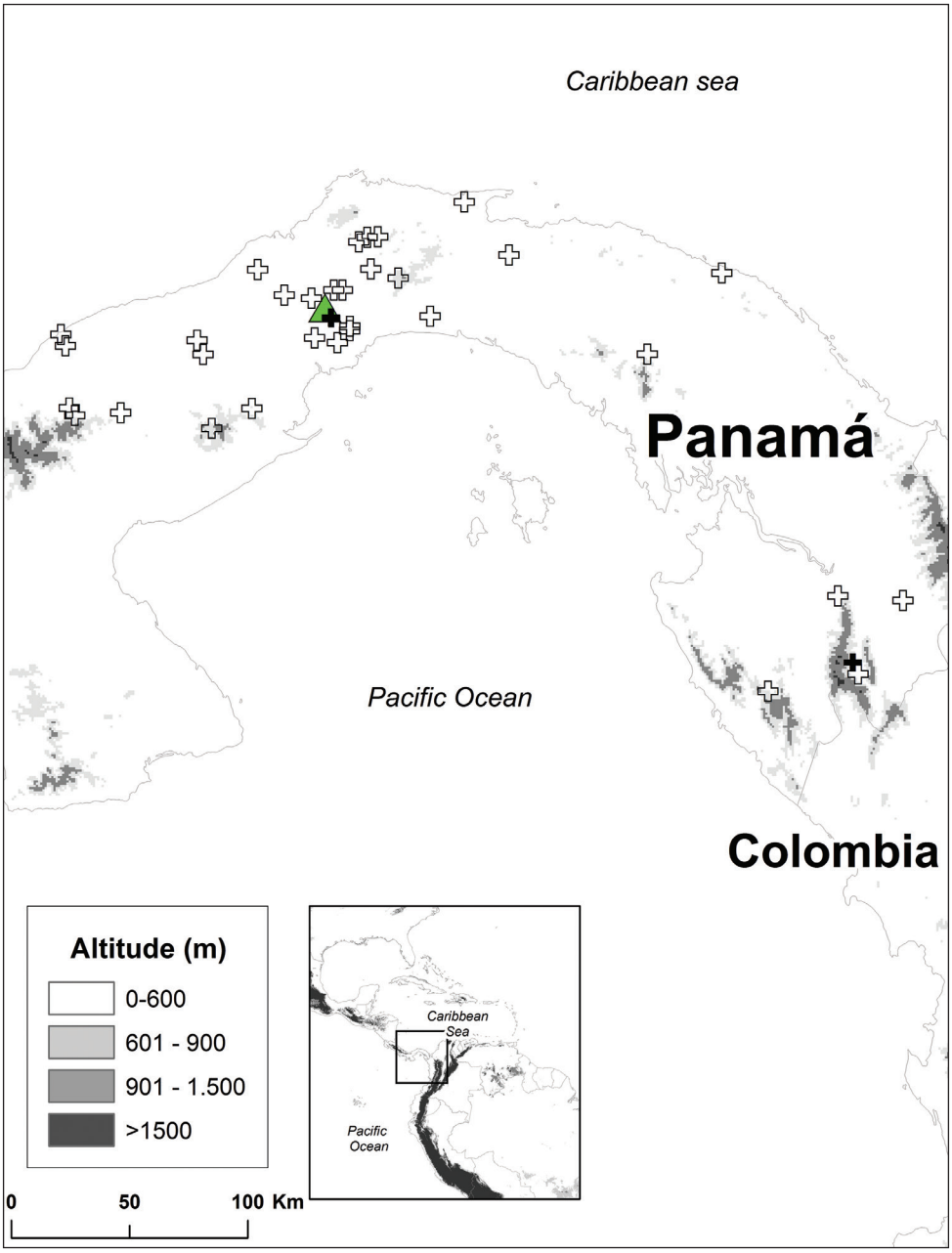

Figure 2. Panamanian populations of the *Rhinella margaritifera* group included in this study. White crosses for specimens analyzed morphologically, black crosses analyzed both morphologically and genetically. The type locality of *R. alata* is shown with a triangle. Specimens (listed in Appendix 1 and Table 1) are deposited at American Museum of Natural History (AMNH), Muséum National d'Histoire Naturelle du Paris (MNHN), Círculo Herpetológico de Panama (CH), and the Museo de Vertebrados de la Universidad de Panama (MVUP).

Table 1. GenBank accession numbers for DNA sequences used in the phylogenetic analysis.

Museum No.	Species	Country	Locality	GenBank Accession No.				Reference
				TYR	16S	12S	COI	
QCAZ10253	*R. alata*	Ecuador	Reserva La Chiquita	KR012523	KR012615	KR012605	KR012568	This study
QCAZ10254	*R. alata*	Ecuador	Reserva La Chiquita	KR012524	KR012616	KR012601	KR012567	This study
QCAZ10255	*R. alata*	Ecuador	Reserva La Chiquita	KR012525	KR012617	KR012602	KR012570	This study
QCAZ11598	*R. alata*	Ecuador	Reserva La Chiquita	KR012526	KR012618	KR012603	KR012550	This study
QCAZ13882	*R. alata*	Ecuador	Manta Real	KR012527	KR012619	KR012597	KR012571	This study
QCAZ13896	*R. alata*	Ecuador	Manta Real	-	DQ158471	DQ158471	-	Pramuk 2006
QCAZ14607	*R. alata*	Ecuador	Borbón	KR012528	KR012620	KR012578	KR012552	This study
QCAZ37244	*R. alata*	Ecuador	Valle Hermoso	KR012539	KR012632	KR012592	KR012576	This study
QCAZ37248	*R. alata*	Ecuador	Valle Hermoso	KR012540	KR012633	KR012595	KR012544	This study
QCAZ 23161	*R. alata*	Ecuador	San Lorenzo	KR012534	KR012626	KR012577	KR012562	This study
QCAZ25023	*R. alata*	Ecuador	La Tortuga	KR012536	KR012629	KR012596	KR012572	This study
QCAZ25025	*R. alata*	Ecuador	La Tortuga	KR012537	KR012630	KR012582	KR012573	This study
QCAZ25032	*R. alata*	Ecuador	La Pedorrera	KR012538	KR012631	KR012604	KR012569	This study
CH9104	*R. alata*	Panama	Cana, Boca Cupé	KR012507	KR012610	KR012598	KR012560	This study
MVUP2299	*R. alata*	Panama	Río Chico Masambí, Parque Nacional Soberanía	KR012511	KR012613	KR012600	KR012561	This study
CH9192	*R. alata*	Panama	Parque Nacional Soberanía	KR012521	KR012611	KR012599	KR012559	This study
QCAZ11597	*R. alata*	Ecuador	Reserva La Chiquita	-	DQ15872	DQ15872	-	Pramuk 2006
104MC	*R. castaneotica*	French Guyana	Tibourou	EF364355	EF364289	EF364263	-	Fouquet et al. 2007b
110PG	*R. castaneotica*	French Guyana	Moint Saint Marcel	EF364353	EF364285	EF364259	-	Fouquet et al. 2007b
QCAZ38477	*R. dapsilis*	Ecuador	Villano B	KR012513	KR012634	KR012586	KR012554	This study
QCAZ38512	*R. dapsilis*	Ecuador	Villano BII	KR012514	KR012635	KR012587	KR012558	This study
QCAZ38560	*R. dapsilis*	Ecuador	Villano B	KR012515	KR012636	KR012588	KR012555	This study
QCAZ38621	*R. dapsilis*	Ecuador	Villano K4	KR012516	KR012637	KR012606	KR012556	This study
QCAZ38688	*R. dapsilis*	Ecuador	Villano K4	KR012517	KR012638	KR012607	KR012575	This study
QCAZ38755	*R. dapsilis*	Ecuador	Villano BII	KR012518	KR012639	KR012589	KR012548	This study
QCAZ38892	*R. dapsilis*	Ecuador	Comunidad Kutintza 2	KR012519	KR012640	KR012608	KR012566	This study

Museum No.	Species	Country	Locality	GenBank Accession No.				Reference
				TYR	16S	12S	COI	
QCAZ38998	*R. dapsilis*	Ecuador	Comunidad Kurintza 3	KR012520	KR012641	KR012590	KR012549	This study
MTR19199	*R. hoogmoedi*	Brazil	Bahia, Camacan	-	JN867571	JN867545	-	Fouquet et al. 2012a
112BM	*R. lescurei*	French Guyana	Litany	EF364343	EF217473	EF364279	-	Fouquet et al. 2007b
3027T	*R. lescurei*	French Guyana	Mitaraka	JN692065	EF364305	EF364279	-	Fouquet et al. 2012b
108MC	*R. margaritifera*	French Guyana	Kaw	EF364333	EF364292	EF364266	-	Fouquet et al. 2007b
136MC	*R. margaritifera*	French Guyana	Crique Margot	EF364335	EF364292	EF364266	-	Fouquet et al. 2007b
389MC	*R. margaritifera*	French Guyana	Camp Canopé	JN692029	-	-	-	Fouquet et al. 2007b
374MC	*R. margaritifera*	French Guyana	Régina	JN692038	JN691389	JN690782	-	Fouquet et al. 2007b
390MC	*R. margaritifera*	French Guyana	St Georges	JN692037	JN691388	JN690781	-	Fouquet et al. 2007b
2559T	*R. margaritifera*	French Guyana	Pic Matecho	JN690780	JN691387	JN690780	-	Fouquet et al. 2012b
4482T	*R. margaritifera*	French Guyana	Angoulème	JN692042	JN691379	JN690772	-	Fouquet et al. 2012b
163BM	*R. margaritifera*	French Guayana	Guatemala	EF364320	EF364292	EF364266	-	Fouquet et al. 2007b
164BM	*R. margaritifera*	French Guyana	Montagne des Singes	EF364321	EF364292	EF364266	-	Fouquet et al. 2007b
176BM	*R. margaritifera*	French Guyana	Crique Grand Leblond	EF364323	EF364292	EF364266	-	Fouquet et al. 2007b
195MC	*R. margaritifera*	French Guyana	Kaw	EF364325	EF364292	EF364266	-	Fouquet et al. 2007b
2034AT	*R. margaritifera*	French Guyana	Nouragues	JN692033	EF364292	EF364266	-	Fouquet et al. 2007b
204MC	*R. margaritifera*	French Guyana	Saul	EF364328	EF364295	EF364269	-	Fouquet et al. 2007b
217MC	*R. margaritifera*	French Guyana	Grant Santi	EF364329	EF364299	EF364273	-	Fouquet et al. 2007b
225MC	*R. margaritifera*	French Guyana	Road St. Elie	EF364330	EF364292	EF364266	-	Fouquet et al. 2007b
284MC	*R. margaritifera*	French Guyana	St Elie	EF364336	EF364292	EF364266	-	Fouquet et al. 2007b
288AG	*R. margaritifera*	French Guyana	St Georges	JN692021	JN691380	JN690773	-	Fouquet et al. 2012b
294MC	*R. margaritifera*	French Guyana	Camp Canope	JN692029	EF364292	EF364266	-	Fouquet et al. 2012b
2BM	*R. margaritifera*	French Guyana	Cisame	EF364313	EF364293	EF364267	-	Fouquet et al. 2007b
307PG	*R. margaritifera*	French Guyana	Lac Toponowini	JN692022	EF364292	EF364266	-	Fouquet et al. 2012b
361MC	*R. margaritifera*	French Guyana	Lucifer	JN692031	EF364292	EF364266	-	Fouquet et al. 2012b
408PG	*R. margaritifera*	French Guyana	Mont Kotika	JN692023	EF364292	EF364266	-	Fouquet et al. 2012b
66MC	*R. margaritifera*	French Guyana	Monts Bakra	EF364334	EF364298	EF364272	-	Fouquet et al. 2007b
74AF	*R. margaritifera*	French Guyana	St Georges	JN692020	EF364266	EF364292	-	Fouquet et al. 2012b

Museum No.	Species	Country	Locality	GenBank Accession No.				Reference
				TYR	16S	12S	COI	
92BM	*R. margaritifera*	French Guyana	Cisame	EF364314	EF364301	EF364275	-	Fouquet et al. 2007b
KU215143	*R. margaritifera*	Peru	Madre de Dios	-	AY819461	AY819331	-	Wiens et al. 2005
13872MTR	*R. margaritifera*	Brazil	Amapá, Lourenço	JN692016	JN691390	JN690783	-	Fouquet et al. 2012b
13873MTR	*R. margaritifera*	Brazil	Amapá, Lourenço	JN692017	JN691391	JN690784	-	Fouquet et al. 2012b
13874MTR	*R. margaritifera*	Brazil	Amapá, Lourenço	JN692018	JN691393	JN690786	-	Fouquet et al. 2012b
13878MTR	*R. margaritifera*	Brazil	Amapá, Lourenço	JN692019	JN691392	JN690785	-	Fouquet et al. 2012b
MRT6313	*R. margaritifera*	Brazil	Pará, Serra do Kukoinhokren	JN692075	JN691394	JN690787	-	Fouquet et al. 2012b
MRT6317	*R. margaritifera*	Brazil	Pará, Serra do Kukoinhokren	JN692076	JN691395	JN690788	-	Fouquet et al. 2012b
KU215146	*R. margaritifera*	Peru	Madre de Dios	-	-	HM563858	JN867978	Mendelson et al. 2011
CORBIDI5840	*R. margaritifera*	Peru	Curupa	KR012522	KR012612	KR012594	KR012564	This study
USNM268828	*R. margaritifera*	Peru	Madre de Dios	-	DQ158490	DQ158490	-	Pramuk 2006
KU215145	*R.* cf. *margaritifera*	Peru	Madre de Dios	-	DQ158491	DQ158491	-	Pramuk 2006
ZUEC-DCC3393	*R.* cf. *margaritifera*	Brazil	Rio de Janeiro, Santo Aleixo	-	-	AY680262	-	Pauly et al. 2004
QCAZ17775	*R. margaritifera*	Ecuador	244 km of Indanza	KR012529	KR012621	KR012581	KR012551	This study
QCAZ17989	*R. margaritifera*	Ecuador	Estación Biológica JatunSacha	KR012530	KR012622	-	KR012565	This study
QCAZ17990	*R. margaritifera*	Ecuador	Estación Biológica JatunSacha	KR012531	KR012623	KR012593	KR012557	This study
QCAZ17991	*R. margaritifera*	Ecuador	Estación Biológica JatunSacha	KR012532	KR012614	-	KR012543	This study
QCAZ23632	*R. margaritifera*	Ecuador	7Km North of Cosanga	KR012535	KR012627	KR012583	KR012542	This study
QCAZ23917	*R. margaritifera*	Ecuador	Gualaquiza-El Ideal	KR012512	KR012628	KR012591	KR012547	This study
QCAZ10601	*R. margaritifera*	Ecuador	Parque Nacional Yasuní	-	DQ15870	DQ15870	-	Pramuk 2006
QCAZ18241	*R. margaritifera*	Ecuador	Shaime	KR012533	KR012625	KR012585	KR012553	This study
10226MSH	*R. margaritifera*	Brazil	Amazonas, Anavilhanas	JN692056	JN691364	JN690757	-	Fouquet et al. 2012b
10339MSH	*R. margaritifera*	Brazil	Amazonas, Anavilhanas	JN692057	JN601365	JN69058	-	Fouquet et al. 2012b
QCAZ42269	*R. margaritifera*	Ecuador	Reserva Yachana	KR012541	KR012642	KR012584	KR012563	This study
111AF	*R. martyi*	French Guyana	Brownsberg	JN692045	EF364303	EF364277	-	Fouquet et al. 2007b
156MC	*R. martyi*	French Guyana	Trijonction	EF364337	EF364303	EF364277	-	Fouquet et al. 2007b
LAJ210	*R. ocellata*	Brazil	Tocantins, Lajeado	-	JN867572	JN867546	-	Fouquet et al. 2012a
MZUSP103261	*R. ocellata*	Brazil	Tocantins, Peixe	-	DQ158479	DQ158479	-	Pramuk 2006
SMF88237	*R.* cf. *paraguayensis*	Bolivia	-	-	JF790186	-	-	Jansen et al. 2011

Museum No.	Species	Country	Locality	GenBank Accession No.				Reference
				TYR	16S	12S	COI	
MNKA9691	*R.* cf. *paraguayensis*	Bolivia	-	-	JF790185	-	-	Jansen et al. 2011
ESTR00173	*Rhinella* sp.	Brazil	Amazonas, Carolina	-	JN867574	JN867548	-	Fouquet et al. 2012a
AF7275337	*Rhinella* sp.	Brazil	Mato Grosso, APM Manso	-	JN867575	JN867549	-	Fouquet et al. 2012a
Outgroup								
QCAZ50698	*Rhinella marina*	Ecuador	Puerto Cayo	KR012508	KR012643	KR012579	KR012545	This study
QCAZ50702	*Rhinella marina*	Ecuador	San Andrés de Rocafuerte	KR012509	KR012644	KR012580	KR012546	This study
QCAZ18203	*Rhinella festae*	Ecuador	Estación Biológica Jatun Sacha	KR012510	KR012624	KR012609	KR012574	This study
KU217501	*Rhinella festae*	Ecuador	Pastaza	-	DQ158423	DQ158423	-	Pramuk 2006
MTD43789	*Rhinella chavin*	Peru	Palma Pampa	-	DQ158441	DQ158441	-	Pramuk 2006
UTA53310	*Rhinella nesiotes*	Bolivia	La Paz	-	DQ158478	DQ158478	-	Pramuk 2006

when all post burn-in values were greater than 200. The first 10% of the sample was discarded as burn-in (Castañeda and Queiroz 2011).

For the ML analysis, we carried out 20 replicate searches and increased the setting "genthreshfortopoterm" until all searches resulted in similar likelihood values, indicating an efficient search (Zwickl 2006; final value was 200,000). Ten replicate searches started from stepwise trees and ten from random trees. The setting "limsprrange" was set to 10 (default = 6). Node support was assessed with non-parametric bootstrapping (Felsenstein 1983) with 100 pseudoreplicates with the same settings of the stepwise full search but with a single replicate per search. The 50% majority rule consensus for the bootstrap trees was obtained with Mesquite 2.75 (Maddison and Maddison 2011).

Uncorrected pairwise (*p*) genetic distances were obtained for gene *16S* using software Mesquite 2.75 (Maddison and Maddison 2011). Missing and ambiguous sites were excluded. Genetic distances comparisons were based on gene *16S* because it has been widely used as a barcode standard in amphibians (e.g. Vences et al. 2005). We assumed that genetic distances > 3% are suggestive of interspecific differentiation (Fouquet et al. 2007c). Genetic distances thresholds are problematic because they can lead to both false negatives and false positives in species identifications (Collins and Cruickshank 2013). We used the threshold only as a working hypothesis that was tested with morphological comparisons.

Results

Phylogenetic analyses

The complete matrix contained up to four genes and 3045 bp for 92 samples. For the complete data set, PartitionFinder chose seven partitions as the best strategy (best model in parenthesis): 12S (GTR + I + G), 16S (GTR + I + G), COI 1st position (TIMef + G), COI 2nd position (TVM + I + G), COI 3rd position (TrN + G), Tyr 1st and 2nd position (TrN + G), Tyr, 3rd position (TrN + I + G). For the mitochondrial analyses, the same five partitions were chosen, one for each ribosomal RNA gene and each codon position in COI. For the nuclear analysis, two partitions were chosen: Tyr, 1st and 2nd position and Tyr, 3rd position.

The tree topologies for the Maximum likelihood and Bayesian phylogenies were similar except for weakly supported nodes (posterior probability < 0.95 and bootstrap < 75). The Maximum Likelihood tree (Fig. 3) shows a basal divergence of *R. castaneotica*, which is sister to two clades containing the remaining species of the *R. margaritifera* species group. One clade is strongly supported in the Bayesian consensus (posterior probability = 1) although it has low bootstrap support (= 63). It contains three groups: Panama (posterior probability = 1.0, bootstrap = 100), Chocó (posterior probability = 1.0, bootstrap = 86) and upper Amazon (posterior probability = 1.0, bootstrap = 68). Chocó and Panama form clade sister to the upper Amazon clade. Both clades, which are on opposite sides of the Andes, are separated by pairwise genetic distances (uncor-

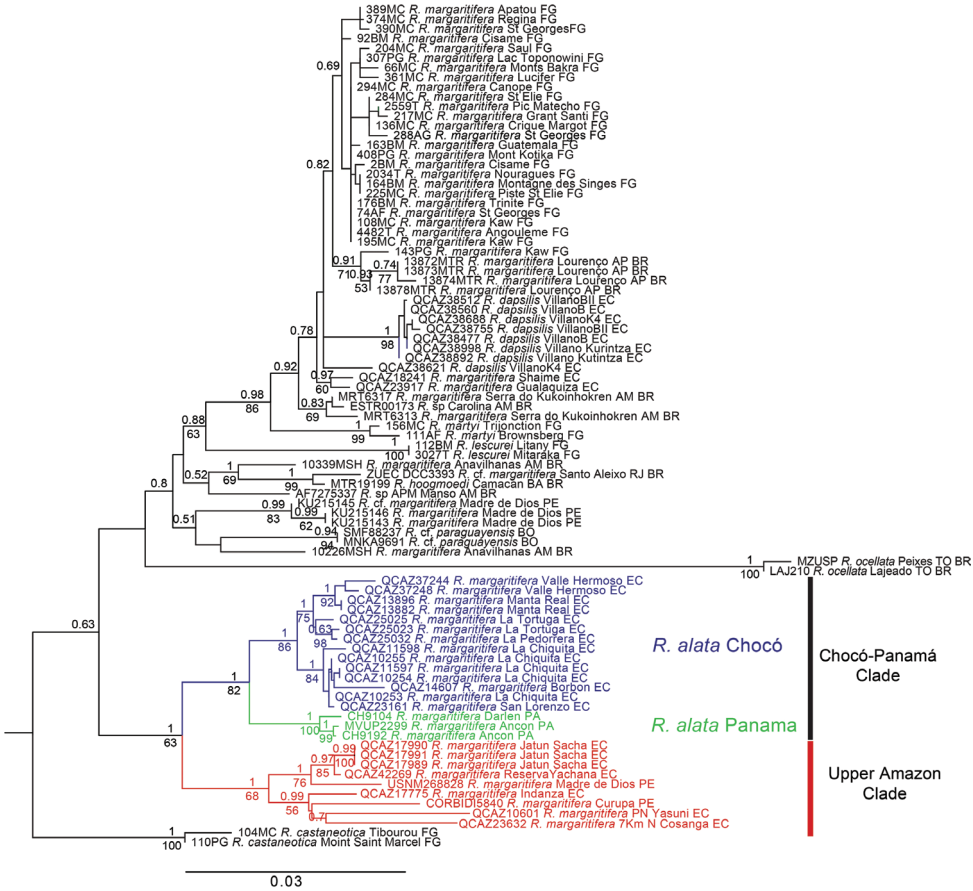

Figure 3. Maximum Likelihood phylogram depicting relationships within the *Rhinella margaritifera* species group. The phylogram was derived from the analysis of 3045 bp of mitochondrial (*12S*, *16S*, *COI*) and nuclear (*Tyr*) genes. Numeric codes on terminals are individual collection numbers (associated data listed in Table 1). Posterior probabilities (above) and bootstrap values (below) are shown on branches except when they are < 0.50 and 50%, respectively. Abbreviations are: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.

rected *p* for the mitochondrial gene *16S*) ranging from 3.01 to 5.5% (average = 4.28, SD = 0.56). The genetic distances and the morphological differences (see next section) between the Chocó-Panama clade and the upper Amazon clade suggest that they are separate species. The *16S* genetic distances between the Chocó and Panama clades range from 1.26 to 1.99% (average = 1.63, SD = 0.19). The relatively low genetic distances and the lack of morphological differences between their populations (see next section) indicate that they are conspecific. The Chocó populations further segregate latitudinally in two well-supported clades. One includes the populations in northern Ecuador (e.g. Reserva La Chiquita and Borbón) while the other includes central and southern populations (e.g. Manta Real and Valle Hermoso, Fig. 3).

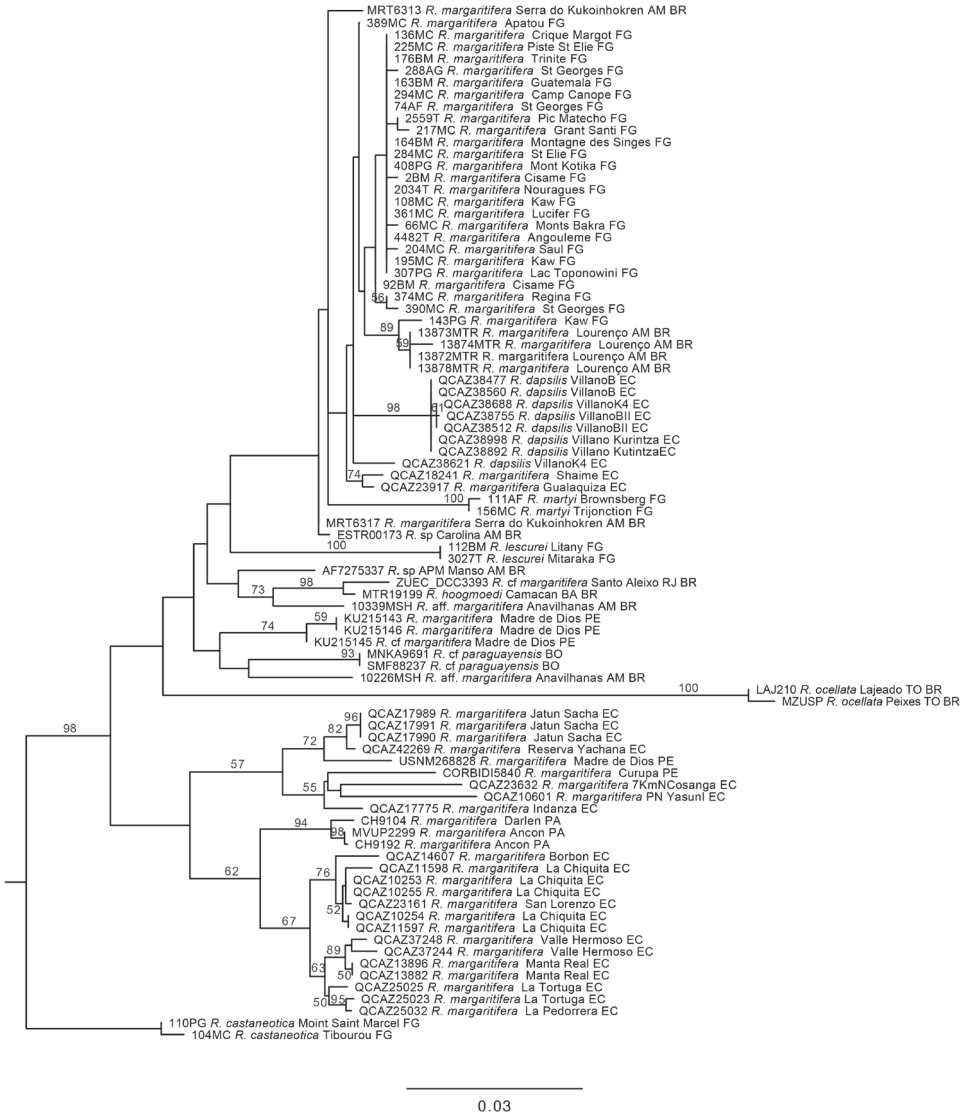

Figure 4. Maximum Likelihood phylogram depicting relationships within the *Rhinella margaritifera* species group. The phylogram was derived from the analysis of 2495 bp of mitochondrial gene fragments (*12S*, *16S*, *COI*). Numeric codes on terminals are individual collection numbers (associated data listed in Table 1). Bootstrap values appear above branches. The branches without numbers have bootstrap values < 50%. Abbreviations: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.

The sister clade to Chocó-Panama + Upper Amazon has weak support and includes other members of the *R. margaritifera* group (*R. dapsilis*, *R. hoogmoedi*, *R. lescurei*, *R. martyi*, *R. ocellata*, *R. paraguayensis* and "*R. margaritifera*") from the Guiana region and

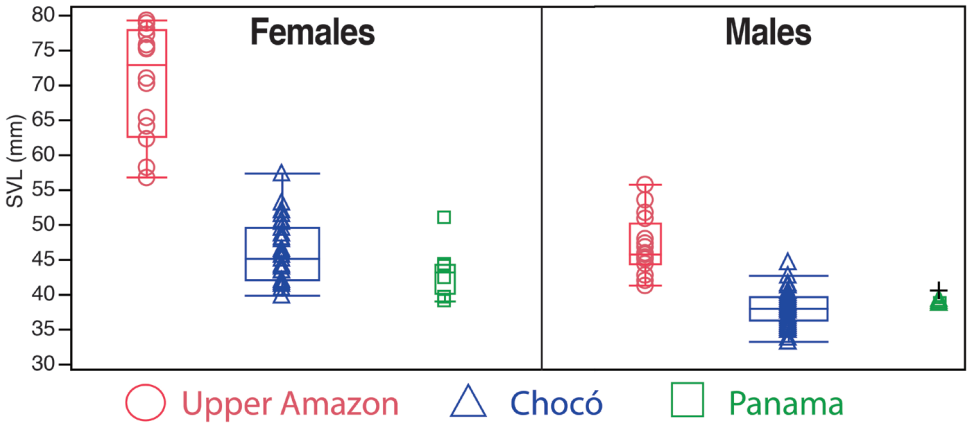

Figure 5. Box and whisker plots showing snout-vent length variation in adult *Rhinella margaritifera* (upper Amazon) and *R. alata* (Chocó and Panama). The central bar indicates the median, the interquartile range is shown by the box length, and the range is shown by the short horizontal lines (whiskers). **SVL** = snout-vent length. The black cross is the holotype of *R. alata*.

Amazonian Brazil, Ecuador and Peru. Relationships among them are weakly supported on most branches.

The Maximum Likelihood tree based on mitochondrial genes (Fig. 4) has similar topology to the Maximum Likelihood tree derived from the analysis of the complete data set (Fig. 3). The Bayesian consensus tree, derived from the Tyrosinase gene, has definitely lower resolution (Appendix 2).

Morphological analyses

Morphometric comparisons. Morphometric data from adults are summarized in Table 2. In the examined series, Amazonian males and females were significant larger than their counterparts from Chocó (Fig. 5; males Student's t = -10.32, DF = 57 $p < 0.001$; females t = -13.12, DF = 45, $p < 0.001$) and Panama (males t = -8.7, DF = 22, $p < 0.001$; females t = -4.43, DF = 20, $p < 0.001$). There are no significant differences in SVL between Chocoan and Panamanian populations (males t = 1.37, DF = 47, p = 0.91; females t = -1.58, DF = 37, p = 0.06).

Significant differences were observed in relative crest size between the Chocó-Panama and upper Amazon clades (Fig. 6). In the former, female supratympanic crest height had a range between 51.6 to 63.5% of head length (n = 39); in the later, range was 68.6 to 95.5% (n = 16). Ranges did not overlap and differences were significant (Wilcoxon's Z = –5.77, $p < 0.001$). Male supratympanic crest height had a range between 49.3 to 59.8% of head length in Chocó-Panama (n = 49); in upper Amazon, range was 50.6 to 78.4% of head length (n = 16). Ranges overlapped but differences were significant (Wilcoxon's Z = 3.11, p = 0.0018).

Table 2. Descriptive statistics for morphometric measurements of adults from *Rhinella margaritifera* from Amazonian Ecuador and *R. alata* from Chocó and Panama. Mean ± SD is given, with the range below. Abbreviations are: **SVL** = Snout-Vent Length; **TL** = Tibia Length; **FL** = Femur Length; **HL**= Head Length; **HW** = Head Width; **STCH** = Supratympanic Crest Height; **SOCH** = Supraorbital Crest Height; **NSD** = Nostril-Snout Distance; **IND** = Inter-Nostril Distance; **TD** = Tympanum Diameter; **FT** = Foot Length. All measurements are in mm.

	R. margaritifera		***R. alata***					
	Amazon		**Chocó**		**Panamá**		**combined**	
Morphometric measurements	**Males (*n* = 16)**	**Females (*n* = 16)**	**Males (*n* = 43)**	**Females (*n* = 31)**	**Males (*n* = 6)**	**Females (*n* = 8)**	**Males (*n* = 49)**	**Females (*n* = 39)**
SVL	45.6 ± 4.11 (54.36–39.88)	68.90 ± 8.26 (77.97–55.42)	36.66 ± 2.42 (43.25 –31.84)	44.82 ± 4.42 (56.19–38.55)	38.03 ± 0.59 (39.20–37.54)	42.38 ± 3.82 (49.69–37.78)	36.83 ± 2.31 (43.25–31.84)	44.27 ± 4.37 (56.19–37.78)
TL	18.73 ± 1.97 (23.13–15.14)	29.36 ± 2.97 (34.26–24.01)	15.98 ± 1.14 (18.72–13.69)	18.26 ± 1.24 (20.73–16.22)	15.86 ± 1.16 (18.12–15.09)	17.79 ± 0.75 (18.99–16.41)	15.97 ± 1.13 (18.72– 13.69)	18.17 ± 1.16 (20.73–16.22)
FL	19.67 ± 1.97 (23.84–16.15)	29.33 ± 3.67 (35.34–22.75)	15.69 ± 1.34 (19.28–13.09)	18.16 ± 1.72 (22.04–15.18)	16.39 ± 0.37 (17.01 –16.03)	17.46 ± 0.67 (18.72–16.72)	15.77 ± 1.27 (19.28–13.09)	18.02 ± 1.58 (22.04–15.18)
HW	16.9 ± 1.59 (19.93–14.77)	25.88 ± 2.73 (30.69–21.01)	12.57 ± 0.95 (15.14–10.31)	15.10 ± 1.6 (18.94–12.49)	12.98 ± 0.17 (13.3–12.8)	14.90 ± 1.12 (17.23–13.79)	12.63 ± 0.91 (15.14–10.31)	15.06 ± 1.50 (18.94–12.49)
HL	14.6 ± 1.28 (17.44–13.27)	22.27 ± 2.71 26.51–17.94)	11.61 ± 0.8 (13.88–10.29)	13.67 ± 1.19 (16.84–11.85)	11.85 ± 0.21 (12.2–11.54)	13.18 ± 1.12 (15.45–11.77)	11.64 ± 0.76 (13.88–10.29)	13.57 ± 1.17 (16.84–11.77)
SOCH	9.46 ± 0.86 (11.13–8.19)	15.43 ± 2.02 (18.33–12.06)	7.71 ± 0.59 (8.87–6.45)	9.28 ± 0.86 (11.40–7.77)	8.39 ± 0.21 (8.67–8.2)	9.13 ± 0.49 (9.87–8.53)	7.79 ± 0.59 (8.87–6.45)	9.25 ± 0.79 (11.4–7.77)
STCH	8.78 ± 1.55 (12.27–6.78)	17.73 ± 3.26 (22.7–12.35)	6.27 ± 0.54 (7.97–5.36)	7.96 ± 0.68 (9.71–6.63)	6.59 ± 0.31 (6.99–6.31)	7.38 ± 0.39 (8.01–6.99)	6.31 ± 0.52 (7.97–5.36)	7.84 ± 0.67 (9.71–6.63)
NSD	2.08 ± 0.44 (2.64–1.41)	2.45 ± 0.42 (3.37–1.79)	1.63 ± 0.29 (2.23–1.05)	1.70 ± 0.21 (2.08–1.25)	1.47 ± 0.17 (1.60–1.18)	1.66 ± 0.16 (1.89–1.35)	1.61 ± 0.28 (2.23–1.05)	1.69 ± 0.20 (2.08–1.25)
IND	3.35 ± 0.35 (3.89–2.70)	3.12 ± 0.37 (3.73–2.59)	2.50 ± 0.33 (3.23–1.86)	2.80 ± 0.43 (3.98–2.16)	2.41 ± 0.11 (2.59–2.31)	2.42 ± 0.23 (2.63–2.08)	2.48 ± 0.32 (3.23–1.86)	2.72 ± 0.43 (3.98–2.08)
TD	3.48 ± 0.24 (3.93–3.18)	4.14 ± 0.21 (4.48–3.65)	3.34 ± 0.47 (4.03–1.95)	3.46 ± 0.59 (4.45–2.5)	3.38 ± 0.20 (3.60–3.13)	3.79 ± 0.25 (4.05–3.31)	3.33 ± 0.45 (4.03–1.95)	3.52 ± 0.55 (4.45–2.5)
FT	16.87 ± 2.145 (21.85–13.76)	24.87 ± 3.64 (28.86 –19.13)	13.46 ± 1.12 (15.88–11.43)	15.33 ± 1.52 (19.40–13.15)	13.72 ± 0.68 (14.70–12.82)	14.96 ± 0.76 (16.54–14.39)	13.48 ± 1.07 (15.88–11.43)	15.25 ± 1.39 (19.4–13.15)

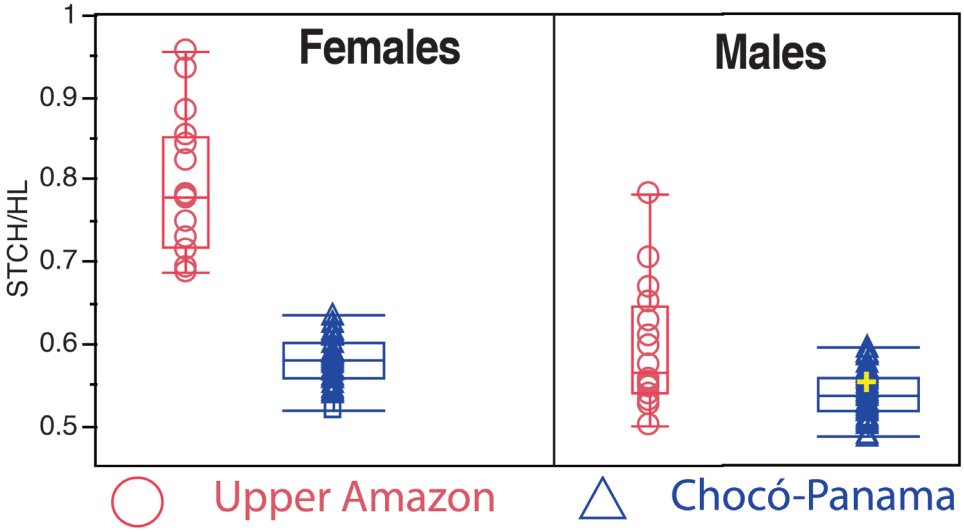

Figure 6. Box and whisker plots showing relative size of supratympanic crests for adult *Rhinella margaritifera* (upper Amazon) and *R. alata* (Chocó-Panama). The central bar indicates the median, the interquartile range is shown by the box length, and the range is shown by the short horizontal lines (whiskers). **STCH** = supratympanic crest height, **HL** = head length. The yellow cross is the holotype of *R. alata*.

Three components with eigenvalues > 1.0 were extracted from the PCA for females (Table 3). The three components accounted for 67.3% of the total variation. The highest loadings of the PCA for females were supratympanic and supraorbital crest height, and tibia length for PC I, inter-nostril distance and tympanum diameter for PC II, and nostril-snout distance and inter-nostril distance for PC III. Three components with eigenvalues > 1.0 were extracted from the PCA in males (Table 3). The three components accounted for 63.3% of the total variation. The highest loadings for the PCA for males were head length and head width for PC I, inter-nostril distance and tympanum diameter for PC II, and tibia length and foot length PC III. The morphometric space of the Chocoan, upper Amazon, and Panamanian populations broadly overlaps in both males and females (Fig. 7).

In the DFA classification for females, 51 out of 55 females were assigned correctly to their geographic region. The four misclassified females from Ecuadorian Chocó were assigned to Panamanian populations. All specimens from the upper Amazon were correctly classified. In the DFA for males, 56 out of 65 males were correctly classified. The eight misclassified males from Ecuadorian Chocó were assigned to Panamanian populations and only one from upper Amazon to Panamanian populations. All males and females from Panama were correctly classified. The DFA analyses indicate that populations from the Ecuadorian Chocó are morphometrically very similar with those from Panama, both groups being markedly different from *R. margaritifera* from the upper Amazon.

Finally, evidence of sexual dimorphism was found in relative crest size: females have larger cephalic crests than males (Fig. 6). The ratio supratympanic crest height/

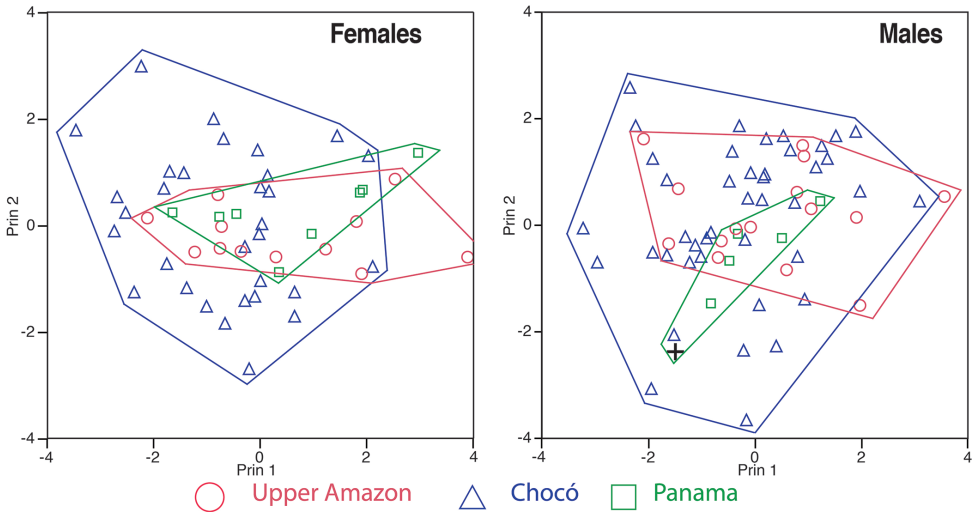

Figure 7. Principal components extracted from the analysis of ten size-corrected morphological variables of adult *Rhinella margaritifera* (upper Amazon) and *R. alata* (Chocó and Panama). The black cross is the holotype of *R. alata*. See Table 3 for character loadings on each component.

Table 3. Character loadings and eigenvalues for Principal Components (PC) Analysis. The analysis was based on ten size-corrected morphometric variables measured in Amazonian, Chocoan and Panamanian populations of the *R. margaritifera* species group. Abbreviations are: **TL** = Tibia Length; **FL** = Femur Length; **HL** = Head Length; **HW** = Head Width; **STCH** = Supratympanic Crest Height; **SOCH** = Supraorbital Crest Height; **NSD** = Nostril-Snout Distance; **IND** = Inter-Nostril Distance; **TD** = Tympanum Diameter; **FT** = Foot Length. Bold figures indicate highest loadings.

Variable	PCA Females			PCA Males		
	PC I	PC II	PC III	PC I	PC II	PC III
FL	0.330	0.165	0.167	0.272	0.159	0.322
FT	0.334	0.214	0.418	0.061	-0.038	**0.661**
HL	0.350	-0.065	0.153	**0.448**	-0.268	-0.078
HW	0.343	0.132	-0.288	**0.446**	-0.222	-0.045
IND	-0.203	**0.381**	**0.512**	0.280	**0.502**	-0.142
NSD	0.217	0.155	**-0.580**	0.262	0.386	-0.186
SOCH	**0.368**	-0.067	0.190	0.423	-0.071	-0.082
STCH	**0.411**	-0.154	-0.039	0.409	-0.290	-0.045
TD	0.071	**0.817**	-0.159	0.099	**0.557**	-0.128
TL	**0.368**	-0.200	0.232	0.134	0.228	**0.610**
Eigenvalue	4.411	1.192	1.128	2.800	1.947	1.585
Cumulative variance (%)	44.11	56.03	67.31	28.00	47.47	63.32

head length (STCH/HL) was significantly different between males and females in the Chocó-Panama clade (Wilcoxon's $Z = 5.15$, $p < 0.001$) and the upper Amazon clade (Wilcoxon's $Z = -4.35$, $p < 0.001$).

Qualitative morphological characters

The upper Amazon clade differs from the Chocó-Panama clade in having protruding vertebral apophyses in the dorsum and bony knobs at angle of jaws (both absent in the Chocó-Panama clade; Figs 8–10). The Chocó-Panama clade differs from other species of the *R. margaritifera* group by a combination of an absence of vertebral apophyses, an absence of bony knob at angle of jaws, low cranial crests, and the tympanum rounded or ovoid (see *Systematic account* section). A large number of specimens were examined (see *Populations sampling* section) and all conform to this characterization. Thus, it seems unlikely that there are additional species of the group in the Chocoan and Panamanian regions.

The holotype of *R. alata* (Thominot, 1884) (Fig. 11) is an adult male with an SVL of 39.2 mm. It has poorly developed supratympanic crests and lacks bony knobs at the angle of jaws. The vertebral apophyses are inconspicuous. These characters and the location of its type locality (within 6 km of one of our examined populations) lead us to conclude that it is conspecific with the Panamanian and Chocoan populations examined herein.

Systematic account of *Rhinella alata*

Rhinella alata (Thominot, 1884)

Bufo alatus Thominot, 1884. Holotype: MNHN 84285, adult male from Obispo, Panama.

Diagnosis. *Rhinella alata* is a small-sized (Table 2; Figs 8 and 9) species of *Rhinella* having the following combination of characters: (1) average SVL of females 44.25 mm (SD = 4.36, *n* = 39), males 36.83 mm (SD = 2.31, *n* = 49); (2) bony knob at angle of jaws absent, corner of mouth angular; (3) supraorbital crests low and thick, continuous with preorbital crests; usually with crenulate texture on vertical surfaces; (4) supratympanic crests concave and small; their posterior edge usually next to the anterior border of parotoid glands; (5) *canthus rostralis* present but inconspicuous, sometimes continuous with preorbital crests; (6) parietal crests usually present, ill-defined; (7) heel reaching posterior margin of eye when hindlimbs adpressed; (8) vertebral apophyses no protruding; (9) snout subacuminate in dorsal view, from rounded to protruding in profile; (10) skin on dorsum bearing a mixture of warts, pustules, and minute tubercles; (11) mid-dorsal line from snout to vent often present; (12) spiculate tubercles on external border of shank, evident especially on females; (13) dorsolateral row of sharply pointed, conical tubercles between posterior border of parotoid glands and groin; (14) tympanic membrane and tympanic annulus distinct; moderately large, ovoid to round; (15) parotoid glands small, elongated posteriorly; (16) upper eyelid warty; (17) tarsal fold absent; (18) digits slender and long, with small knobs at tip; lateral fringes present; finger lengths 3 > 4 > 2 > 1; toe lengths 4 > 5 > 3 > 2 > 1; (19) nuptial pads present.

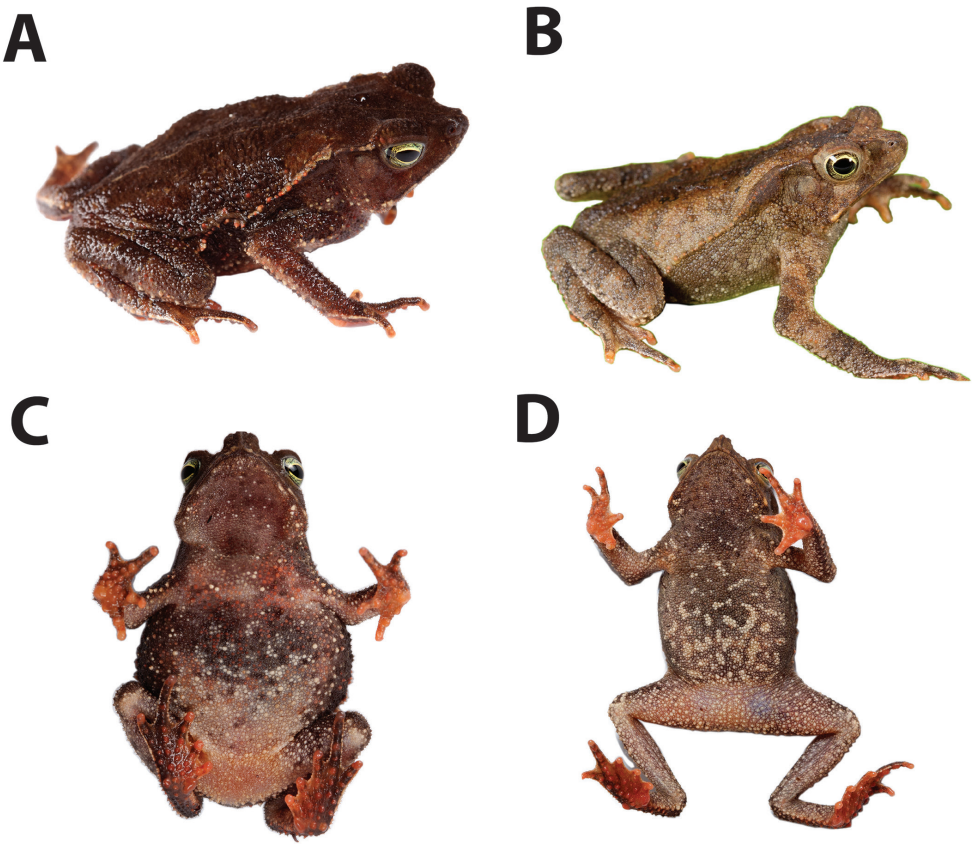

Figure 8. Dorsolateral and ventral views of *Rhinella alata* from the Chocó region. **A** and **C** QCAZ 50568 (SVL 40.37 mm), adult female, La Concordia, Santo Domingo Province, Ecuador **B** and **D** QCAZ 37248 (SVL 40.23 mm), adult male, Valle Hermoso, El Oro Province, Ecuador. Not shown at the same scale. Photos by S.R. Ron.

Rhinella alata is most similar to *R. acutirostris*. Both species differ from other members of the *R. margaritifera* group by the absence of protruding vertebral apophyses, canthus rostralis not raised, snout projected, and low cranial crests. *Rhinella acutirostris* differs from *R. alata* in having a bony knob at the angle of jaws (bony knob absent in *R. alata* [Hoogmoed 1986, Lötters and Köhler 2000]). *Rhinella alata* differs from the holotype of *R. proboscidea* (ZSM 1145/0) in having a less protruding snout and skin on dorsum bearing a mixture of warts, pustules, and minute tubercles (smooth skin in *R. proboscidea*). *Rhinella dapsilis* is much larger than *R. alata* (*R. dapsilis* holotype SVL = 77 mm, adult male; Myers and Carvalho 1945) and has a fleshy proboscis in the snout (proboscis absent in *R. alata*). *Rhinella alata* differs from *R. yunga* in having tympanic membrane and annulus distinct (tympanic membrane and annulus absent in *R. yunga*; Moravec et al. 2014). *Rhinella hoogmoedi*, *R. magnussoni*, *R. martyi*, *R. paraguayensis*, *R. scitula*, *R. sclerocephala*, and *R. stanlaii* have a bony knob at angle of jaws (Caramaschi and Pombal 2006, Lima et al. 2007, Fouquet et al. 2007a, Ávila et al. 2010,

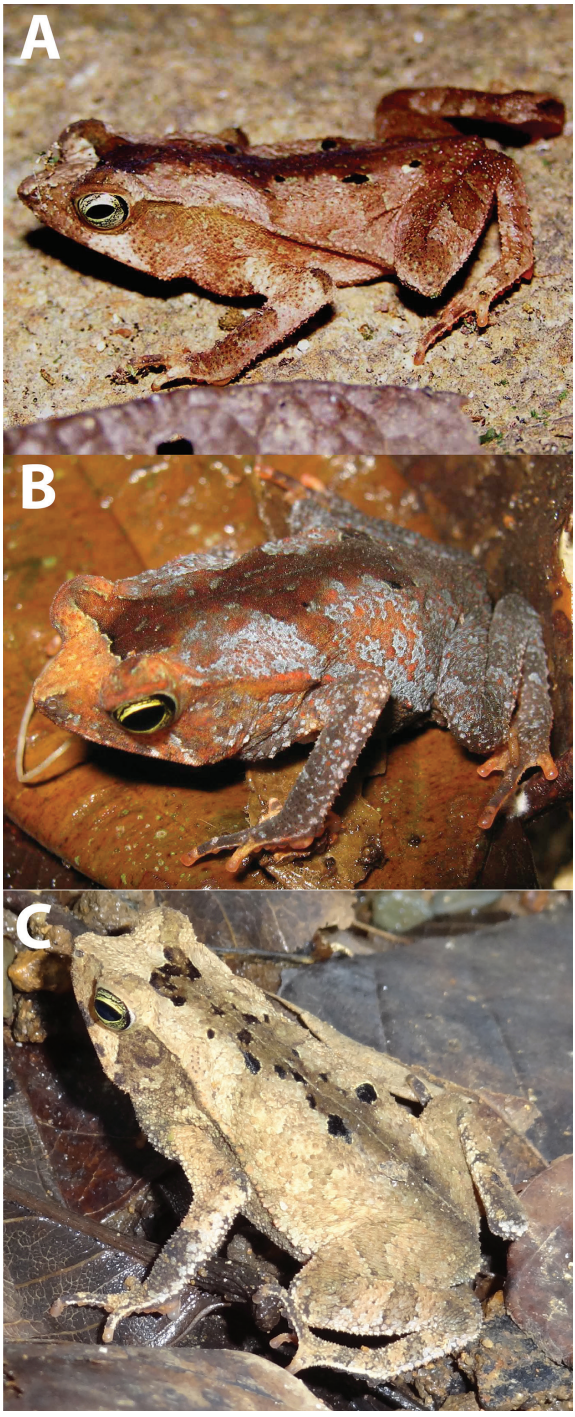

Figure 9. Dorsolateral views of *Rhinella alata*. **A** Cerro Azul, Parque Nacional Chagres, Panama Province, Panama. Photo by Ángel Sosa **B** Cerro Bruja, Parque Nacional Portobelo, Colón Province, Panama. Photo by Ángel Sosa **C** Gamboa, Colón Province, Panama. Photo by Roberto Ibáñez.

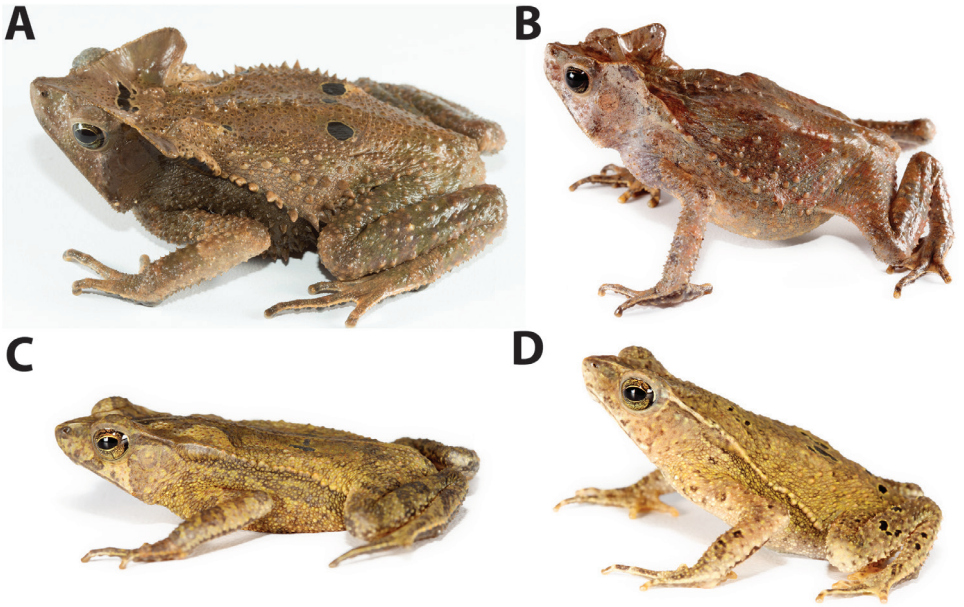

Figure 10. Dorsolateral views of *Rhinella margaritifera* from the Ecuadorian Amazon. Females: **A** QCAZ 55930 (SVL 80.15 mm) **B** QCAZ 55914 (SVL 72.49 mm), Lorocachi, Pastaza Province, Ecuador; males: **C** QCAZ 52343 (SVL 37.59 mm) **D** QCAZ 52344 (SVL 36.66 mm), Cascada San Rafael, Sucumbíos Province, Ecuador. Photos by S.R. Ron. Not shown at the same scale.

Caramaschi and Niemeyer 2003, Mijares-Arrutia and Arends-R 2001, Lötters and Köhler 2000; bony knob absent in *R. alata*). *Rhinella alata* differs from *R. castaneotica*, *R. margaritifera* (sensu stricto) and *R. roqueana*, by the absence of protruding vertebral apophyses (present in *R. castaneotica* [Caldwell 1991], *R. margaritifera* [Lavilla et al. 2013], and *R. roqueana* [Melin 1941]).

Rhinella alata is most closely related to populations of *R. margaritifera* from the upper Amazon basin in Ecuador and Peru. They can be easily distinguished by differences in body size (Fig. 5; see morphometric comparisons section) and relative size of cranial crests (Fig. 6).

Holotype. The holotype is an adult male with SVL = 39.2 mm (Fig. 11). Descriptions of the holotype have been provided by Leavitt (1933) and Hoogmoed (1989). The bony knob at angle of jaws and vertebral apophyses are absent. The crests are low and thick. There is a dorsolateral row of conical tubercles from the posterior border of the parotoid gland to the groin. There is a clear mid-dorsal line from the snout to the vent. The tympanum is rounded.

Variation. Variation in dorsal and ventral coloration of preserved specimens is shown in Figures 12 and 13. Background dorsal coloration varies from light gray (QCAZ 37244, AMNH 88689), light brown (QCAZ 14607, AMNH 104454) to dark gray (QCAZ 6733) or dark brown (QCAZ 11598, AMNH 52744), with irregular black and yellowish marks (QCAZ 4444, AMNH 88690). Some specimens

Figure 11. Dorsal (**A**), ventral (**B**), and lateral (**C**) views of the holotype of *Rhinella alata*. MNHN 84285, adult male, SVL = 39.2 mm.

Figure 12. *Rhinella alata* from Ecuador showing variation in dorsal and ventral coloration of preserved specimens. Left to right, males: QCAZ 6733 (SVL 38.23 mm), QCAZ 10279 (SVL 35.08 mm); females, QCAZ 11598 (SVL 42.13 mm), QCAZ 14607 (SVL 50.95 mm), QCAZ 10439 (SVL 47.06 mm). See Appendix 1 for locality data. Not shown at the same scale.

have nearly uniform brown dorsum without marks (QCAZ 31603, 10296, AMNH 10296). A clear mid-dorsal line is often present (e.g. QCAZ 3502, QCAZ 12233).

Ventral surfaces of preserved specimens have a cream to yellowish-cream background color with irregular darker marks arranged in diverse patterns; marks can

Figure 13. *Rhinella alata* from Panama showing variation in dorsal and ventral coloration of preserved specimens. Left to right, male: AMNH 89459 (SVL 37.54 mm); females, AMNH 88694 (SVL 41.21 mm), AMNH 55476 (SVL 41.19 mm), AMNH 104454 (SVL 49.69 mm), AMNH 88689 (SVL 42.75 mm), AMNH 20896 (SVL 42.98 mm). See Appendix 1 for locality data. Not shown at the same scale.

be light gray (QCAZ 6734, AMNH 88689), light brown (QCAZ 6732, AMNH 104454), dark gray (QCAZ 31606) or dark brown (QCAZ 6733, AMNH 89459), and vary from being restricted to the anterior half of the body (QCAZ 31604, AMNH 89459) to being present over the entire venter (QCAZ 4445, AMNH 88694). A longitudinal mid-ventral cream thin stripe can be present in the gular region (QCAZ 31602, 31606) or from the gular region to the mid-venter (QCAZ 6731, 11598).

Head shape in dorsal view varies from elongated (QCAZ 11598, AMNH 89459) to subtriangular (QCAZ 4447, AMNH 55475); in lateral view it varies from rounded (QCAZ 31605, AMNH 52749) to protruding (QCAZ 11393, AMNH 55475). Canthal region coloration varies from light gray or light brown to dark gray or dark brown. In some individuals the area below the eye and tympanum is yellowish cream (QCAZ 4447, AMNH 20896) or brown (QCAZ 31603, AMNH 88694) and differs from the color of the dorsum. Cloacal tubercles vary from yellowish cream (QCAZ 4441, AMNH 20896), to gray (QCAZ 31606) or brown (QCAZ 31602, AMNH 88695).

Color in life. Based on digital photograph of an adult female QCAZ 50568 (Fig. 8). Dark brown dorsum with irregular light brown and yellowish marks; there is a clear mid-dorsal line. Dorsal surfaces of tights and shanks are dark brown with transversal brown bands. Dorsal surfaces of forelimbs are dark brown with irregular light brown marks. Dark brown tubercles are abundant on the dorsum. Ventral surfaces vary from light brown to dark brown, with some irregularly distributed white and orange spots. The fingertips and the subarticular tubercles on fingers and toes are red-orange. Canthal region and tympanum are dark brown; iris greenish yellow with black reticulation.

Based on a digital photography of an adult male QCAZ 37248 (Fig. 8). Light brown dorsum with black spots and light brown and light gray marks. Dorsal surfaces of tights, shanks and forelimbs are light brown with transversal dark brown bands. Brown tubercles are abundant on the dorsum. Ventral surfaces are dark brown with irregularly distributed yellowish marks; the posterior part of the venter is cream. The subarticular tubercles of palms, soles, and fingertips are red-orange. Canthal region and tympanum are dark brown; iris greenish yellow with black reticulation.

Distribution and ecology. *Rhinella alata* has been recorded at 37 localities in the Ecuadorian Chocó (Cañar, Carchi, El Oro, Esmeraldas, Manabí, Pichincha, and Santo Domingo Provinces; Fig. 1), one locality in the Colombian Chocó (Barbacoas, Nariño; see *Taxonomic remarks*) and 35 localities in Panama (Comarca Guna Yala and Provinces Coclé, Colón, Darién and Panama; Fig. 2). It has a wide elevation range, from 19 to 1500 m above sea level.

The examined specimens from Chocoan populations contain 21 gravid females (average SVL = 45.37 mm, SD = 4.05 mm): QCAZ 4262, QCAZ 4441, QCAZ 4442, QCAZ 4443, QCAZ 7065, QCAZ 10296, QCAZ 11597, QCAZ 11598 collected in January; QCAZ 50568 collected in February; QCAZ 11392, QCAZ 31601, QCAZ 31603, QCAZ 31605 collected in April; QCAZ 25023 collected in June; QCAZ 10439 collected in August; QCAZ 14607 collected in November; QCAZ 10301 collected in December. This suggests year round reproductive activity with a peak between January and April, a period that corresponds to the rainy season in the Ecuadorian Chocó.

In Panamanian populations gravid females were found in January (AMNH 104454), September (AMNH 55461), November (AMNH 88689), and December (AMNH 53699). In central Panama, *R. alata* breeds in ponds and pools along permanent streams or swamps. Reproduction is explosive and most takes place from the middle of the rainy season to early dry season (Wells 1979, Ibáñez et al. 1999). Choruses last less than 24 hours with males usually calling at night and oviposition occurring by day, especially in the early afternoon (Wells 1979). Otherwise, individuals are primarily diurnal, found active on the leaf litter of the forest floor during daytime, and often found asleep on leaves of low vegetation at night (Ibáñez et al. 1999). Diet is specialized on ants (Toft 1981).

Most of the Ecuadorian specimens are from Reserva Mayronga and Reserva Ecológica Cotacachi-Cayapas. They were found in the leaf litter of secondary forest and in agricultural lands. Some adults were observed at night within the forest in vegetation above the ground and some were found in amplexus (QCAZ 10271, QCAZ 10274, QCAZ 10275 in November 1996, and QCAZ 31604, QCAZ 31605 in February 1996). All the specimens collected in Reserva Ecológica Cotacachi-Cayapas were found in secondary forest. At some collecting sites, the forest has been cleared for cacao plantations (QCAZ specimen database).

According to the classification of Sierra et al. (1999) the vegetation types for Ecuadorian localities are: (1) Lowland Evergreen Forest of Coastal Range, characterized by abundant epiphytes, climbers and herbaceous plants, with a canopy of 30 m (e.g. Reserva La Chiquita, Durango); (2) Semideciduous Lowland Forest of Coastal Range, defined by

the presence of broad canopy trees up to 20 m and curved shafts; the tree stratum is characterized by the presence of spiny, deciduous species with epiphytes while the forest floor has herbaceous plants (e.g. Bilsa, La Tortuga); (3) Evergreen Foothill Forest of Coastal Range, characterized by a canopy that can reach 30 m or more and trunks of trees covered with orchids, bromeliads, ferns and aroids (e.g. Manta Real, Alto Tambo); (4) Deciduous Lowland Forest of Costal Range, characterized by losing leaves during part of the year with a great varieties of cactus and thorny plants; the most conspicuous trees are the family Bombacaceae have curved trunks and broad crown. (e.g. El Progreso); (5) Semideciduos Foothill Forest of Coastal Range, characterized by having slightly dispersed vegetation, with trees over 20 m and dense herbaceous layers of ferns (e.g. Valle Hermoso).

The main vegetation types for Panamanian localities are (following Hogan 2010): (1) Isthmian-Atlantic Moist Forests, characterized by tall tropical evergreen forest with buttressed canopy trees reaching 40 m and with an extremely rich epiphyte flora (e.g. Cruces Trail, Punta Rincón); (2) Eastern Panamanian Montane Forest, at elevations from 500 to 1800 m above sea level, includes marshes, swamp forests, semi-deciduous tropical moist forests, premontane wet forest, cloud forests and elfin forests (e.g. Cana, Cerro Tacarcuna); (3) Chocó-Darién Moist Forests, at elevations between 0 and 1000 m above sea level, between the Pacific Ocean and the western range of the Andes (e.g. Dad Nakue Dubpir, Udirbi).

Taxonomic remarks. Based on morphological characters, Vélez-Rodriguez (2004) ascribed four populations from Panama and Colombia to *R. alata*: Isthmus of Panama (Panama; 15 males, 10 females); Parque Nacional Los Katíos (Colombia; 12 males, 15 females); Gorgona and Güape Island (Colombia; 7 males, 8 females); Municipio Restrepo (Colombia; 7 males, 8 females). Based on data from Vélez-Rodriguez (2004), these populations differ from the holotype of *R. alata* and populations of *R. alata* in Ecuador and Panama (in parentheses) in having: (1) a *canthus rostralis* protruding in females and ill-defined in males (inconspicuous in males and females), (2) parietal crests well defined in females, ill-defined in males (ill-defined in males and females), (3) vertebral apophyses slightly visible externally (absent). The differences suggest that those specimens are not *R. alata* and may belong to a different species. Alternatively, differences between *R. alata* described by Vélez-Rodriguez (2004) and our study could be an artifact resulting from the use of distinct terminology for similar character states.

In contrast, Mueses-Cisneros and Moreno-Quintero (2012) reported two species of the *R. margaritifera* group form Barbacoas, Nariño, Colombia (*Rhinella* sp. 9 and *Rhinella* sp. 10). Two photographs of live individuals (pp. 45) show morphological features that fall within the observed variation of *R. alata*. We tentatively assign them to *R. alata* but direct specimen examination is required to confirm this identification.

Discussion

The taxonomic status and phylogenetic position of populations traditionally ascribed to *R. margaritifera* (= *Bufo typhonius*; e.g. Anderson 1945, Miyata 1982, Ortega-An-

drade et al. 2010) from western Ecuador and Central America were reviewed. The examination of the holotype of *R. alata* in combination with the morphological and genetic information from 72 populations from the Chocó region and Panama indicate that those populations should be referred to *R. alata*. The similarity between Chocoan and Panamanian populations was previously noted by Hoogmoed (1990).

Systematics and morphology

Hoogmoed (1990), Lescure and Marty (2000) and Fouquet et al. (2007b) considered that *R. margaritifera* from French Guyana, with hypertrophied crests, corresponds to *R. margaritifera* sensu stricto. In a recent review, however, Lavilla et al. (2013) assigned a neotype with the type locality in "Humaitá, State of Amazonas, Brazil". In our phylogeny (Fig. 3), the sister clade of *R. ocellata* include the closest localities to the new type locality for *R. margaritifera* and are likely to contain populations of *R. margaritifera* sensu stricto. Our phylogeny and previous reviews (e.g. Fouquet et al. 2007b) indicate that species diversity in the *R. margaritifera* group is greatly underestimated. In our phylogeny, two *R. margaritifera* from the southern Amazon in Ecuador (QCAZ 18241 and QCAZ 23917) are more closely related to *R. margaritifera* from French Guyana and *R. dapsilis* than to other *R. margaritifera* from Amazonian Ecuador. They probably represent an undescribed species, characterized by the presence of vertebral apophyses, bony knobs at the angle of jaws, and poorly developed crests. More studies are needed to define the status of these populations, as well as that of *R.* cf. *paraguayensis* from Bolivian and Brazilian Amazon and *R.* cf. *hoogmoedi* from Brazilian Atlantic Forest.

The identity of the upper Amazon clade (Ecuador-Peru) remains unresolved. It was not possible to ascribe it unequivocally to any described species of the *R. margaritifera* species group and it is unlikely to be *R. margaritifera* sensu stricto (as defined by Lavilla et al. 2013). Thus, these populations may belong to an undescribed species characterized by having prominent supratympanic crests, conspicuous vertebral apophyses in the dorsum and bony knobs at angle of jaws (Fig. 10). We refrain from describing this species until genetic samples of *R. margaritifera* sensu stricto are available and a comprehensive review of the group is carried out. For now, we suggest that these populations are referred as *R. margaritifera* sensu lato.

These results raise some rather interesting questions. For instance, the complete distribution range of *R. alata* is yet to be determined. Extensive and explicit studies are necessary to reveal whether the species is continuously distributed from Ecuador to Panama or if it consists one, two (or more) disjoint population nuclei. This would be an indispensable step before planning further studies on the evolutionary history or conservation status of the species. Moreover, future studies including a larger number of samples, more representative of the geographic range of each species within the *R. margaritifera* group, from Colombia, Venezuela and Suriname, will help to clarify their evolutionary identity. It will also be necessary to re-evaluate, using molecular, mor-

phological, ecological, behavioral, and phylogenetic analyses, the taxonomic status of species that have been previously described only morphologically such as *R. acutirostris*, *R. magnussoni*, *R. proboscidea*, *R. roqueana*, *R. sclerocephala*, *R. scitula* and *R. stanlaii*. Integrative approaches like the one we pursued in this study will help to disentangle the complex evolutionary history, systematics, and taxonomy of this species group.

Biogeographic implications

Because all species in the *R. margaritifera* species group are distributed in South America, it is reasonable to assume that the presence of *R. alata* in Central America is the result of a single dispersal event from South America. The genetic distances between Chocoan and Panamanian populations are low (range 1.2–1.9%) and suggest that their divergence was recent and occurred after the closure of the Panamanian isthmus during the late Pliocene. Assuming a rate of evolution of the gene *16S* of 0.00249–0.00277 substitutions per site per lineage per Myr (Evans et al. 2004; Lemmon et al. 2007), the divergence between these populations occurred ~ 2.16 to 3.42 Myr ago (under the 0.00277 rate) or ~ 2.41 to 3.81 Myr ago (under the 0.00249 rate). Thus, it is likely that the divergence between Panama and Chocó took place after the completion of the Panamanian Isthmus (~ 3.5 Myr ago; Coates et al. 1992, Coates and Obando 1996). These estimates of time of divergence, however, should be considered with extreme caution because they assume a molecular clock at a rate estimated for species in different families. Further explicit studies will be necessary to estimate divergence times with more confidence.

Rhinella alata is sister to populations of *R. margaritifera* from the Ecuadorian and Peruvian Amazon and the eastern Andean slopes, up to 2000 m of elevation, forming altogether a robust clade. The two lineages are highly divergent from each other (uncorrected *p* distances 3.0–5.5%, mitochondrial gene *16S*) and are morphologically distinctive. Therefore, both clades clearly represent separate species. Previously, *R. margaritifera* was considered to occur on lowland rainforests east and west of the Andes of Ecuador. This distribution was atypical because out of 174 amphibian species inhabiting the Amazonian rainforests of Ecuador below 600 m of elevation, only three also occur in the rainforests of the Chocó region west of the Andes: *Hypsiboas boans*, *Rhinella marina* and *Trachycephalus typhonius* (Ron et al. 2014). Despite having similar environmental conditions and being geographically close (as low as 100 km of airline distance), rainforests on both sides of the Andes share few amphibian species, a result of the barrier effect of the Andes. Our results showing that *R. margaritifera* only occurs on the eastern side demonstrate that their unusual distribution was an artifact of the incorrect delimitation of species boundaries. We suspect that the same problems could explain the disjunct distributions of *Rhinella marina*, *Trachycephalus typhonius* and *Hypsiboas boans*. Therefore, tropical rain forests of the Amazon and the Chocó may not share amphibian species.

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Appendix 1

Examined material. Numbers in bold indicate specimens analyzed genetically and morphometrically.

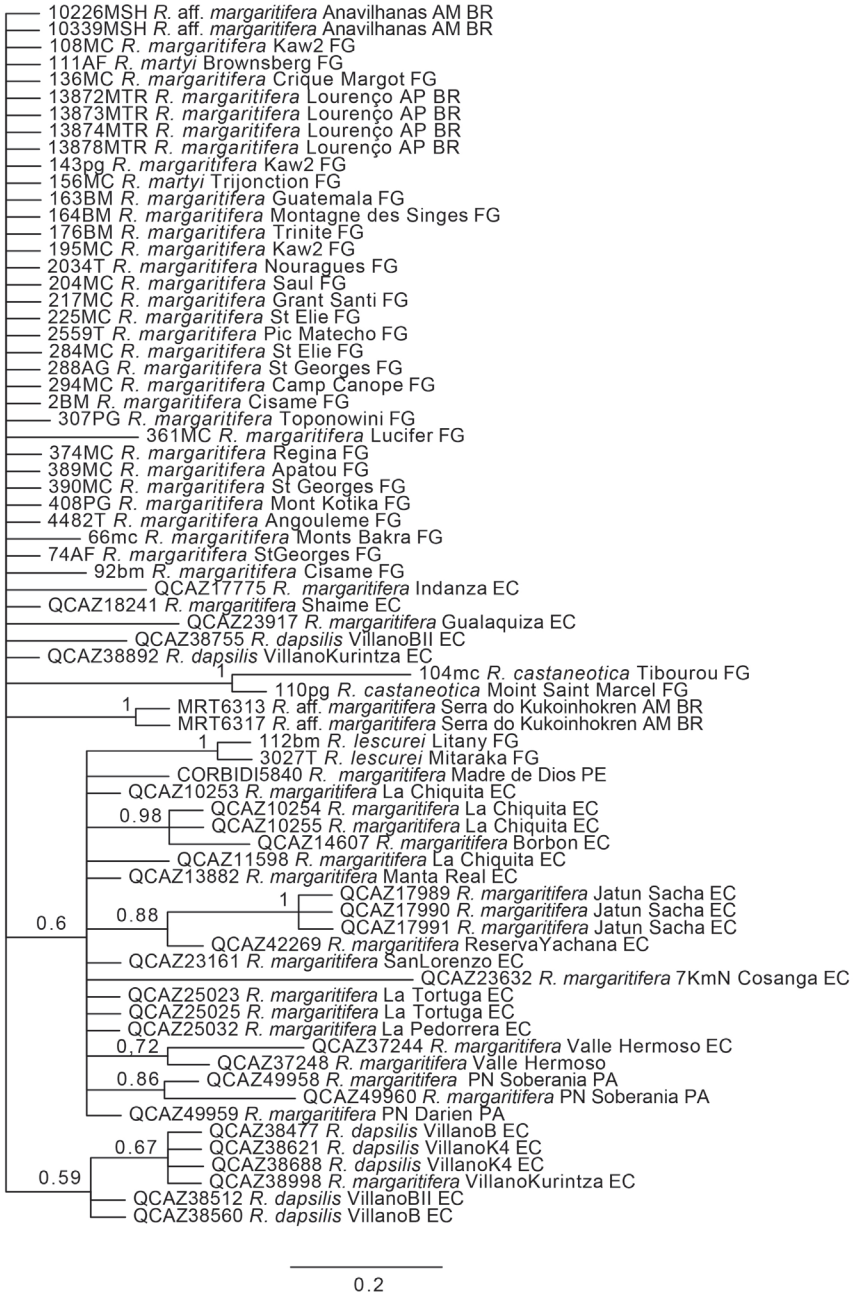
Rhinella alata.— ECUADOR: PROVINCIA CAÑAR: Manta Real, Río Patul (2.5679°S, 79.3666°W), 350–400 m (QCAZ 3437, 3551, 4757–758); Manta Real (2.5537°S, 79.3642°W), 500 m (QCAZ 12778–779). PROVINCIA CARCHI: Vía Zumba–El Chota, 1500 m (QCAZ 12233). PROVINCIA EL ORO: Valle Hermoso, Parroquia Bella María (3.5019°S, 79.8172°W), 379 m (**QCAZ 37244, 37248**); El Progreso, vía Pasaje–Pan de Azúcar (3.2883°S, 79.7581°W), 180 m (QCAZ 10366). PROVINCIA ESMERALDAS: Lagarto, Mayronga Reserve (1.042°S, 79.28°W), 100 m (4262–4264, 4441–4451, 4709–4717, 6637–6642); Reserva Ecológica Bilsa (0.6202°S, 79.931°W), 534 m (QCAZ 6731–6743); Corriente Grande, Río Cayapas (0.6895°S, 78.9589°W), 70 m (QCAZ 10271, 10274–281, 10289, 10290, 10292, 10295–299, 10299, 10301); Reserva Ecológica Cotacachi Cayapas, Charco Vicente (0.6962°S, 78.9109°W), 60 m (QCAZ 3338–3339, 11391–396); Pichiyacu, Comunidad Chachi, Río Cayapas (0.9081°S, 78.998°W), 260 m (QCAZ 31602–609); Reserva Ecológica Cotacachi–Cayapas o Playa de Oro (0.8285°S, 78.722°W), 179 m (QCAZ 49381–382, 49387, 49391); Las Golondrinas near Río Canandé (QCAZ 12651–652); Durango, Río San José (1.054°S, 78.625°W), 33 m (QCAZ 24968–978); Río Onzole (0.712°S, 79.092°W), 110 m (QCAZ 10440–443); Comunidad Loma Linda, Río Onzole (0.8754°S, 79.0511°W), 95 m (QCAZ 10439); La Concordia (0.0022°S, 79.4105°W), 144 m (QCAZ 50573, 50568); San Lorenzo, Protectora La Chiquita (1.2333°S, 78.76°W), 60 m (**QCAZ 10253, 10254**–255, **11597,**

11598); San Lorenzo, La pera del Guarapo (1.2684°S, 78.8067°W), 253 m (**QCAZ 23161**); La Pedorrera (0.4667°S, 79.9833°W), 53 m **(QCAZ 25032);** La Tortuga (0.591°S, 79.957°W), 86 m (**QCAZ 25023**); Borbón (1.0667°S, 79.05°W), 70 m (**QCAZ 14607**); Viche (0.6615°S, 79.5387°W), (QCAZ 4674); Durango (1.0427°S, 78.6245°W), (QCAZ 8549, 35250); 7 km western of Durango (1.0133°S, 78.6682°W) 220 m, (QCAZ 23164, 23623); Viruela, Rio Cayapas (1.1142°S, 78.9936°W), 45 m (QCAZ 10289); Al Tambo (0.9169°S, 79.5662°W) 253 m, (QCAZ 21138); El Milagro, La Mayronga (1.003°S, 79.326°W). PROVINCIA MANABÍ: El Carmen (0.274°S, 79.459°W). 300 m (QCAZ 7038–7039, 7065). PROVINCIA PICHINCHA: Reserva Forestal ENDESA (0.1667°S, 79,1667°W), 720 m (QCAZ 1659); Río Canoi (0.075°S, 79.051°W), 570 m (QCAZ 2745); 1 km E of Pedro Vicente Maldonado (0.0833°S, 79.039°W), 670 m, (QCAZ 2752); San Miguel de los Bancos (0.0166°S, 78.8833°W), (QCAZ 3813, 3815–818); San Miguel de los Bancos, Río Pitzará, 130 m (QCAZ 50846); km 9 San Miguel de los Bancos–Puerto Quito road (0.072°S, 78.9599°W), (QCAZ 5860); Puerto Quito, ENDESA (0.098°S, 79.117°W), (QCAZ 36827). PROVINCIA SANTO DOMINGO: Bosque Protector La Perla (0.057°S, 79.359°W), (QCAZ 3500–504); km 8 road to Santo Domingo (0.2005°S, 79.1924°W), 528 m (QCAZ 23621). PANAMA: COMARCA GUNA YALA: Dad Nakue Dubpir, Río Ogandí (9.2477°N, 78.1744°W), 150 m (CH 8842); Udirbi, Reserva Forestal (9.3167°N, 78.9833°W), 342 m (CH 1706); PROVINCIA COCLÉ: La Mina, Río Indio (8.9382°N, 80.1469°W), 48 m (CH 4922); near Río Tife cascade, Parque Nacional General de División Omar Torrijos Herrera (8.7065°N, 80.6352°W), 460 m (CH 0065); Obispo (9.1167°N, 79.6833°W) (MNHN 84285); Quebrada La Tiburcia, Cascajal (8.7158°N, 80.4605°W), 180 m (CH 5042); Quebrada La Varona, near Palmarazo (8.7342°N, 80.6565°W), 125 m (CH 5139). PROVINCIA COLÓN: Chitra, Santa Isabel (9.5186°N, 79.1534°W), 90 m (CH 7783); El Limón, Río Indio (8.9919°N, 80.1701°W), 19 m (CH 4967); Rinconcito, Punta Rincón (9.0135°N, 80.6884°W), 52 m (CH 1412); Río Caimito, Petaquilla (8.9706°N, 80.671°W), 54 m (CH 5476); Río Boquerón (9.3857°N, 79.4826°W), 150 m (AMNH 89459); Río Frijoles, Camino del Oleoducto, Parque Nacional Soberanía (9.1523°N, 79.7347°W), 67 m (CH 0307); road to Piña, after the represa Gatún (9.2603°N, 79.94°W), 34 m (CH 1679); Sta. Rosa and Guayabalito (9.1833°N, 79.65°W), 36 m (AMNH 55475); PROVINCIA DARIÉN: between Dos Bocas de Antaral and campsite on Serranía de Jingurudó (7.6564°N, 77.9986°W), <675 m (CH 4641); Cerro Tacarcuna, Río Pucuro (8.0011°N, 77.4852 °W), 640 m (AMNH 104454); Cana, trail to Boca de Cupé, Pinogana (7.7661°N, 77.6752°W), 518 m (CH 9104); Estación Pirre, Río Peresénico (8.0192°N, 77.7325°W), 90 m (CH 4057); Laguna Purriche (7.7222°N, 77.6555°W), 475 m (CH 6376); PROVINCIA PANAMA: Altos de Majé (AMNH 88689–8690, 88694); Barro Colorado (9.1636°N, 79.8378°W), 79 m (AMNH 20896, 5274, 55461–462); Parque Nacional Soberanía, Ancón (9.0764°N, 79.6594°W), 130 m (CH 9192); Chiva Chiva Road, Parque Nacional Camino de Cruces (9.0284°N, 79.5899°W), 41 m (CH 0491); Cruces trail (9.0453°N, 79.5892°W), 77 m (AMNH 55460); Finca Santa Bárbara, Nuevo Empe-

rador, Arraiján (9.0011°N, 79.7235°W), 135 m (CH 1158); near Boquerón, Candelaria and Peluca (9.3671°N, 79.5546 °W) (AMNH 53699); near entrance to Chilibrillo Cave (9.1833°N, 79.6167°W) (AMNH 55476); Pacora (9.0833°N, 79.2833°W), 20 m (QCAZ 55481); Río Arraijancito (8.983°N, 79.6361°W), 110 m (CH 3980); Río Chico Masambí, Parque Nacional Soberanía, Ancón (9.0787°N, 79.6601°W), 135 m (MVUP 2299); Río Indio Arriba (8.6562°N, 80.1144°W), 645 m (CH 5005); San Juan de Pequení (9.3841°N, 79.5227°W), 100 m (CH 3702); stream near ACP Estación Río Chico (9.2636°N, 79.5097°W), 116 m (CH 6825); Tortí (8.9389°N, 78.4573°W), 95 m (MVUP 2256); Trinidad (8.7321°N, 79.9617°W), 420 m (CH 4313); Altos de Cerro Azul, Cerro Jefe (9.2284°N, 79.4046°W), 800 m (CH 3441).

Rhinella margaritifera.— ECUADOR: PROVINCIA ORELLANA: Parque Nacional Yasuní, Estación Científica Yasuní (0.6772°S, 76.4012°W), 230 m (QCAZ 8415, 17736, 17740, 41011); Parque Nacional Yasuní, Bloque 31 (0.942°S, 75.905°W), (QCAZ 11909); Parque Nacional Yasuní, Rio Yasuní (0.9248°S, 75.9152°W), 206 m (QCAZ 11940); Parque Nacional Yasuní, Via Pompeya-Iro (0.6536°S, 76.4536°W), 287 m (QCAZ 17216, 17329, 43011, 22401); Parque Nacional Yasuní, Apaika (0.8656°S, 75.9245°W), (QCAZ 33545); Estación Biológica Tiputini (0.0639°S, 76.1493°W), 250 m (QCAZ 10207); Nuevo Rocafuerte (0.8967°S, 75.437°W),186 m (QCAZ 39466); Añangu (0.5249°S, 76.3844°W), 255 m (QCAZ 43952–953); Chiroisla (0.58°S, 75.9177°W), 207 m (QCAZ 44318–319; Huiririma (0.7116°S, 75.6239°W), 194 m (QCAZ 44563–565). PROVINCIA PASTAZA: Río Bobonaza (1.8056°S, 77.3313°W), 250 m (QCAZ 10650); Kapawi Lodge (2.5387 °S, 76.8583°W), 239 m (QCAZ 25476, 25488–489); Pomona (1.625°S, 77.9072°W), 846 m (QCAZ 25631). PROVINCIA SUCUMBIOS: Reserva Limoncocha (0.4062°S, 76.6195°W), 261 m (QCAZ 43104, 43108); Pañacocha (0.4712°S, 76.0667°W), 255 m (QCAZ 44098–099). PROVINCIA NAPO: Reserva Yachana (0.8333°S, 77.1667 °W), 350 m (**QCAZ 42269**); Cascada de San Rafael (0.1036°S, 77.5808°W), 1300 m (QCAZ 31708). PROVINCIA MORONA SANTIAGO: Plan de Milagro (3.0011 °S, 78.5052°W), 1950 m (QCAZ 48242).

Appendix 2



Bayesian consensus phylogram depicting relationships within the *Rhinella margaritifera* species group. The phylogram was derived from the analysis of 550 bp of nuclear gene Tyrosinase. Museum catalog numbers are shown in Table 1. Abbreviations are: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.