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The Vermetidae of the Gulf of Kachchh, western coast of India (Mollusca, Gastropoda)

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Abstract

Coral reefs are often termed underwater wonderlands due to the presence of an incredible biodiversity including numerous invertebrates and vertebrates. Among the dense population of benthic and bottom-dwelling inhabitants of the reef, many significant species remain hidden or neglected by researchers. One such example is the vermetids, a unique group of marine gastropods. The present study attempts for the first time to assess the density and identify preferred reef substrates in the Gulf of Kachchh, state of Gujarat, on the western coast of India. A total of three species of the family Vermetidae were recorded during the study and their substrate preferences identified.

Keywords

Coral reefs, Vermetidae, Paga Reef, Gulf of Kachchh

Introduction

The coral reef ecosystem is well known for its prodigious density and diversity of inhabitants, hence sometimes referred to as marine rain forests (Mulhall 2008). However, many species in the reef ecosystem remain overlooked due to their non-charismatic and/or cryptic nature as well as smaller size (Shima et al. 2010). Such species include

one of the sessile gastropods, the vermetids. Vermetids do not have a normal coiling shell and become untwisted; in post-larval stages, the irregularly coiled shells are firmly attached to rock or any other hard substrates. These meandering tubes resemble the polychaete worm tubes (Rudiger 1995), hence the name vermetids. It is thought that there are more than 160 living species in warm temperate and tropical environments (Golding et al. 2014). The Gulf of Kachchh is situated on the western coast of India and located between 22°15'N and 23°40'N latitude and 68°20'E and 70°40'E longitude. The Gulf of Kachchh is the only site in Gujarat bestowed with one of the four major coral reef formations of the country (Singh et al. 2004). Patchy coral formations are evident on intertidal sandstones in the Gulf. The present study was carried out on Paga Reef, a submerged reef situated at the western part of the Gulf (Figure 1). The shape of the reef is oval, extending east to west with prominent fringes on northern side.

Vermetids are distributed in the marine habitats of the pan-tropical and subtropical bands around the globe (44°S to 44°N latitude) usually close to coral reefs diffusion boundaries (Safriel 1975). Present-day vermetids build dense aggregates in high-energy environments (Brachert et al. 2007). These biogenic structures are mainly consisted by *Dendropoma* species together with coralline algae, serpulids and encrusting foraminiferans (Brachert et al. 2007). The largest vermetid, *Dendropoma maximum* Sowerby, 1825, is common and widespread throughout the Indo-Pacific (Hadfield et al. 1972; Hughes and Lewis 1974; Zvuloni et al. 2008).

In terms of biodiversity, coral reefs are an exceptionally diverse ecosystem showcasing a large number of species interactions (Menge and Sutherland 1976). Such interactions, such as predation and competition, have often been studied in order to understand the positive and negative impacts of each organism on the others (Abrams 1987). For example, a study at Moorea revealed a negative correlation between the density of vermetids and the percentage cover of live coral (Shima et al. 2010). Furthermore, the incidence of flattened coral growth forms was associated with the presence of vermetids on French Polynesia (Shima et al. 2010). The strength of these deleterious interactions argues strongly for an urgent need to improve our knowledge regarding the ecological role of vermetid gastropods as well as their interactions with corals. Therefore, the present study is aimed at describing the density, diversity and association preferences of vermetids in combination with various biotic and abiotic factors. It should be noted that the present study was focused only on field surveys for baseline data collection; it did not involve laboratory experiments or collections.

In the Gulf of Kachchh, the presence of vermetids were recorded at Adatra reef, where they form thick encrustations over hard substrata and can survive in the extreme physical conditions such as prolonged exposure due to high tidal amplitudes (Pillai et al. 1979). In addition to this, the Zoological Survey of India (2004) recorded the single vermetid species *Thylacodes variabilis* Hadfield & Kay, 1972 (as *Serpulorbis*), from various locations inside and outside the Gulf of Kachchh *i.e.*, Adatra, Okha, Porbandar, Bedi, Mithapur and Danda (ZSI 2004). However, these studies did not emphasize the density of vermetids, nor their association with live corals. The vermetids are found in

Figure 1. Map showing the study site in the Gulf of Kachchh, Gujarat, west coast of India.

association of various coral species which give rise to a number of alterations to the growth and survival of corals (Shima et al. 2010). Hence, the present study is the first of its kind to assess the density and association preferences of the vermetids on Paga reef, in the Gulf of Kachchh (Figure 1).

Paga reef has abundant reef biodiversity including both fauna and flora except for the sand patch exposed at the western end of the reef. The coral assemblages show very diverse forms on this reef, including a variety of sponges, sea anemones, zoantharians, tube worms, crabs, gastropods, bivalves and echinoderms. Altogether, they contribute more than 200 species of marine invertebrates in this locality (Singh et al. 2004). It has been observed that the density of coral decreases from the edge to the centre of the reef with some exceptional tidal pools. During the present survey, a total of 11 species of coral were recorded, the majority of which were *Favia speciosa* (Dana, 1846), *F. favus* (Forskal, 1775), *Porites lutea* (Quoy & Gaimard, 1833), *P. compressa* (Dana, 1846), *Turbinaria peltata* (Esper, 1794) and *Goniopora planulata* (Ehrenberg, 1834) among others.

Vermetids were evident as soft-bodied organisms with a bright orange, pale red or dark magenta colouration and covered in an upright calcareous tube, which was sometimes slightly elevated. The vermetid shells, originally dull white to earthy grey in colour, were observed to be well-encrusted by epibionts such as calcareous algae and live coral. The length of the vermetid tube emerging out of the live coral substratum was 2–3 mm and ranged from 3–8 mm in other substrates, like rock covered by silt or sand. In some places, the irregularly coiled tubes of the vermetids were also found in completely exposed conditions. The present study summarises the occurrence of three genera of vermetids *i.e.*, *Ceraesignum*, *Thylacodes* and *Petaloconchus* that showed densities of 7.7, 5.3 and 0.4 individuals/100 m^2 respectively.

Method

Paga reef is located between 22°28.8' to 22°30.0'N latitude and 69°11.6' to 69°15.0'E longitude covering an area of 1472.4 ha which remains submerged during high tide and gets exposed only during low tides. Therefore, the field work was carried out during a small window of low spring tides falling in daylight. The reef area was surveyed using ten belt transects measuring 100 m × 1 m in the intertidal zone (English et al. 1997). The transects were orientated perpendicular to the reef edge in order to cover a variety of substrates prevailing at reef edge and reef flat. The coordinates were recorded with an E-trex Garmin hand-held GPS navigator. The observations include survey of the vermetids along with the associated coral species or any other substratum. The study involved four major substrate types *viz.*, live coral, silt over rock, rubble, algae over rubble. One-way ANOVA was performed to evaluate the variation in vermetid densities among substrate types.

Results

A total of three genera of vermetids belonging to Family Vermetidae (Subclass Caenogastropoda, Order Littorinimorpha, Superfamily Vermetoidae) was recorded from the study sites namely, *Ceraesignum* Golding, Bieler, Rawlings & Collins, 2014, *Thylacodes* Guettard, 1770 and *Petaloconchus* Lea, 1843 (Figure 2). The organisms were present in all reef zones across the intertidal belt of Paga reef but primarily concentrated on available hard substrata. Among the three genera, *Ceraesignum* spp. (7.7 individuals/100 m^2) showed the highest density followed by *Thylacodes* spp. (5.3 individuals/100 m^2) and *Petaloconchus* spp. (0.4 individuals/100m^2). The generic composition in the survey is 57% of *Ceraesignum* spp., 40% *Thylacodes* spp. and 3% *Petaloconchus* spp. Furthermore, the association of each genus with the substrate type was different, illustrated in the histogram (Figure 3). *Ceraesignum* was mostly associated with silt on rock while *Thylacodes* was associated with the coral *Porites*.

Overall, the occurrence of vermetids was recorded on seven different substrates, *i.e.*, silt on rock (thin veneer of silt on rock, SoR), rubble, *Favia* Milne Edwards, 1857, *Platygyra* Ehrenberg, 1834, *Porites* Link, 1807, *Goniastrea* Milne Edwards & Haime, 1848 and algae over rubble (AoR). Maximum numbers of individuals were recorded on SoR and a minimum on AoR. However, there is no significant difference for substrate preference among the three genera ($F = 1.923$, $df = 9.445$, $p = 0.1993$). The species-specific preference of each vermetid species for a substrate is described below. All vermetid species were solitary: no gregarious species were found during the survey.

Ceraesignum (Golding et al., 2014) (synonym *Dendropoma*)

The density of the large vermetid *Ceraesignum* was 7.7 individuals/100 m^2 found on six different substrates. It remained embedded in the massive and submassive coral

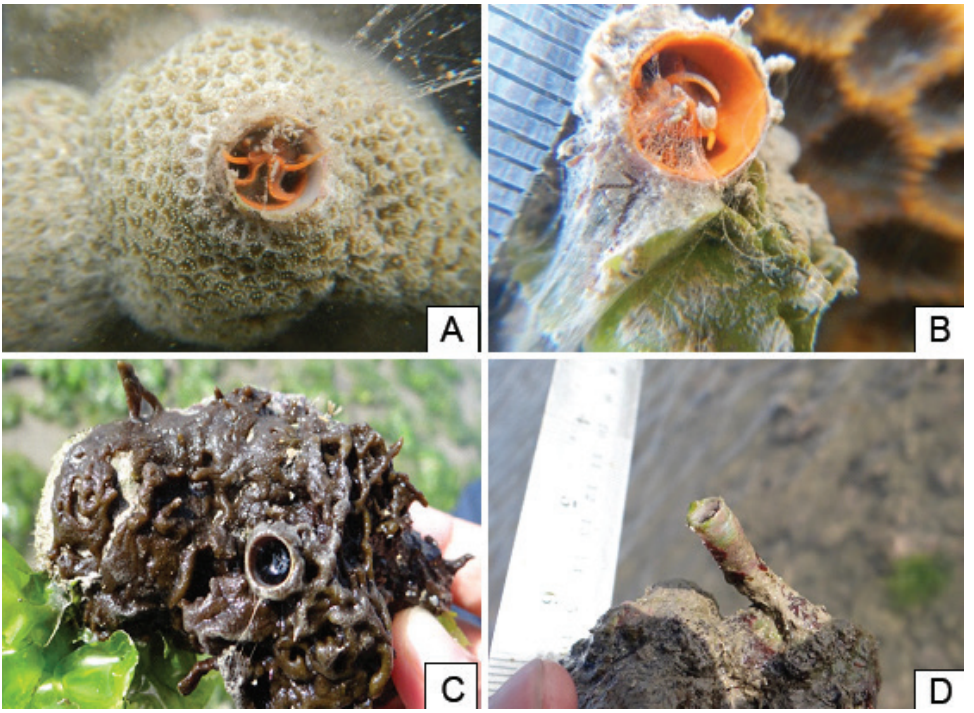

Figure 2. Showing association of vermetids with various substrata and epibionts **A** *Thylacodes* sp. attached with *Porites lutea* showing mucus net and pedal tentacle **B** a brightly coloured *Thylacodes* sp. with active pedal tentacles at positions 11 o'clock and 4 o'clock **C** *Ceraesignum* sp. embedded in an algae covered rubble **D** erect, uncovered *Petaloconchus* sp. tube attached to rubble.

colonies or rock, except a short part of the tube opening. The individuals of this genus showed the highest association with SoR (73%) and were recorded the least number of times on *Favia* Milne Edwards, 1857 (1%) and AoR (Figure 4). If the substrates are divided as per the biotic and abiotic categories, 73% of the total *Ceraesignum* has been recorded on abiotic substrates and 27% on live coral and algae. Moreover, among the biotic substrates, 26% of the individuals were found embedded in living coral colonies. *Ceraesignum* showed association with a total of four genera of live corals: *Platygyra* (12%), *Goniastrea* (4%), *Favia* (1%) and *Porites* (9%). It is noteworthy that three of the four coral genera belong to family Faviidae; among all the live corals, maximum individuals were found on the live colonies of *Platygyra*.

Thylacodes (Guettard, 1770)

The density of *Thylacodes* was 5.3 individuals/100 m^2, which is less compared to *Ceraesignum* spp. The individuals of this genus were found associated with six different substrates:

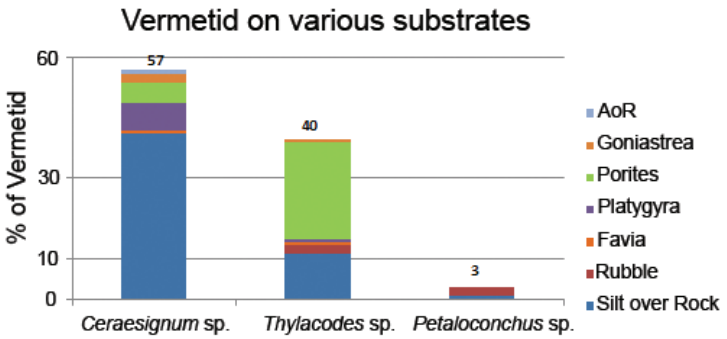

Figure 3. Percentage of vermetids on various reef substrates.

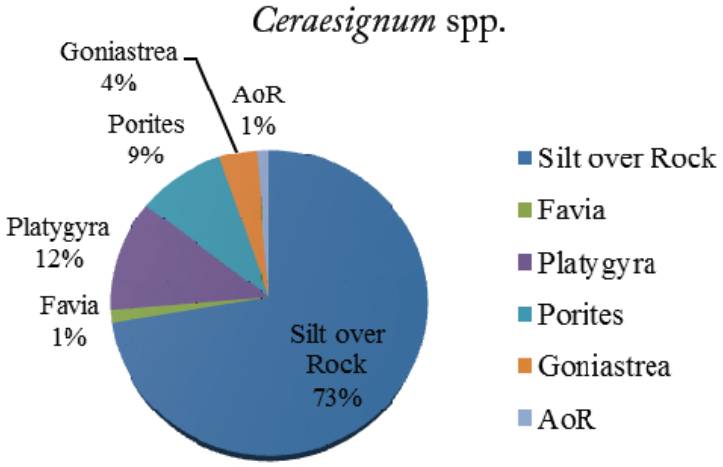

Figure 4. Percentage of *Ceraesignum* spp. on various reef substrates.

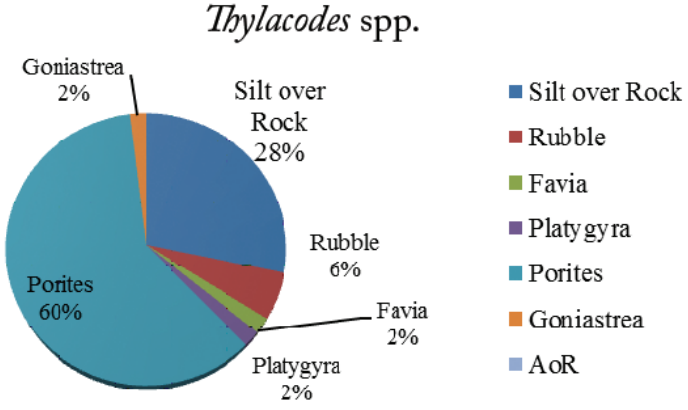

Figure 5. Percentage of *Thylacodes* spp. on various reef substrates.

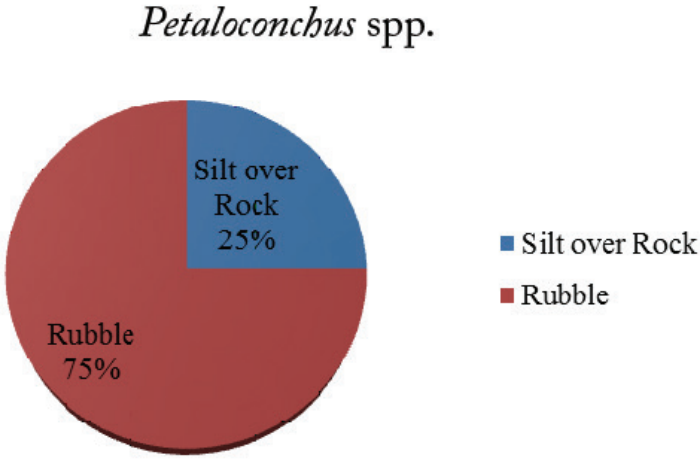

Figure 6. Percentage of *Petaloconchus* spp. on various reef substrates.

SoR, rubble, *Porites*, *Platygyra*, *Favia* and *Goniastrea* (Figure 5). Among these, 34% of the animals were recorded on some abiotic substrates *viz.*, SoR and rubble, and 66% of the individuals were recorded on live corals. A total of four live coral genera were recorded: *Porites* (60%), *Platygyra* (2%), *Goniastrea* (2%) and *Favia* (2%). The highest density of *Thylacodes* was recorded on *Porites* spp. It implies that more than 50% of the animals were found embedded in the live coral colonies of *Porites*. The animals were recorded without an operculum as bright orange "spots" in the dark green *Porites*. *Thylacodes* spp. shells, originally dull white to earthy grey in colour, were observed well-encrusted by epibionts such as calcareous algae and live coral. The length of the vermetid tube emerged out of the live coral substratum was 2–3 mm and ranges approximately 3–8 mm in records of presence in other substrates like rock covered by silt or sand.

Petaloconchus (Lea, 1843)

The genus *Petaloconchus* was represented by only four individuals and the density was 0.4 individuals/100 m^2. The majority (75%) of the individuals were recorded on rubble and the remaining 25% on SoR (Figure 6). The animal shells were evident as erect tubes growing on the rubble and sometimes encrusted by epibionts like calcareous algae. Not a single animal was recorded on live coral colonies.

Discussion

The present study revealed that vermetid density on Paga reef varies with the genera; however, the results of ANOVA shows that there is no significant difference in the

density of the vermetids on different substrates. Hence, individuals of all the three genera are distributed evenly on seven different substrates on Paga reef. Additionally, it was observed that not a single individual of *Ceraesignum* spp. was recorded on barren rubble and no *Petaloconchus* spp. were recorded on live coral colonies. A total of 17% *Ceraesignum* spp. were observed on Faviidae corals with large corallite sizes, whereas 60% of *Thylacodes* were recorded on *Porites*, a genus with small corallite size. The association of the genus *Thylacodes* with live coral is higher than *Ceraesignum*; however, there is no significant difference ($p > 0.05$) between the means of both the genera.

The density of *Ceraesignum* spp. has resulted as 7.7 individuals/100 m^2, which is much less compared to other studies worldwide. Hadfield (1972) recorded the density of another species of *Ceraesignum* (as *D. gregarium* Hadfield & Kay, 1972) as high as 60,000/m^2 on a water-levelled bench at Diamond Head, Oahu. Smalley (1984) studied the effects of various factors on population density of the solitary vermetid *Ceraesignum maxima* on Luminao Barrier Reef, Guam. These studies revealed that the vermetid density between the coral heads ranged from 0–520 individuals/m^2 and on the *Porites lutea* coral heads it ranged from 15–520 individuals/m^2, which are far higher than the records of the present study. This may be attributed to the large range? of substrate availability on Luminao Barrier Reef and Oahu. Additionally, Smalley (1984) recorded 78% of the population attached to the exposed sides of the coral heads whereas the present study revealed that 73% of the population attached to various type of dead substratum on reef flat.

Shima et al. (2010) recorded that the presence of vermetids was strongly associated with growth anomalies of the reef-building coral, *P. lobata*. As well as providing the geological survey of a vermetid reef of the Salento Peninsula, Brachert et al. (2007) showed occurrences within two different settings of the Messinian reef complex: along the shallower seaward portion of the platform edge and in the upper part of the slope, in the transition zone between *Porites* colonies and bio-clastic accumulations. Smalley (1984) also studied the population density of vermetids over the coral heads of *Porites lutea.* This association preference is concordant with the present study consisting of an association of vermetids with the massive coral *Porites lutea* and additionally, the new associations of vermetids with *Favia speciosa*, *Goniastrea pectinata* and *Platygyra* sp., which has revealed novel research. Moreover, the authors have also observed a stronger association of vermetids with *Porites lutea* and *Porites compressa* on other reefs of the Gulf of Kachchh (unpublished work). Hadfield (1972) also recorded the strong association of *Petaloconchus keenae* with *Porites i.e.*, 590 individuals/m^2 of the *Porites* colony, whereas *Petaloconchus* spp. was recorded only on abiotic substrates in the present study.

The impact of the largest species of vermetid gastropod, *Dendropoma maximum*, on the corals revealed that the vermetid gastropod reduces skeletal growth of corals by up to 81% and coral survival by up to 52%, presumably by an unknown mechanism involving the mucus nets (Shima et al. 2010). These significant effects suggest that the interaction between vermetids and corals (regardless of the underlying mechanisms) has the potential to reduce coral cover and alter coral reef community structure (Shima et al. 2010). Hence, it is required to identify the interaction of vermetid-coral asso-

ciation in the GoK. Among all the living coral genera, some vermetids (*Thylacodes*) showed the highest association with *Porites* on Paga Reef. *Porites* is a dominant and frequently encountered coral genus and plays a significant role in community composition on this reef. It is frequently distributed at a variety of reef zones., starting from tidal pools at back reef areas and the reef flat to up to the reef edge with massive as well as submassive growth forms.

Conclusions

The present work brings forth the density, diversity and habitat preferences of three vermetid genera at Paga Reef, in the Gulf of Kachchh, India. The study reveals the occurrence of vermetids on variety of substances. The organisms are distributed in all the reef zones across the intertidal belt of the reef. In spite of the adaptability of vermetids to a large range of substrates and reef zones, their density remains limited on Paga Reef compare to other reef areas worldwide. The study recommends an urgent need to identify the organisms at more detailed taxonomic levels with their respective habitat preferences.

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A new monster from southwest Oregon forests: *Cryptomaster behemoth* sp. n. (Opiliones, Laniatores, Travunioidea)

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Abstract

The monotypic genus *Cryptomaster* Briggs, 1969 was described based on individuals from a single locality in southwestern Oregon. The described species *C. leviathan* Briggs, 1969 was named for its large body size compared to most travunioid Laniatores. However, as the generic name suggests, *Cryptomaster* are notoriously difficult to find, and few subsequent collections have been recorded for this genus. Here, we increase sampling of *Cryptomaster* to 15 localities, extending their known range from the Coast Range northeast to the western Cascade Mountains of southern Oregon. Phylogenetic analyses of mitochondrial and nuclear DNA sequence data reveal deep phylogenetic breaks consistent with independently evolving lineages. We use discovery and validation species delimitation approaches to generate and test species hypotheses, including a coalescent species delimitation method to test multi-species hypotheses. For delimited species, we use light microscopy and SEM to discover diagnostic morphological characters. Although *Cryptomaster* has a small geographic distribution, this taxon is consistent with other short-range endemics in having deep phylogenetic breaks indicative of species level divergences. Herein we describe *Cryptomaster behemoth* **sp. n.**, and provide morphological diagnostic characters for identifying *C. leviathan* and *C. behemoth*.

Keywords

Short-Range Endemic, DNA barcoding, cryptic species, Bayes Factor Delimitation, genealogical congruence

Introduction

With more than 4100 described species (Kury 2013), the Opiliones suborder Laniatores is incredibly diverse, and many more species likely await discovery. Despite their diversity, Laniatores is understudied, and many aspects of their phylogeny and evolution remain unknown (Giribet and Sharma 2015). An example is the monotypic genus *Cryptomaster* Briggs, 1969 and its large-bodied (~4 mm) but cryptic species, *C. leviathan* Briggs, 1969. *Cryptomaster leviathan* was described from a single locality near the coastal town of Gold Beach, Oregon in the Pacific Northwest (PNW) of the United States. Briggs noted that this new species is remarkably large in size relative to most other Nearctic Laniatores. However, *C. leviathan* did not receive further study and remained known only from the type locality until recent published records from the Cascade Range (Derkarabetian et al. 2010) and north of the type locality in the Coast Range (Shear et al. 2014). Given that many Laniatores taxa show high species diversity in small geographic regions (e.g., Ubick and Briggs 1989, Briggs and Ubick 1989, Ubick and Briggs 2002, Derkarabetian and Hedin 2014), these extensions of the known distribution across different mountain ranges indicate the potential for additional species within *Cryptomaster*.

In this study we increase the number of *Cryptomaster* localities to 15, all from mountainous southwestern Oregon. We use multi-locus DNA sequence data to investigate population structure and divergence from samples throughout this range. We recover a deep and concordant phylogenetic split for five loci that is indicative of species level divergence. Discovery and validation species delimitation approaches are used to assess support for multiple species within *Cryptomaster*. Also, we examine morphological differentiation between the divergent genetic groups to provide diagnostic characters for species identification. We delimit two species within *Cryptomaster*, and here describe *Cryptomaster behemoth* sp. n. This research highlights the importance of short-range endemic arachnids for understanding biodiversity (Harvey 2002, Harvey et al. 2011, Keith and Hedin 2012), and further reveals mountainous southern Oregon as a hotspot for endemic animal species (e.g. Shelley 1995, Cokendolpher et al. 2005, Mead et al. 2005, Leonard et al. 2011, Griswold et al. 2012).

Methods

Specimen collection

We collected 77 *Cryptomaster* individuals from 14 localities in the Coast and Cascade Mountains of southern Oregon (Fig. 1, Suppl. material 1: Table S1), including from near the type locality of *C. leviathan* (Gold Beach, OR). *Cryptomaster* are primarily found in mature coniferous forests under woody debris. We attempted collections specifically targeting *Cryptomaster* further north and south of our samples, but these were unsuccessful. Individuals were preserved in 100% EtOH (Koptec) or 80% EtOH

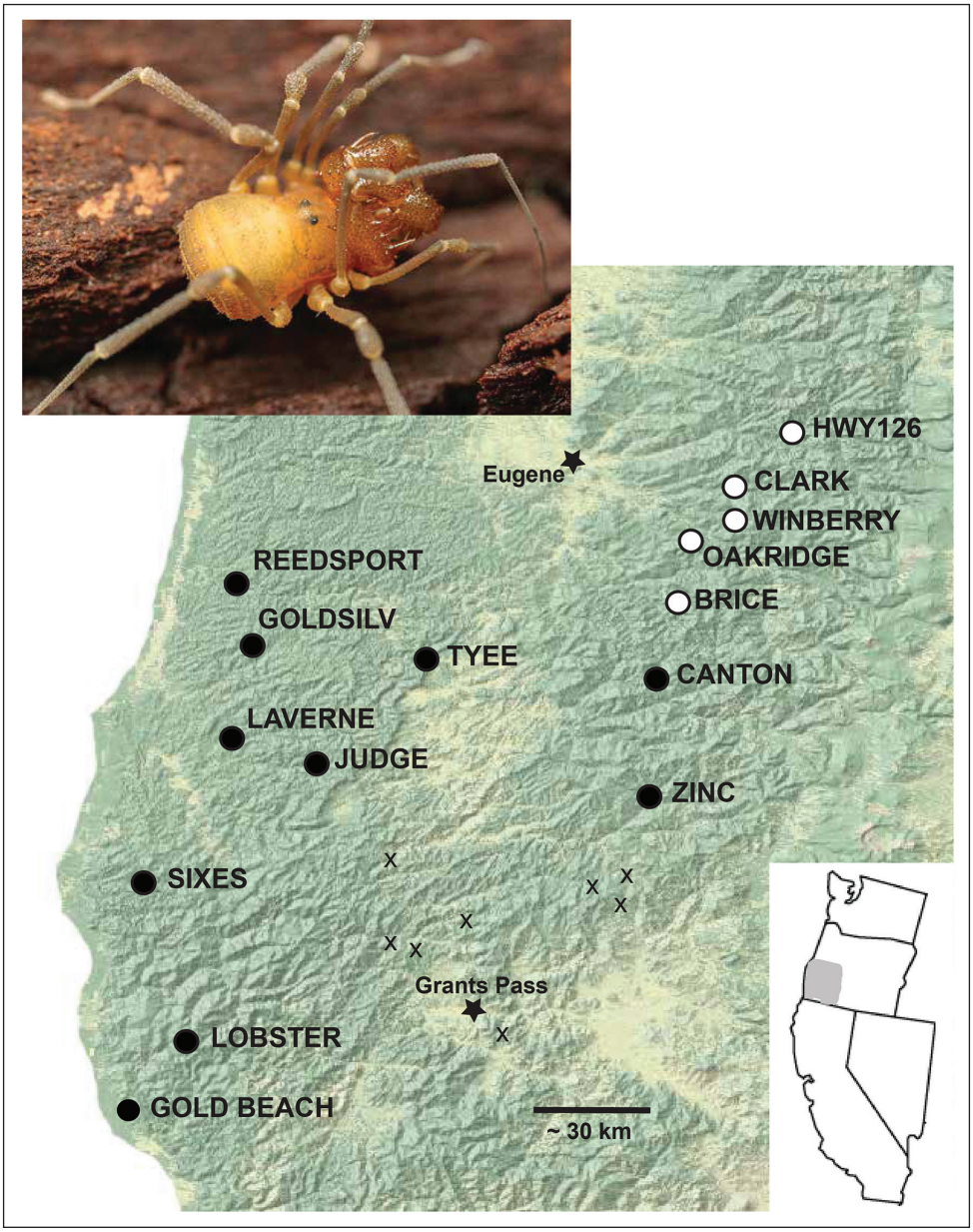

Figure 1. Distribution of *Cryptomaster leviathan* (closed circles) and *C. behemoth* (open circles) in southwestern Oregon. Small X's represent locations in a potential Cascade to Coast corridor where we have collected other travunioids, but not *Cryptomaster*. Inset: male *C. leviathan* from the Sixes River location.

for genetic or morphological analysis, respectively. *Speleomaster lexi* Briggs, 1974 was used as an outgroup, following both morphological (Briggs 1974) and molecular evidence (Derkarabetian et al. 2010) that indicate a *Speleomaster* + *Cryptomaster* sister

group relationship. Locality data for all specimens are available on the Symbiota Collections of Arthropods Network (http://symbiota4.acis.ufl.edu/scan/portal/index.php). Specimens are housed in the San Diego State Terrestrial Arthropod Collection (SDSU_TAC); type specimens are deposited at the California Academy of Sciences (CASENT9039221).

Genetic sampling

Genomic DNA was extracted from multiple legs per specimen using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and the manufacturer's protocol. Sequence data were obtained for the mitochondrial gene cytochrome c oxidase subunit I (COI), and four nuclear loci (Toll, putative; F-box/LRR-repeat protein, putative; Protein phosphatase 2A regulatory subunit A, putative; Neuromusculin, putative). DNA amplification was performed in a total volume of 25 μL with 1.6 units Platinum Taq (Invitrogen, Carlsbad, CA), 2.2 mM $MgCl_2$, 1X PCR buffer, 0.2 mM each dNTP, and 0.4 μM of each primer (Suppl. material 1: Table S2). For COI, cycling conditions consisted of 94 °C for 2 minutes, then 30 cycles of 94 °C for 30 seconds, 50 °C (+ 0.2 °C/cycle) for 40 seconds, and 72 °C for 1.5 minutes, followed by 5 cycles of 94 °C for 30 seconds, 56 °C for 40 seconds, and 72 °C for 1.5 minutes, and then a final 72 °C for 5 minutes. For the nuclear loci, cycling conditions consisted of 94 °C for 3 minutes, then 10 cycles of 94 °C for 1 minute, 63 °C (-0.5 °C/cycle) for 1 minute, and 72 °C for 1 minute, followed by 30 cycles of 94 °C for 15 seconds, 58 °C for 1 minute, and 72 °C for 1 minute, and then a final 72 °C for 5 minutes. PCR products were purified using Montage filter plates and sequenced in both directions by Macrogen USA using the amplification primers. Sequences were edited in Geneious Pro 6 (Kearse et al. 2012) and aligned using MAFFT (Katoh 2013). For heterozygous nuclear sequences, Phase v.2.1.1 (Stephens et al. 2001, Stephens and Scheet 2005) was used to bioinformatically infer alleles.

Species discovery

Phylogenetic and genetic distance based discovery analyses were used to generate species hypotheses. Maximum likelihood (ML) analyses of individual loci were conducted with RAxML BlackBox v.8.1.11 (Stamatakis et al. 2008) with the GTR + Γ model on the CIPRES web server (Miller et al. 2010), with automatic bootstrap termination. For ML analysis of each locus and *BEAST analyses, the COI dataset was partitioned by codon position, while the less-variable nuclear loci datasets were not partitioned. Genetic diversity statistics were calculated using MEGA v6.06 (Tamura et al. 2013). Automatic Barcode Gap Discovery (ABGD) was applied to the COI data using default settings and the transition/transversion ratio set at 2 (Puillandre

et al. 2011). POFAD was run with all nuclear loci using standardized distances calculated with the genpofad option (Joly and Bruneau 2006). POFAD distance results were imported into SplitsTree4 (Huson and Bryant 2006) for reconstruction of a NeighborNet network.

Species validation

Based on the results of gene tree and species discovery approaches, we compared four alternative species hypotheses using Bayes Factor Delimitation (Grummer et al. 2014). *BEAST v1.8.1 (Drummond et al. 2012) was run with the four nuclear loci under four different species models (see Results, Table 1). Briefly, these species models consisted of: 1) a single species (*C. leviathan*), 2) two species, following POFAD results, 3) three species, following the COI gene tree, and 4) four species, following ABGD results. Analyses were run with a strict molecular clock and sequence models determined by jModeltest2 (Guindon and Gascuel 2003, Darriba et al. 2012). Analyses were run for 100,000,000 generations with data stored every 10,000 generations. Log files for all *BEAST runs were visualized in Tracer v.1.6 (Rambaut et al. 2014). Analyses run with GTR sequence models failed to converge, and thus HKY models were applied with the other model parameters as determined by jModeltest2. The Marginal Likelihood Estimate (MLE) was generated based on path sampling (Lartillot and Philippe 2006) and stepping stone (Xie et al. 2011) methods with a chain length of 100,000 generations and pathSteps set at 100. Analyses were run twice and the average MLE was taken for each species model. The Bayes factor was determined by $2*(-\ln_{HypA} - -\ln_{HypB})$, with values greater than 10 indicating decisive support for a hypothesis (Kass and Raftery 1995).

Morphological methods

Linear measurements were taken as in Derkarabetian and Hedin (2014) using an Olympus SZX12 dissecting microscope with an ocular micrometer. For SEM imaging, genitalia were extended from the body by opening the genital and anal opercula, and inserting a small blunt insect pin through the anal operculum and pushing the genitalia out. Specimens were dried using a critical point dryer, mounted on Ted Pella aluminum SEM stubs using copper conductive tape and coated with a 0.6 nm platinum coat. Multiple coats were applied in order to ensure proper coverage and to prevent charging. Coated specimens were imaged on a Quanta 450 SEM at the San Diego State University Electron Microscope Facility. Additionally, genitalia were examined using an Olympus BX40 compound microscope with a drawing tube. Genitalia were dissected from the body as above, then cleared in 10% KOH before viewing.

Results

Gene trees and species discovery

Genetic sampling results, GenBank accession numbers, and genetic diversity statistics are summarized in Suppl. material 1: Table S3. Sequence alignments of phased data have been submitted to DRYAD doi:10.5061/dryad.76rb1. Maximum likelihood analysis of each locus revealed a deep and concordant phylogenetic split within *Cryptomaster* (Figs 2, 3, Suppl. material 2: Figs S1–5). All loci show strong support (bootstrap >83%) for a clade distributed in the Coast Range and southwestern Cascade Range. A second clade with a relatively restricted distribution occurs further northeast

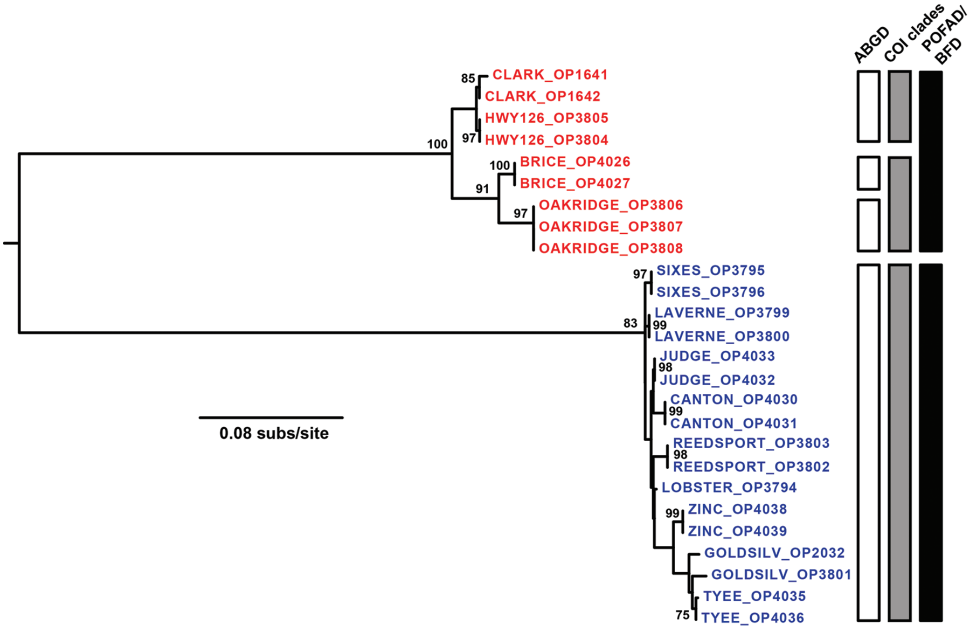

Figure 2. Maximum likelihood COI gene tree with vertical bars showing results of discovery and validation analyses. Blue text = *C. leviathan*, red text = *C. behemoth*. Numbers adjacent to nodes indicate bootstrap support greater than 70%. The tree was rooted with *Speleomaster lexi* (not shown, see Suppl. material 2: Fig. S1).

Table 1. Results of Bayes Factor Delimitation analysis. Species hypotheses are indicated in Fig. 2. Marginal likelihood estimates (MLE) from path sampling (PS) and stepping stone (SS) analyses are shown, with corresponding Bayes Factor (BF) values.

	MLE (PS)	BF	MLE (SS)	BF
4 Species	-4939.87	12.14	-4942.67	13.03
3 Species	-4940.62	13.63	-4943.17	14.03
2 Species	-4933.80	-	-4936.16	-
1 Species	-5010.68	153.75	-5014.01	155.70

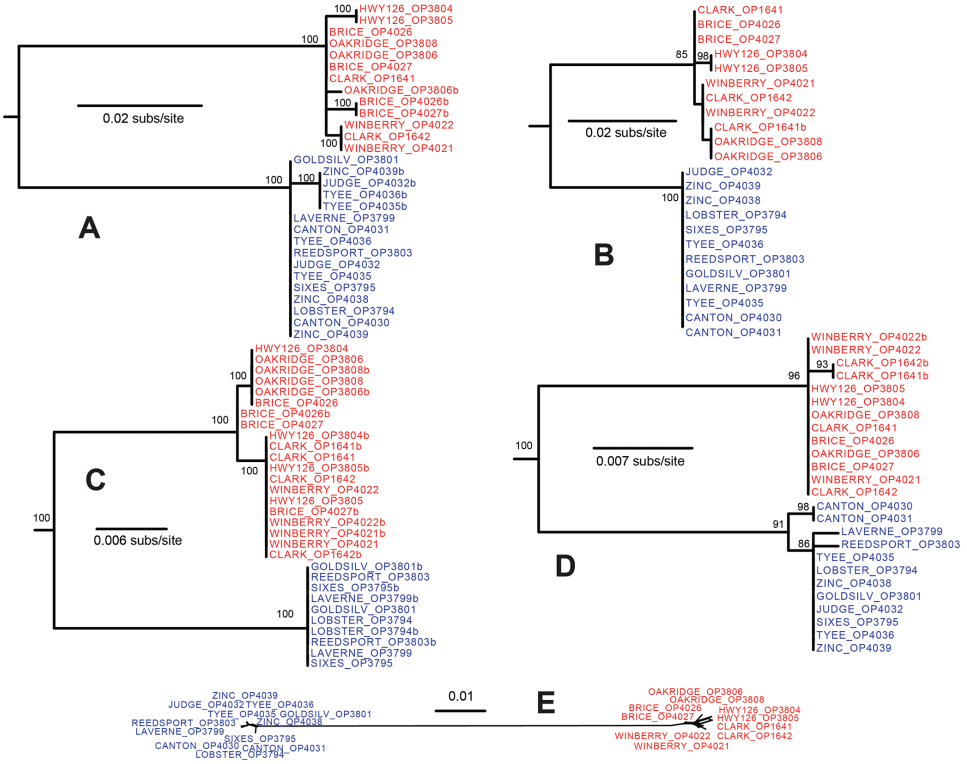

Figure 3. A–D Maximum likelihood nuclear gene trees; numbers adjacent to nodes indicate bootstrap support greater than 70%. Trees were rooted with *Speleomaster lexi* (not shown, see Suppl. material 2: Fig. S2–5). Blue text = *C. leviathan*, red text = *C. behemoth*. **A** Toll **B** F-box/LRR-repeat protein **C** phosphatase 2A protein **D** Neuromusculin **E** NeighborNet network generated from summary distances (ingroup nuclear data only) calculated in POFAD.

in the western Cascade Range (Figs 1–3). Within these clades there is little supported phylogenetic structure with the exception of COI, which further divides the northern Cascade group into a well-supported clade (BS = 91%) consisting of Oakridge+Brice, and a clade with low support (BS = 57%) consisting of Clark+HWY126. The mean p-distance between the two primary clades for COI was 15.5%, and ranged from 3.6-8.2% for the nuclear loci (Suppl. material 1: Table S3).

ABGD analysis of the COI data supported a four species hypothesis (Fig. 2). One species consists of the well-supported COI clade that is distributed in the Coast Range and southwestern Cascades (= *C. leviathan*). The remaining three ABGD species occur further north in the Cascade Range and consist of Clark+HWY126, Oakridge, and Brice. POFAD analysis of the nuclear data resulted in two clusters with minimal internal divergence, consistent with the deep phylogenetic split found in all nuclear loci (Fig. 3).

Species validation

Multiple species hypothesis models were tested using Bayes Factor Delimitation (Fig. 2, Table 1). These hypotheses consisted of 1) a single species of *Cryptomaster*, following Briggs 1969, 2) two species, following POFAD results, 3) three species following the major splits observed in the COI gene tree (*C. leviathan*, Clark+HWY126, Oakridge+Brice), and 4) the four species indicated by the ABGD analysis. BF results based on MLE from both path sampling and stepping stone methods (Table 1) indicate decisive support for the two species hypothesis over alternative hypotheses, with a single-species hypothesis most strongly rejected.

Taxonomy

We note that within each species of *Cryptomaster* two forms are present, a larger and a smaller form, that show a bimodal size distribution (Fig. 4). The basis for these

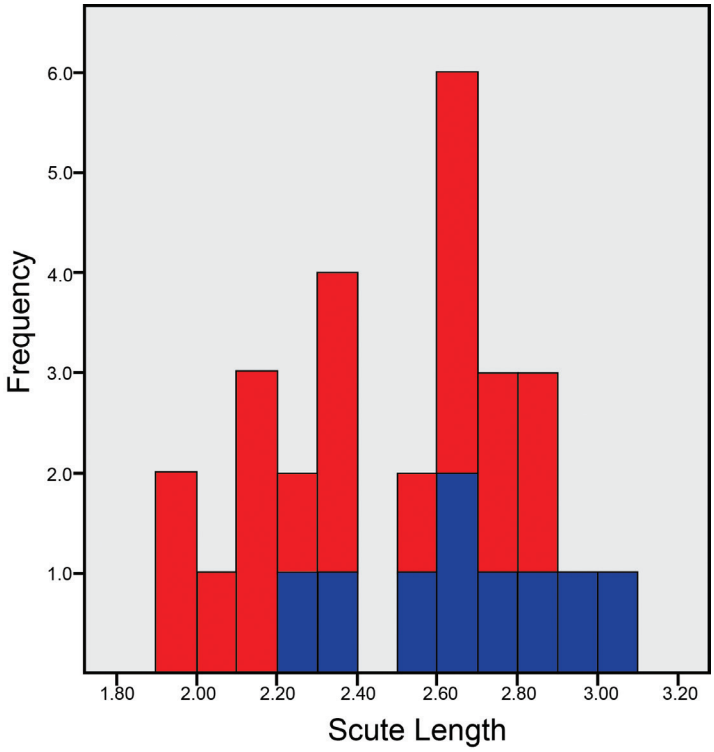

Figure 4. Scute length (mm) distribution of *Cryptomaster*. Figure made using SPSS 22 (IBM Corp. 2013). Blue = *C. leviathan*, red = *C. behemoth*.

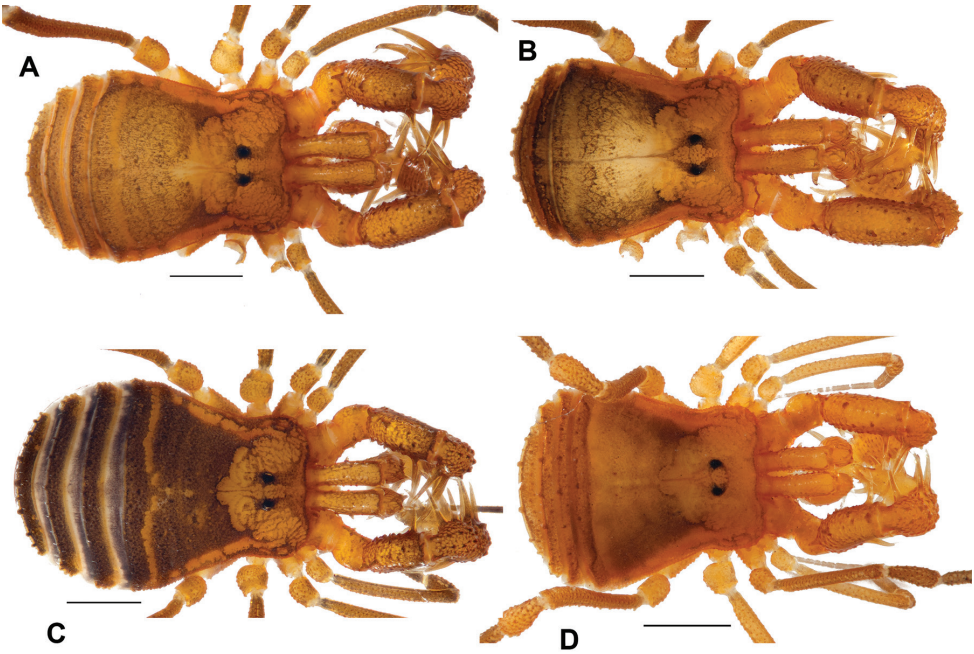

Figure 5. *Cryptomaster* dorsal coloration. **A** Male *C. leviathan* (SDSU_OP4039) **B** Holotype male *C. behemoth* (CASENT9039221, SDSU_OP4026) **C** Female *C. leviathan* (SDSU_OP4037) **D** Allotype female *C. behemoth* (CASENT9039221, SDSU_OP4029). Scale bars: 1 mm.

two forms is unknown – the different forms can be found in both sexes, in both species, and from the same localities. Additionally, the two forms are not genetically divergent as COI sequences from different individuals from the same locality are typically most closely related (Fig. 2), and little intraspecific variation exists for the nuclear genes (Fig. 3). Since the original diagnostic characters for the genus *Cryptomaster* (hind claws meeting in 180° opposition, distal swelling on tibia of second leg of males) apply to all examined specimens, we do not redescribe the genus. Here we redescribe *C. leviathan* (Fig. 5A, C) from type specimens held at the California Academy of Sciences (CAS) and newly collected material, increasing our understanding of *C. leviathan* with new localities (Fig. 1, Suppl. material 1: Table S1) and morphological data (Suppl. material 1: Table S4) from across its range. Additionally, we describe the new species *C. behemoth* sp. n. (Fig. 5B, D), providing diagnostic characters for both species (Fig 6).

Morphological abbreviations: DCS = distal cheliceral segment, GO = genital operculum, LII = leg II, OC = ocularium, PCS = proximal cheliceral segment, PF = pedipalpal femur, PT = pedipalpal trochanter, SBT = seta-bearing tubercle. All measurements are in millimeters. Morphological images have been submitted to Morphbank.

Cryptomaster leviathan Briggs, 1969

Figures: map 1; habitus 5A, C, 7A, C, E; somatic 6A, 8A, C, E; penis 6C, E, 9A, C, E; ovipositor 10A–E

Cryptomaster leviathan Briggs, 1969: 41–43, figures 15–25.

Type material examined. Holotype male and five female paratypes from 4.5 miles south of Gold Beach, Curry County, Oregon, 29 January 1967, under spruce bark in virgin spruce forest, coll. T. Briggs, V. Lee, and K. Hom.

Diagnosis. This species differs from *C. behemoth* in having the enlarged SBT of PT acute (Fig. 6A), and keel-shaped protrusion of dorsal plate of penis with apical pair of spines fully erect and directed along the longitudinal axis of the penis (Fig. 6C, E).

Genetic data. GenBank Accession numbers: KU059639-KU059655, KU059667-KU059678, KU059690-KU059701, KU059713-KU059717, KU059729-KU059740.

Morphbank images.

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<http://www.morphbank.net/?id=856245>, 4 SEM images

Redescription. MALE: Measurements of holotype male, with the average and range of all three specimens measured in parentheses (Suppl. material 1: Table S4).

Body length 3.44, scute length 2.75 (2.71; 2.5–2.89), scute width 3.06 (2.75; 2.5–3.06), prosoma width 2.05 (1.95; 1.81–2.05). Shoulder tubercles present but small. Scute microgranulate. Holotype discolored due to preservation; for other specimens, integument color contrasts dorsally at midline between prosoma and opisthosoma, although not as strongly as in females. OC width 0.59 (0.55; 0.49–0.59). Ventral surface microgranulate. GO missing in holotype, length 0.3–0.34, width 0.28–0.31.

PT with acute mesal SBT. PF length 2.01 (1.98; 1.8–2.13), PF depth 0.72 (0.69; 0.64–0.72), with 7 (sometimes 5–6) spines, with the basal pair prominent, 3 (sometimes 4) enlarged dorsal spines, and 2 enlarged prolateral spines distally. PCS with 3 anterior spines dorsally and with 2 or 3 small retrolateral spines; DCS with 2 rows of small, dorsal, forward-facing acute SBTs. PCS width 0.42, DCS length 1.6, DCS width 0.46.

Trochanter 0.57, femur 0.92, patella 0.7, tibia 1.48, metatarsus 1.72, tarsus 1.04. LII length 11.19 (11.31; 10.69–12.06): trochanter 0.62 (0.6; 0.58–0.62), femur 2.73 (2.73; 2.55–2.91), patella 0.94 (0.89; 0.77–0.95), tibia 2.3 (2.37; 2.28–2.55), meta-

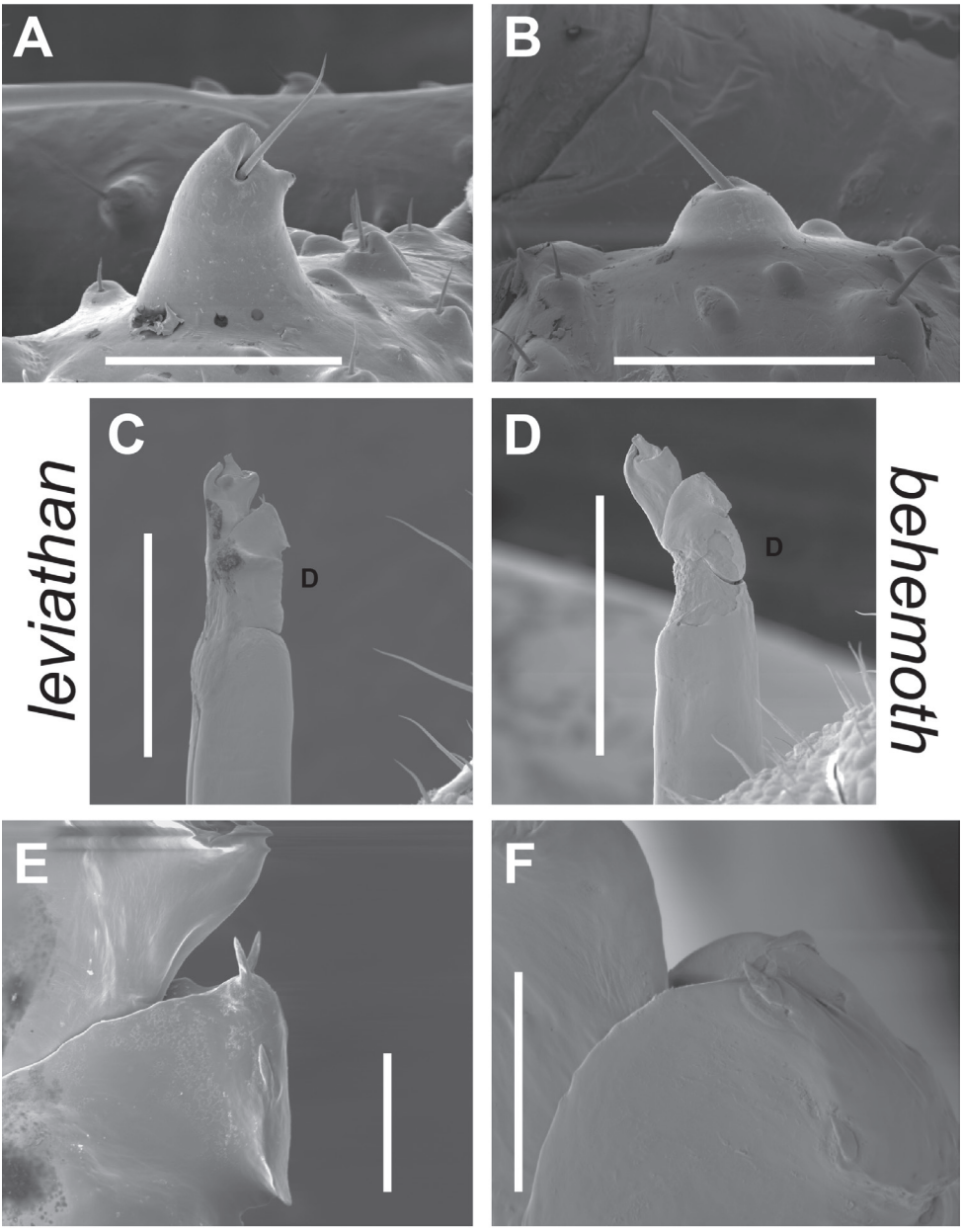

Figure 6. *Cryptomaster* diagnostic morphological characters. **A–B** Lateral view of seta-bearing tubercle found on ventral side of palpal trochanter **A** *C. leviathan*, Judge Hamilton **B** *C. behemoth*, Brice Creek **C–D** Lateral view of male penis ("D" indicates dorsal side) **C** *C. leviathan*, Laverne County Park. **D** *C. behemoth*, Oakridge **E–F** Close-up lateral view of penis tip showing spines on dorsal plate **E** *C. leviathan*, Laverne County Park, detail showing erect apical spines **F** *C. behemoth*, Oakridge, detail showing appressed apical spines. Scale bar: 200 μm (**A, B**); 300 μm (**C–D**); 40 μm (**E–F**).

tarsus 2.49 (2.61; 2.49–2.8); tarsus 2.1 (2.11; 1.99–2.25); tibia distally and ventrally swollen, with 5 rounded SBTs, 1–3 with setae twisted. Tarsal count 5-[11–15]-5–6.

Penis elongate; glans laterally compressed, dorsal plate extending outward into a more angled and acute keel shaped protrusion, with two pairs of spines, apical pair erect (pointing along the longitudinal axis of the penis), subapical pair appressed to dorsal plate; ventral plate cultriform with dorsally curved apical process.

FEMALE: Nineteen total individuals examined, including five paratypes; average measurements taken for subset (Suppl. material 1: Table S4), with range of all seven specimens measured in parentheses. Descriptive characters as for males unless otherwise noted.

Scute length 2.82 (2.69–3.03), scute width 3.01 (2.84–3.13), prosoma width 1.96 (1.8–2.14). Relative to males very dark, with strong contrast at midline between light-brown prosoma and dark-brown opisthosoma. OC width 0.56 (0.51–0.6). GO length 0.37 (0.33–0.39), width 0.38 (0.34–0.4).

PT mesal SBT acute. PF length 1.84 (1.68–1.94), PF depth 0.63 (0.58–0.67), usually with 5 (up to 7) ventral spines, 4 dorsal spines (2 to 6), and 2 distal prolateral spines. PCS with 2–3 anterior spines dorsally, with 1–4 small retrolateral spines.

LII length 10.23 (9.61–10.81): trochanter 0.58 (0.54–0.67), femur 2.53 (2.35–2.63), patella 0.83 (0.76–0.89), tibia 2.16 (1.99–2.29), metatarsus 2.39 (2.16–2.55), tarsus 1.92 (1.81–2.0); tibia without distal ventral swelling.

Ovipositor with four lobes, lateral lobes largest with seven apical setae, and a single large spine with a bifurcate tip, ventral lobe smallest.

Other material examined. See Suppl. material 1: Table S1 and S4 for locality information of all specimens examined.

Distribution and habitat. For specific localities, habitats, and microhabitats see Suppl. material 1: Table S1. This species is distributed in southwestern Oregon including throughout the southern Oregon Coast Range from the Umpqua River to the Coquille River, and south into the Klamath Mountains to the Rogue River. The range extends east to the southern Oregon Cascade Mountains in the South Umpqua and North Umpqua River Basins. *Cryptomaster leviathan* is typically associated with mature coniferous or mixed coniferous and hardwood forests, but has also been found in disturbed forests and forests with few conifers. Specimens are most often found under large woody debris associated with decaying logs and stumps, and in *Acer* and *Polystichum* leaf litter.

Cryptomaster behemoth **Starrett & Derkarabetian, sp. n.**

http://zoobank.org/6F8DCC84-A59D-4FEC-AB7C-0A05678D8223

Figures: map 1; habitus 5B, D, 7B, D, F; somatic 6B, 8B, D, F; penis 6D, F, 9B, D, F; ovipositor 10F

Cryptomaster leviathan [partim] Derkarabetian et al. (2010)
Cryptomaster leviathan [partim] Shear et al. (2014)

Etymology. The specific epithet is a noun in apposition, which refers to the large size of this species. Like *leviathan*, the specific epithet *behemoth* is derived from Hebrew; these are the names of two large and powerful beasts mentioned in the Book of Job.

Type material. Holotype male and allotype female (deposited in CAS, CASENT9039221; SDSU_OP4026, SDSU_OP4029) from near Brice Creek, Brice Creek Road, 3.3 miles southeast of Forest Service Road 17, Umpqua National Forest, Lane County, Oregon; N43.6749°, W122.7290°; elevation 418 m; 29 March 2015; habitat: *Acer macrophyllum*, *Thuja plicata*, *Pseudotsuga menziesii*, *Polystichum munitum* forest; in and under large woody debris and other forest litter; collectors: J. Starrett, S. Derkarabetian, A. Cabrero, C. Richart. **Paratypes:** One female (deposited in CAS) from identical locality and information as holotype and allotype. Three females (two deposited in CAS, 1 deposited in SDSU_TAC; SDSU_TAC0000023) from Goodman Creek Road, off OR 58, northwest of Oakridge, Lane County, Oregon; N43.8429°, W122.6854°; elevation 340 m; 19 August 2014; habitat: old growth Douglas fir forest/woody debris; collectors: M Hedin, E Ciaccio, A Cabrero, J Starrett, S Derkarabetian. Two females (one each deposited in CAS and SDSU_TAC; SDSU_TAC0000234) from Brice Creek, Brice Creek Road, 3.3 miles southeast of FS 17, Umpqua National Forest, Lane County, Oregon; N43.6760°, W122.7290°; elevation 418 m; 29 March 2015; habitat: *Acer macrophyllum, Thuja plicata, Pseudotsuga menziesii, Polystichum munitum* forest; woody debris and litter; collectors: J Starrett, S Derkarabetian, A Cabrero, C Richart. One female (deposited in SDSU_TAC; SDSU_TAC0000028) from Highway 126, near Quartz Creek Road, Lane County, Oregon; N44.1248°, W122.3846°; elevation 300 m; 19 August 2014; habitat: decent *Pseudotsuga menziesii* forest; woody debris; collectors: M Hedin, E Ciaccio, A Cabrero, J Starrett, S Derkarabetian.

Diagnosis. This species differs from *C. leviathan* by having the enlarged SBT of PT rounded (Fig. 6B), and keel-shaped protrusion of dorsal plate of penis with apical pair of spines appressed and perpendicular to the longitudinal axis of the penis (Fig. 6D, F).

Genetic data. GenBank Accession numbers: HM056724, KU059631-KU59638, KU059657-KU059666, KU059680-KU059689, KU059703-KU059712, KU059719-KU59728.

Morphbank images.

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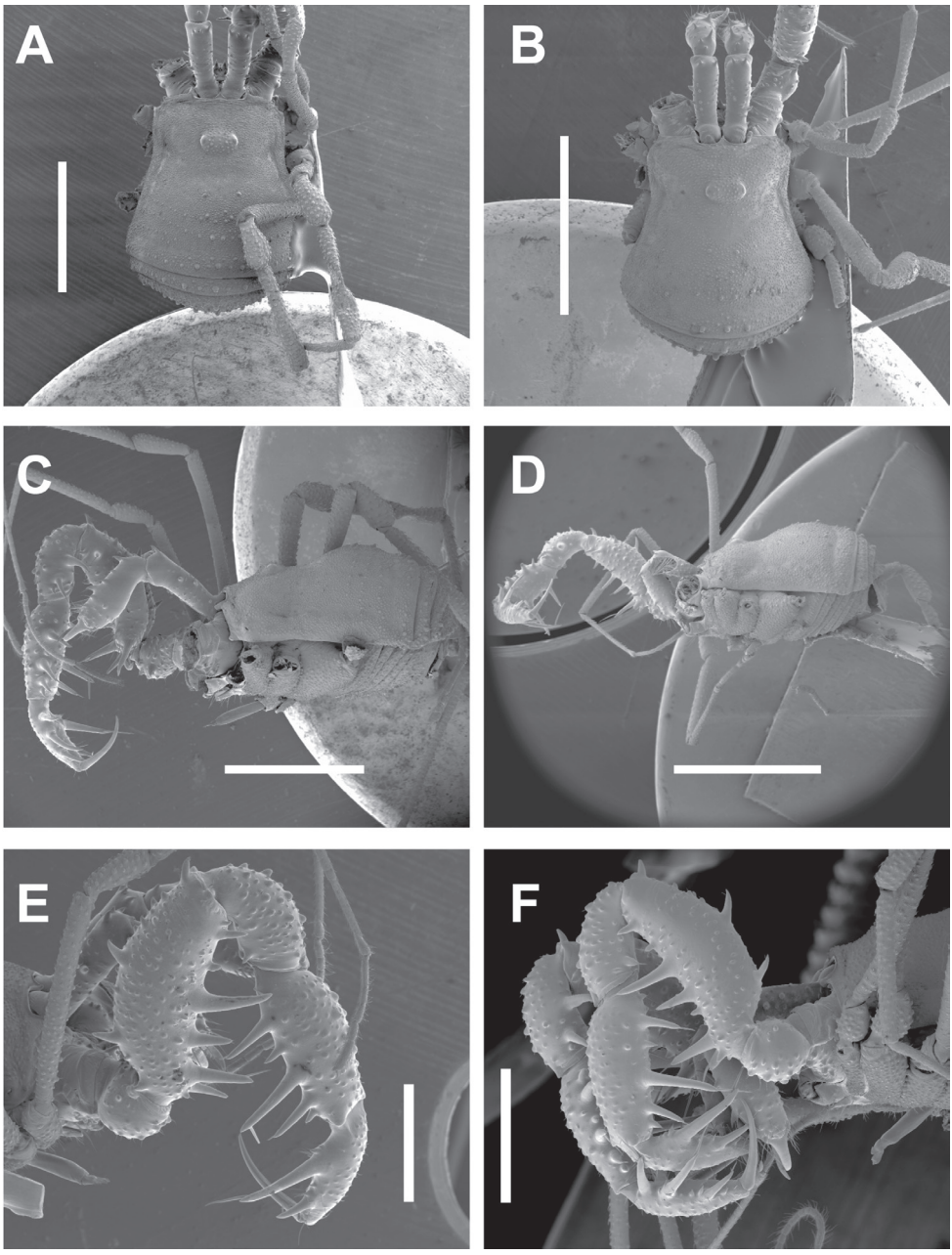

Figure 7. *Cryptomaster* habitus. **A–B** Habitus, dorsal **A** *C. leviathan*, Judge Hamilton **B** *C. behemoth*, Brice Creek **C–D** Habitus, lateral **C** *C. leviathan*, Judge Hamilton **D** *C. behemoth*, Oakridge (female) **E–F** Pedipalp, retrolateral **E** *C. leviathan*, Judge Hamilton **F** *C. behemoth*, Oakridge. Scale bar: 2 mm (**A, B, C, D**); 1 mm (**E–F**).

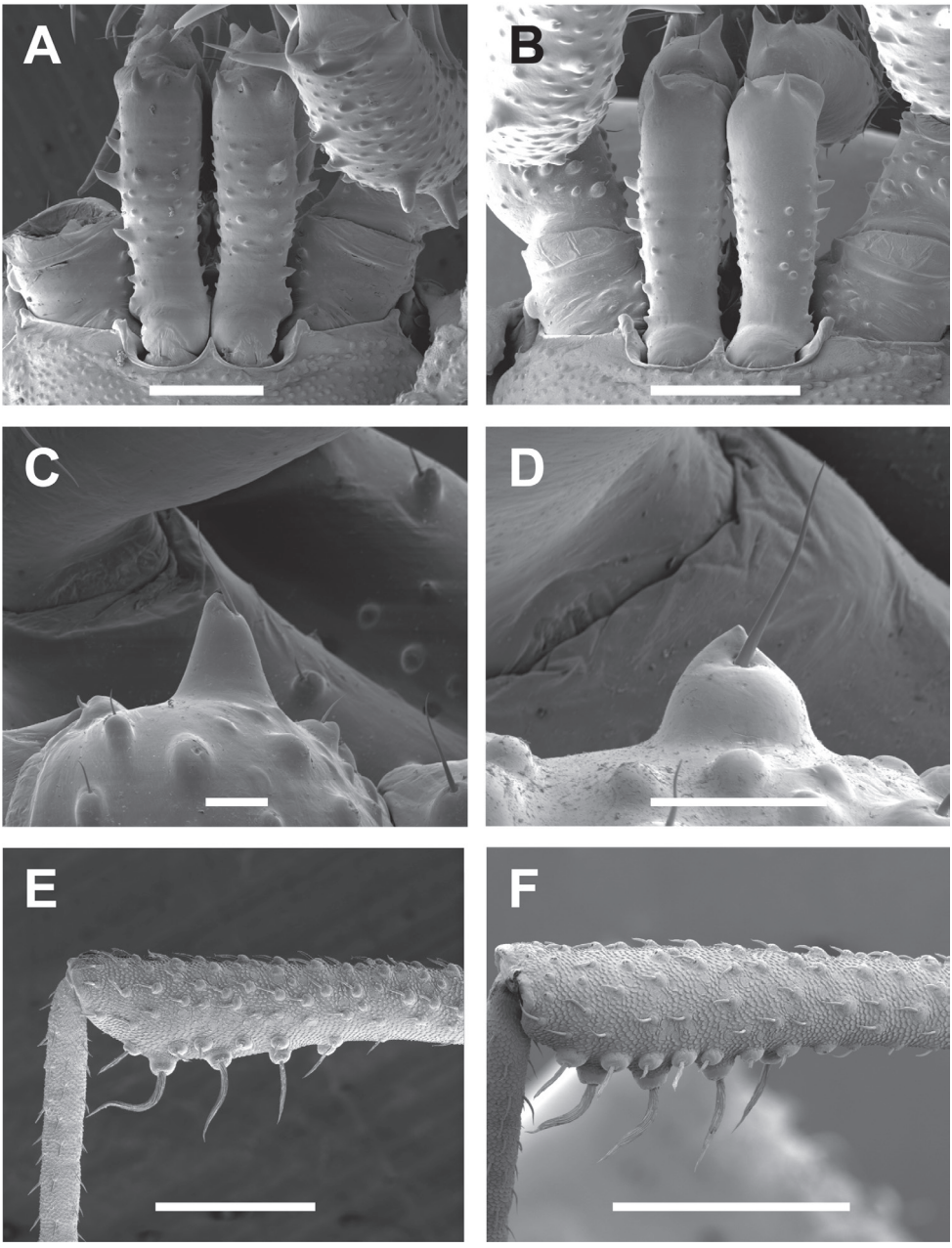

Figure 8. *Cryptomaster* appendages. **A–B** Proximal cheliceral segment, dorsal **A** *C. leviathan*, Reedsport **B** *C. behemoth*, Oakridge **C–D** SBT of palpal trochanter, lateral **C** *C. leviathan*, Laverne County Park **D** *C. behemoth*, Oakridge **E–F** Leg II tibia, distal swelling, retrolateral **E** *C. leviathan*, Judge Hamilton **F** *C. behemoth*, Oakridge. Scale bar: 500 μm (**A, B**); 100 μm (**C, D**); 400 μm (**E, F**).

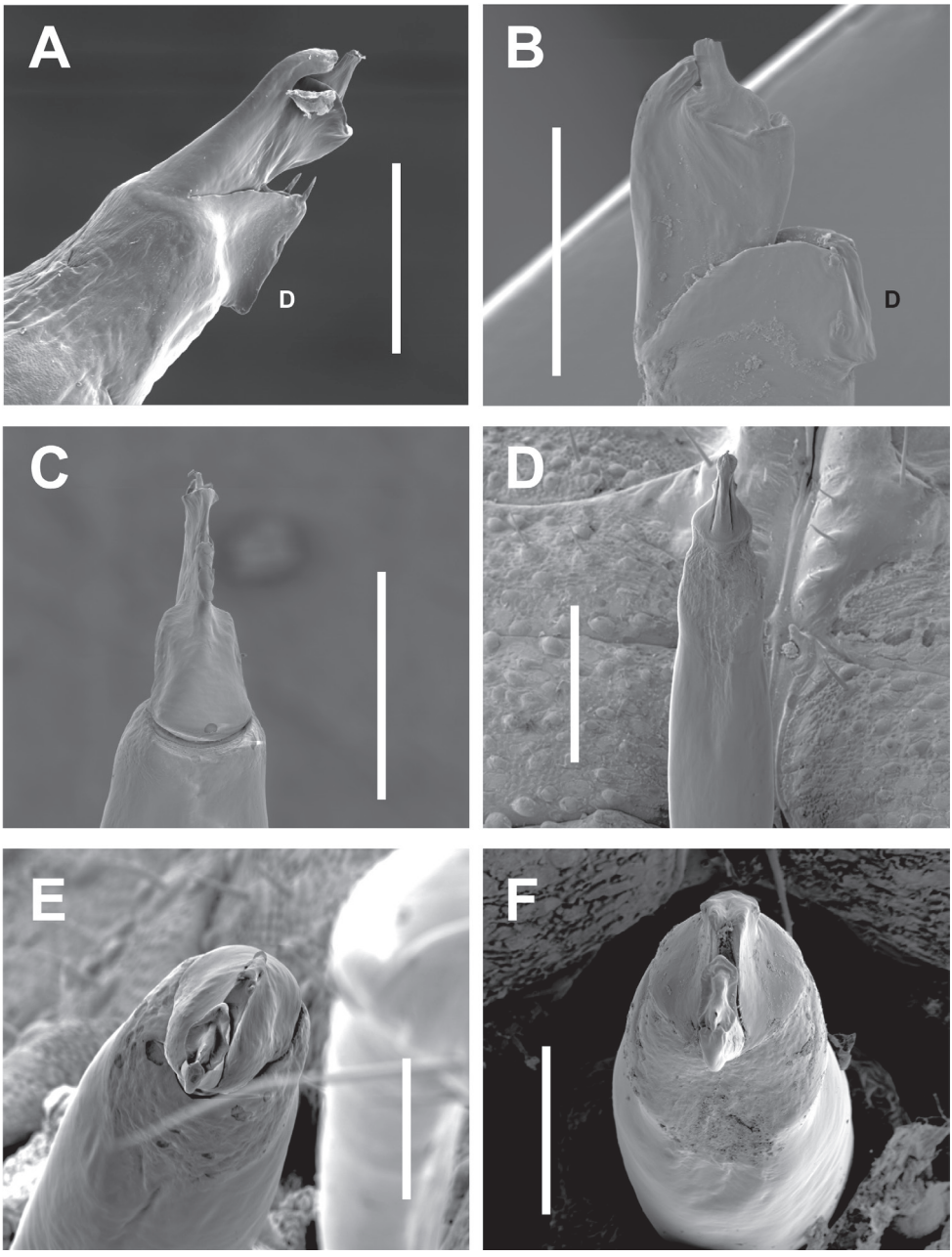

Figure 9. *Cryptomaster* penises. **A–B** Apicolateral ("D" indicates dorsal side) **A** *C. leviathan*, Laverne County Park **B** *C. behemoth*, Brice Creek **C–D** Apicodorsal **C** *C. leviathan*, Judge Hamilton **D** *C. behemoth*, Oakridge **E–F** Apical **E** *C. leviathan*, Judge Hamilton **F** *C. behemoth*, Brice Creek. Scale bar: 100 μm (**A, B, E, F**); 200 μm (**C, D**).

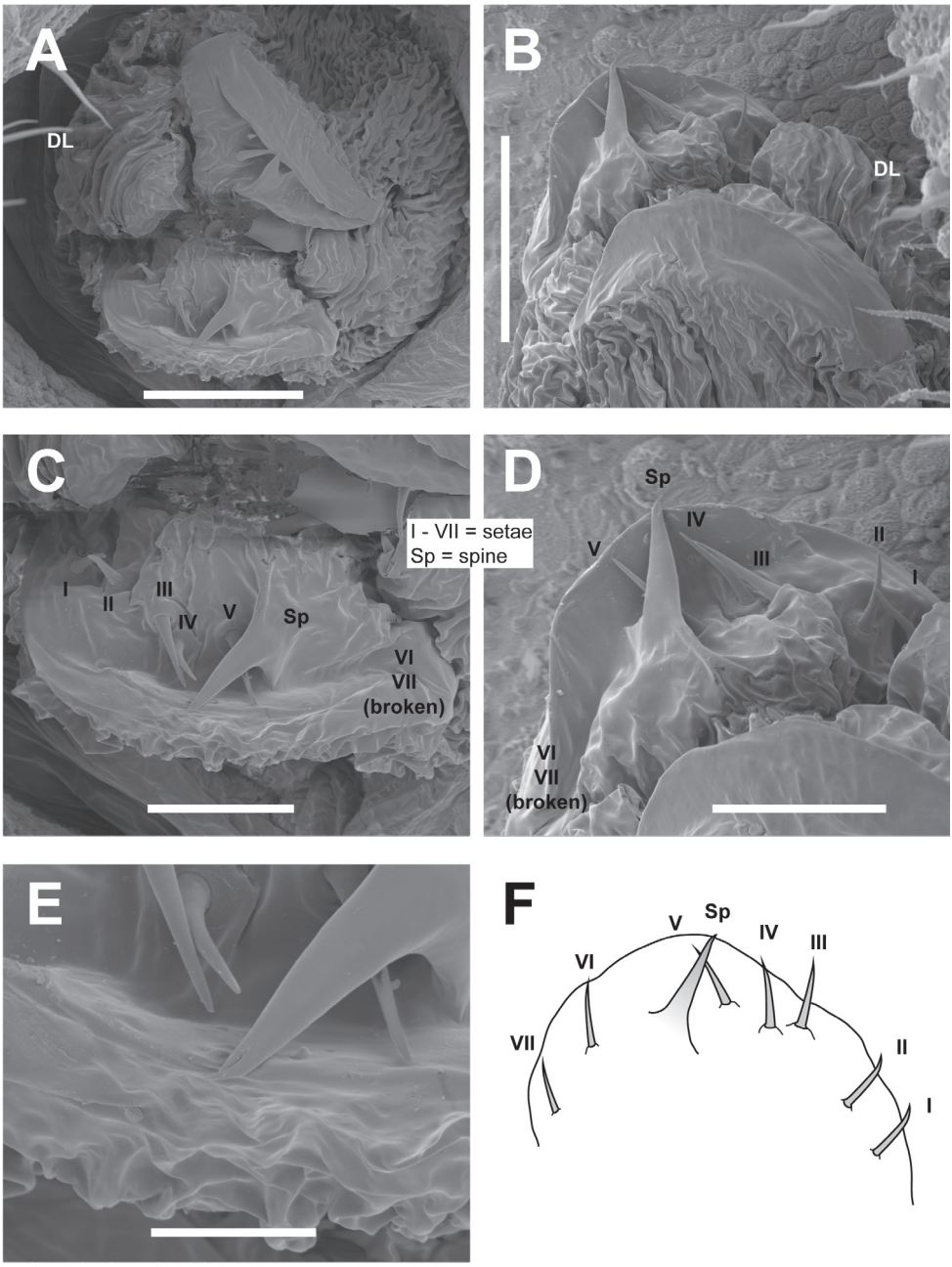

Figure 10. *Cryptomaster* ovipositors. **A–E** *C. leviathan*, Reedsport **A** Apical ("DL" indicates dorsal lobe) **B** Lateral **C** Apical, emphasizing setae and spine arrangement **D** Lateral, emphasizing setae and spine arrangement **E** Apical, spine **F** *C. behemoth*, Oakridge, sagittal view, emphasizing setae and spine arrangement, 40×. Scale bar: 100 μm (**A, B**); 50 μm (**C, D**); 25 μm (**E**).

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SDSU_OP1642, GUID: 8558ef80-a8c7-439d-bd93-dba8ec8d11d4
<http://symbiota4.acis.ufl.edu/scan/portal/collections/individual/index.php?occid=14702250>, 1 image

Description. MALE: Measurements of holotype male, with the average and range of all nine specimens measured in parentheses (Suppl. material 1: Table S4).

Body length 3.40, scute length 2.69 (2.46; 1.97–2.75), scute width 2.58 (2.41; 1.97–2.75), prosoma width 1.88 (1.77; 1.48–1.94). Shoulder tubercles present but small. Scute microgranulate. Integument color without contrast dorsally at midline between prosoma and opisthosoma in all individuals. OC a low broad mound; height 0.14; width 0.59 (0.52; 0.39–0.59). Eye color dark brown; surrounding integument with black pigment. Ventral surface microgranulate. GO length 0.32 (0.31; 0.27–0.32), width 0.28 (0.27; 0.26–0.29).

Mesal SBT of PT relatively low, rounded, with seta near apex of tubercle. PF length 2.0 (1.78; 1.38–2.03), PF depth 0.76 (0.65; 0.44–0.8), with 4–6 ventral spines, with the basal pair prominent, usually 3 enlarged dorsal spines (sometimes 4), and 2 enlarged distal prolateral spines. Pedipalp patella with 2 (one specimen with 3) enlarged prolateral spines and 1 ventroretrolateral spine; tibia with rows of 5 enlarged pro- and retrolateral spines; tarsus with 3 prolateral and 2 retrolateral enlarged spines. PCS with 2 dorsal anterior spines (sometimes 1–3); and with 2 small retrolateral spines (sometimes 1); DCS with 2 rows of small, dorsal, forward-facing acute SBTs. PCS width 0.37, DCS length 1.63, DCS width 0.47.

Leg II length 10.59 (9.88; 8.15–11.0); trochanter 0.58 (0.53; 0.41–0.59), femur 2.66 (2.45; 1.99–2.72), patella 0.84 (0.8; 0.64–0.89), tibia 2.26 (2.14; 1.73–2.4), metatarsus 2.33 (2.22; 1.82–2.48), tarsus 1.93 (1.81; 1.54–1.97); tibia distally and ventrally swollen, with 3–5 rounded SBTs, 2–4 with setae twisted. Tarsal claw as for genus. Tarsal count 5–13–5–6; variation exists in the number of LII tarsal segments.

Penis elongate; glans laterally compressed, dorsal plate extending outward into a more rounded keel shaped protrusion, with two pairs of spines, both pairs appressed to plate (perpendicular to the longitudinal axis of the penis); ventral plate cultriform with dorsally curved apical process.

FEMALE: Measurements of allotype female, with the average and range of all 10 specimens measured in parentheses (Suppl. material 1: Table S4). Descriptive characters as for males unless otherwise noted.

Body length 2.94, scute length 2.35 (2.29; 2.05–2.69), scute width 2.5 (2.52; 2.23–2.75), prosoma width 1.62 (1.61; 1.49–1.76). Integument color darker than males, usually with light contrast dorsally at midline between prosoma and opisthosoma (in 7 of 8 individuals). OC height 0.12; width 0.47 (0.47; 0.4–0.52).

Mesal SBT of PT relatively low, rounded, with seta near apex of tubercle. PF length 1.53 (1.48; 1.39–1.64), PF depth 0.54 (0.53; 0.47–0.61), with 6 or 7 ventral spines (sometimes 5), with the basal pair prominent, with 3 enlarged dorsal spines (sometimes 4), and 2 enlarged distal prolateral spines (sometimes 1). Pedipalp patella with 2 enlarged prolateral spines and 1 ventroretrolateral spine. PCS with 2 anterior spines dorsally, with 2 small retrolateral spines; PCS length 0.30; DCS length 1.19, width 0.28.

LII length 8.66 (8.51; 8.15–9.38); trochanter 0.5 (0.49; 0.44–0.59), femur 2.15 (2.06; 1.95–2.29), patella 0.68 (0.69; 0.66–0.76), tibia 1.78 (1.79; 1.68–1.96), metatarsus 1.95 (1.91; 1.82–2.1), tarsus 1.6 (1.57; 1.55–1.69); Tarsal count 5–11–5-6; variation exists in the number of LII tarsal segments.

GO length 0.3 (0.3; 0.27–0.34), width 0.29 (0.3; 0.27–0.36).

Ovipositor with four lobes, lateral lobes largest with seven apical setae, and a single large spine with a bifurcate tip, ventral lobe smallest.

Other material examined. See Suppl. material 1: Tables S1 and S4 for locality information of all specimens examined.

Distribution and Habitat. For specific localities, habitats, and microhabitats see Suppl. material 1: Table S1. This species is distributed in the central Cascade Mountains of Oregon east and southeast of Eugene from Brice Creek in the Row River Drainage north to the north side of the McKenzie, with all known localities in Lane County. It is possible that populations occur further north in the western Cascades (Fig. 1). Habitats and microhabitats do not obviously differ from *C. leviathan*, found in mature coniferous or mixed coniferous and hardwood forests, most often associated with large woody debris and bark.

Discussion

Species delimitation and short range endemic taxa

Cryptomaster exhibits a deep molecular phylogenetic break consistent with species level divergence, similar to that observed in many other harvestmen taxa (Boyer et al. 2007a, Thomas and Hedin 2008, Hedin and Thomas 2010, Schönhofer and Martens 2010, Derkarabetian et al. 2011, Richart and Hedin 2013, Fernández and Giribet 2014). Based on analyses of genetic data using discovery and validation approaches, we conclude that *Cryptomaster* consists of two species. We found complete genealogical concordance in the mitochondrial and nuclear loci sampled, indicating strong evidence for long-term reproductive isolation between *C. leviathan* and *C. behemoth* (Avise and Ball 1990). While gene tree and ABGD analyses of COI data indicate additional potential species, mitochondrial datasets are known to over-split short range endemic arachnid taxa (e.g., Keith and Hedin 2012, Satler et al. 2013, Derkarabetian and Hedin 2014, Fernández and Giribet 2014, Hamilton et al. 2014, Leavitt et al. 2015, Hedin et al. 2015, Wachter et al. 2015). Thus, we favor the more conservative two-species hypothesis, which is supported by the multi-species coalescent validation approach using four independent nuclear loci.

Our sampling efforts greatly increased the known range of *Cryptomaster*, yet both species still appear to have limited distributions. Interestingly, the range for *C. leviathan* extends across multiple mountain ranges, yet little to no genetic structure exists between these ranges. Conversely, *C. behemoth* has a particularly small range, yet harbors higher population genetic structure. This could be due to the greater topographic complexity of the central Cascade Range, or these populations may have persisted in their current locations for a longer time compared to *C. leviathan* populations. A pattern of deep population structure in topographically complex regions is consistent with numerous other arachnid taxa (Hendrixson and Bond 2005, Boyer et al. 2007a, Thomas and Hedin 2008, Bryson et al. 2013, Hedin et al. 2013, Esposito et al. 2015).

Biogeographic Uncertainty

Short-range endemic taxa have been shown to help elucidate ancient biogeographic processes (Boyer et al. 2007b, Bryson et al. 2013, Hedin et al. 2013). However, given the deep genetic break between *C. leviathan* and *C. behemoth*, contrasting with minimal population structure within these taxa, it is difficult to decipher the processes that led to the division of these species. *Cryptomaster leviathan* is comparatively much more widespread with populations in the Klamath Mountains, southern Oregon Coast Ranges, and the Cascade Mountains, although these ranges appear to be connected by corridors of possibly suitable habitat (Fig. 1). Speciation may have occurred while *C. leviathan* and *C. behemoth* inhabited separate mountain ranges and *C. leviathan* later dispersed from the coast northeast to the Cascade Range. Alternatively, the two species may have diverged within the Cascade Range. Under this scenario, *C. leviathan* could have already been present in the Coast Range or dispersed west from the Cascades subsequent to speciation. These species currently occupy different river drainage systems, separated by the relatively high-elevation Calapooya Divide, but it remains unclear whether this represents a primary or secondary barrier to dispersal. Sampling of faster evolving loci and additional fine-scale geographic sampling is needed to test these alternative hypotheses.

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Supplementary material 1

Supplementary Table 1–4

Authors: James Starrett, Shahan Derkarabetian, Casey H. Richart, Allan Cabrero, Marshal Hedin

Data type: Excel Table

Explanation note:

Table S1. Locality data

Table S2. PCR primer information

Table S3. GenBank accession information and genetic diversity statistics

Table S4. Morphological measurements

Supplementary material 2

Supplementary Figures 1–5

Authors: James Starrett, Shahan Derkarabetian, Casey H. Richart, Allan Cabrero, Marshal Hedin

Data type: figures

Explanation note:

Figure S1. Maximum likelihood gene tree for cytochrome c oxidase 1 (COI) locus. Numbers adjacent to nodes indicate bootstrap support greater than 70%.

Figure S2. Maximum likelihood gene tree for Toll locus. Numbers adjacent to nodes indicate bootstrap support greater than 70%.

Figure S3. Maximum likelihood gene tree for F-box/LRR-repeat locus. Numbers adjacent to nodes indicate bootstrap support greater than 70%.

Figure S4. Maximum likelihood gene tree for phosphatase 2A locus. Numbers adjacent to nodes indicate bootstrap support greater than 70%.

Figure S5. Maximum likelihood gene tree for Neuromusculin locus. Numbers adjacent to nodes indicate bootstrap support greater than 70%.

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RESEARCH ARTICLE

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Tsukiyamaia, a new genus of the tribe Baorini (Lepidoptera, Hesperiidae, Hesperiinae)

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Abstract

Skippers of the tribe Baorini are evidently a monophyletic group in the subfamily Hesperiinae. In this study, a new Baorini member *Tsukiyamaia albimacula* **gen. n.** et **sp. n.** is described from north Myanmar, southwest China and north Vietnam. Despite its peculiar and striking wing-pattern, this new genus has some important characters of Baorini, such as a broad and bifid uncus and a well-developed gnathos. Based on an analysis of male genitalia and the molecular phylogenies inferred from both mitochondrial and nuclear genes (28 taxa, total aligned length: 2968 bp), it is proposed that the genus *Tsukiyamaia* is closely related to the genus *Polytremis*, which has high species diversity in China. This study not only describes a new skipper but also highlights that *Tsukiyamaia* is important in clarifying phylogenetic relationship of *Polytremis* and its allies.

Keywords

Polytremis, new species, *cox1*, *cox2*, *EF-1α*

Introduction

Phylogenetic relationships and higher classifications of Hesperiidae at tribal level were primarily settled by Warren et al. (2008, 2009) based on morphological and molecular evidence. In this phylogenetic framework, the tribe Baorini is a well-supported monophyletic group belonging to the subfamily Hesperiinae (Warren et al. 2008). This tribe was established by Doherty (1886) as Baorinae and is currently composed of eleven genera: *Brusa* Evans, 1937, *Zenonia* Evans, 1935, *Gegenes* Hübner, 1819, *Parnara* Moore, 1881, *Borbo* Evans, 1949, *Pelopidas* Walker, 1870, *Polytremis* Mabille, 1904, *Baoris* Moore, 1881, *Caltoris* Swinhoe, 1893, *Iton* de Nicéville, 1895 and *Prusiana* Evans, 1937 (Warren et al. 2009) and 99 valid species (Evans 1937, Evans 1949, Chiba and Eliot 1991, Koiwaya 1996, Tsukiyama et al. 1997, Huang 1999, Sugiyama 1999, Devyatkin and Monastyrskii 2002, Huang 2003, Vane-Wright and de Jong 2003, Yuan et al. 2010, Zhu et al. 2012). Two genera, *Brusa* and *Zenonia* are endemic to the Ethiopian region, and the other nine genera are mainly Indo-Australian and south Palaearctic (Mediterranean and Manchurian). Evans (1937, 1949) completed the most recent revision of the world's fauna of Hesperiidae, and arranged phenotypically similar genera into informal groups in his systematics. However, phylogenetic relationship of genera within the group is not clear. The above-mentioned genera were classified in the Gegenes-group, except for *Prusiana* which was treated as a genus in the Taractrocera-group (Evans 1949). Subsequently, the Pelopidas-group (Eliot 1978) and Gegenini (Chou 1994) were proposed based on the Malaysian and Chinese faunas respectively.

The members of the tribe Baorini are brown with small semi-hyaline white spots, except for two genera, *Zenonia* and *Prusiana*, which have extensive orange markings resembling those of Taractrocerini (Warren et al. 2009). Warren et al. (2009) stated that the male genitalia were distinctive in Evans' Gegenes-group: a relatively broad, bifid uncus, a well-developed gnathos, and the harpe terminating in an upward-pointing, serrate hook.

Recently most of newly described Baorini taxa were discovered in the range from the south boundary of Himalayas to South China (Koiwaya 1996, Tsukiyama et al. 1997, Huang 1999, Sugiyama 1999, Huang 2003, Yuan et al. 2010, Zhu et al. 2012), where species richness and endemism are obviously higher than in other regions in East Asia (Chiba 2009). Some male specimens of an undescribed species were obtained from Myanmar, which were of uncertain taxonomic position due to only male characters. Subsequently, a female and some male specimens were added from southwest China and Vietnam, and molecular phylogenies based on mitochondrial and nuclear genes were inferred. This investigation suggests that this new species belongs to a new genus of the tribe Baorini, which is sister to *Polytremis*.

Methods

Sampling

For morphological comparison, eight male and one female specimens of this new taxon were examined. For inferring phylogenetic relationships of tribe Baorini to investigate the position of the new genus, 28 species were sampled (Table 1). A total of seven out of eleven genera in Evan's Gegenes-group were sampled and they are all distributed with *Tsukiyamaia* in Indo-Australian and the south Palaearctic region. Data of ten taxa were obtained from previous studies (Warren et al. 2008, 2009; Table 1).

Morphological procedures

We employed the standard method in Lepidoptera research to examine the male and female genitalia as well as other morphological characters of *Tsukiyamaia* (Zhu et al. 2012). The terminology for wing patterns followed Evans (1949) and for genitalia Shirôzu (1960) and Ehrlich (1958).

The holotype and one female paratype of the new taxon were deposited in Department of Biology, Shanghai Normal University, China. One male paratype was deposited in the private collection of Jia-Qi Wang. The other paratype from China is in the collection of Kadoorie Conservation China, Kadoorie Farm and Botanic Garden, Hong Kong. The rest of the paratypes are in Hiroshi Tsukiyama's collection (Chiba-pref., Japan).

Molecular procedures

Genomic DNA was extracted from the thoracic or leg tissue via using the Purgene DNA Isolation kit (Gentra Systems, Minnesota, USA), following the manufacturer protocol. The primers used for amplifying the mitochondrial cytochrome c oxidase I and II (*cox1* and *cox2*) and nuclear elongation factor 1 alpha (*EF-1α*) genes were adopted from previous studies (Caterino and Sperling 1999; Kandul et al. 2004; Simonsen et al. 2010; Lu et al. 2009). Each PCR reaction was carried out in a final volume of 30 μL with 0.32 μM dNTP, 1.5 mM MgCl2, 0.2 μM of each primer, 1X Taq buffer, 1U Taq DNA polymerase, and finally added dH_2O up to 30 μL. The PCR program was setting as 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50–55 °C, and 1–2 min at 72 °C. The final elongation step was continued for 7 min at 72 °C, and stopped at 4 °C. The PCR products were checked on 1.0 % agarose gels in 1X TBE buffer to ensure the PCR fragments were correctly amplified. DNA sequences were obtained by an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular sequences of the *cox1-cox2* and *EF-1α* genes were checked and assembled into contiguous arrays using Sequencher 4.8 (GeneCode, Boston, USA). After

Table 1. List of the skippers used in this study.

Name	Voucher	Locality	Accession number		Reference
			cox1-cox2	*EF-1α*	
Calpodes ethlius	144-ADW		EU364494	EU364289	Warren et al. 2008
Dubiella belpa	458-ADW		EU364051	EU364249	Warren et al. 2008
Ochlodes bouddha	H1-0635	Taiwan	KT240162	KT240144	this study
Pyrrhopygopsis crates	64-ADW		EU364503	EU364298	Warren et al. 2008
Saliana esperi	514-ADW		EU364501	EU364296	Warren et al. 2008
Suastus gremius	H1-1548	Taiwan	KT240163	KT240145	this study
Synapte silius	634-ADW		EU364431	EU364226	Warren et al. 2008
Talides sinois	512-ADW		EU364457	EU364252	Warren et al. 2008
Thracides phidon	451-ADW		EU364502	EU364297	Warren et al. 2008
Udaspes folus	H1-1546	Taiwan	KT240164	KT240146	this study
Baoris farri	H1-0260	Sichuan, China	KT240165	KT240147	this study
Brobo cinnara	H1-0684	Fujian, China	KT240166	KT240148	this study
Caltoris bromus	H1-1645	Taiwan	KT240167	KT240149	this study
Caltoris cahira	H1-1644	Taiwan	KT240168	KT240150	this study
Iton watsonii	600-MCZ		EU364490	EU364285	Warren et al. 2008
Parnara guttata	H1-1008	Sichuan, China	KT240169	KT240151	this study
Pelopidas conjuncta	H1-1565	Taiwan	KT240170	KT240152	this study
Pelopidas mathias	H1-0617	Taiwan	KT240171	KT240153	this study
Pelopidas thrax	570-ADW		EU364492	EU364287	Warren et al. 2008
Polytremis gotama	H1-1019	Yunnan, China	KT240172	KT240154	this study
Polytremis kiraizana	H1-1437	Taiwan	KT240173	KT240155	this study
Polytremis lubricans	H1-0052	Taiwan	KT240174	KT240156	this study
Polytremis matsuii	H1-0982	Sichuan, China	KT240175	KT240157	this study
Polytremis nascens	H1-0321	Sichuan, China	KT240176	KT240158	this study
Polytremis pellucida	234-ADW		EU364493	EU364288	Warren et al. 2008
Polytremis zina	H1-0607	Taiwan	KT240177	KT240159	this study
Pseudobrobo bevani	H1-0888	Yunnan, China	KT240178	KT240160	this study
Tsukiyamaia albimacula	H1-1661	Yunnan, China	KT240179	KT240161	this study

primer regions were cropped, the sequence dataset was aligned according to amino sequence similarity with the default settings by MUSCLE (Edgar 2004) in MEGA 5.1 software package (Tamura et al. 2011). Missing data and ambiguities were designated to IUPAC codes, and all the sequences were submitted to GenBank (Assession No. KT240144-KT240179; Table 1)

To evaluate species differentiation among Baorini skippers, genetic distance between species was calculated via MEGA 5.1. Pairwise distance with Kimura-2-parameter (Kimura and Ohta 1972) was performed, and bootstrap method was used to estimate its variance. For reconstructing phylogenies, two methods were used: Bayesian

inference (BI) was carried out by using MrBayes v. 3.2.1 (Ronquist et al. 2012), and Maximum Likelihood (ML) was performed in RAxML Pthreads-based SSE3 version 7.4.2 (Ott et al. 2007; Stamatakis 2006). In BI method, the substitution model was set to GTR+Γ (GTR: General Time Reversible; Γ: gamma distribution), and the taxa *Udaspes folus* was set as functional outgroup for investigate genus relationship among Baorini based on the latest phylogenetic relationship of skippers (Warren et al. 2009). To evaluate the effect of different partition strategies, four different datasets were executed: (1) no partition (combined dataset); (2) two gene region partitions (mitochondrial and nuclear genes); (3) four gene partitions (*cox1*, tRNA-Leu, *cox2*, and *EF-1α*), and (4) both gene and codon partition (ten partitions). Each partition matrix has its independent substitution model if partition was setting. Each dataset has run with six chains (five heated and one cold) for one million generations and sampled trees every 100 generations. The log-likelihood scores were plotted against generation time, and then burn-in the first 25% trees and the remaining trees were used for representing the posterior probability if the stationarity was reached. In ML method, datasets were processed with the non-default settings as follows: substitution model was set to GTR-GAMMA. Outgroup and the four partition datasets were set as the BI method. The node support values of ML topology were evaluated by 1000 bootstrap (BS) replicates with ten additional searches per replicate to improve the confidence of each bootstrap search.

Results

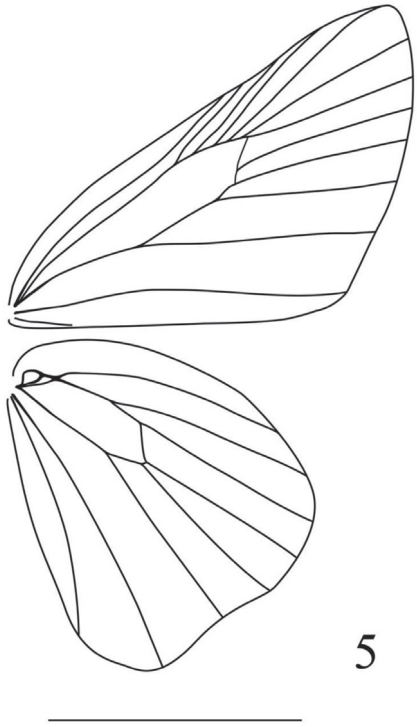
Morphological systematics

***Tsukiyamaia* gen. n.**
http://zoobank.org/23C5AC28-1908-4451-A2E6-3A4A8FA39CA4
Figs 1–4

Type species. *Tsukiyamaia albimacula* sp. n.; designated by monotypy.

Description. Antennae: 9.5–10 mm in length, half-length of forewing, nudum 13–14 on apiculus; Labial palpi: Second segment stout and erect, with brown hairs dorsally and yellowish hairs ventrally; third segment short, pointed and erect. Legs: middle tibiae unspined. Wing-shape: Forewing 19–20 mm in length, triangular in shape; costa about 1.4 times as long as dorsum, approximately straight, weakly arched on anterior half; apex angulated; termen lightly curved on anterior half; inner dorsum almost straight. Hindwing nearly triangular in shape; costa slightly longer than dorsum, obviously arched; termen curved on anterior half; tornus concave; dorsum almost straight.

Wing venation (Fig. 5)**.** Forewing: vein 2A very short not reaching dorsum; vein Cu_2 arising before the origin of vein R_1 and slightly nearer the origin of Vein M_3 than to base; Vein M_2 obviously closer to Vein M_3 than to Vein M_1 at origin; cell longer than half the wing length. Hindwing: Vein Cu_1 arising beyond the origin of Vein M_1;



Figures 1–5. *Tsukiyamaia albimacula*. **1** holotype, ♂, upperside **2** holotype, ♂, underside **3** paratype, ♀, upperside **4** paratype, ♀, underside **5** wing venation. Scale bar: 10 mm.

Vein Cu_2 arising beyond the origin of Vein Rs; Vein M_2 absent. Discocellular veins on both wings obvious.

Wing markings (Figs 1–4)**.** without stigma or secondary sexual characters; forewing with semi-hyaline spots in spaces Cu_2, M_3, M_2, R_3, R_4, R_5 and cell; hindwing upperside with a cigar-shaped spot in space M_2, underside centrally with a large white marking restricted from vein 2A to vein Rs.

Male genitalia (Figs 6–10)**.** Tegumen swollen; uncus U-shaped bifurcated; gnathos bifurcated, slightly turned inside at tip and outwardly spined; valva approximately rectangle; dorsal process of harpe well produced; ventral process of harpe weakly protruded; phallus deeply bifid distally, well protruded and heavily spined outwardly; cornuti absent; manica membranous; juxta U-shaped.

Etymology. The generic name is derived from Hiroshi Tsukiyama, whose outstanding contribution to the taxonomy of Hesperiidae is noteworthy.

***Tsukiyamaia albimacula* sp. n.**
http://zoobank.org/6ED6C0E1-0571-4536-BF4B-B00F857B19FE

Description. Antennae 9.5–10 mm in length, about 1/2 the length of forewing, black brown except club gray dorsally and grayish yellow ventrally; nudum 13-14 on apiculus. Palpi erect, with brown hairs dorsally and yellowish hairs ventrally. Thorax and abdomen covered with brown hairs. Forewing 19–20 mm in length. Both wings ground color black brown at each sides, with white spots and marking; costal area of forewing and entire hindwing covered with brown scales underside; cilia of both wings brown. Upperside forewing: three apical spots in spaces R_3–R_5, arranged linear; one discal spot present at the middle of the space M_2; in space M_3, a reduced spot present in the holotype, and absent in two paratypes; cell spots conjoined as trapezium-shaped, which also conjoined with the Cu_1 spot. Underside forewing markings same as upperside. Upperside hindwing: only with a cigar-shaped spot in space M_1. Underside hindwing: Discal area with a very large, rectangle white marking extending from vein Rs to the middle of space Cu_2. Inward margin smooth, upward to the end of the discal cell. Outward margin lightly serrated, and evidently elongated in space M_1.

Male genitalia (Figs 6–10)**.** Tegumen swollen; uncus U-shaped, bifurcated dorsally, pointed at tip laterally; gnathos bifurcated, longer and wider than uncus, slightly turned inside at tip and outwardly spined; saccus short, pointed distally; valva approximately rectangle; ampulla slightly elongate upward, harpe dorsally with a long and straight elongated process and ventrally with a relatively short and small process, outward margin concave and covered with dense hairs; costa smooth dorsally, sacculus concave ventrally; phallus 1.4 times as long as valva; subzonal about 1.3 times as the length of suprazonal, distally deeply bifid as two protruded processes, equal in length and heavily spined outwardly; without cornuti; manica membranous; juxta U-shaped.

Female genitalia (Figs 11–12)**.** Papilla analis nearly rectangle, covered with hairs on the surface; apophysis posterioris slender and short; Lamella postvaginalis oblong

Figures 6–10. Male genitalia of *Tsukiyamaia albimacula*. **6** lateral view of ring **7** dorsal view of tegumen **8** outer view of left valva **9** ventral view of phallus **10** lateral view of phallus. Scale bar: 1 mm.

Figures 11–12. Female genitalia of *Tsukiyamaia albimacula*. **11** ventral view **12** lateral view. Scale bar: 1 mm.

with outer margin arched; lamella antevaginalis with triangular parts laterally, slightly sclerotized; ductus bursae short, wide as ostium bursae, strongly sclerotized; bursa copulatrix oval, membranous with no signum.

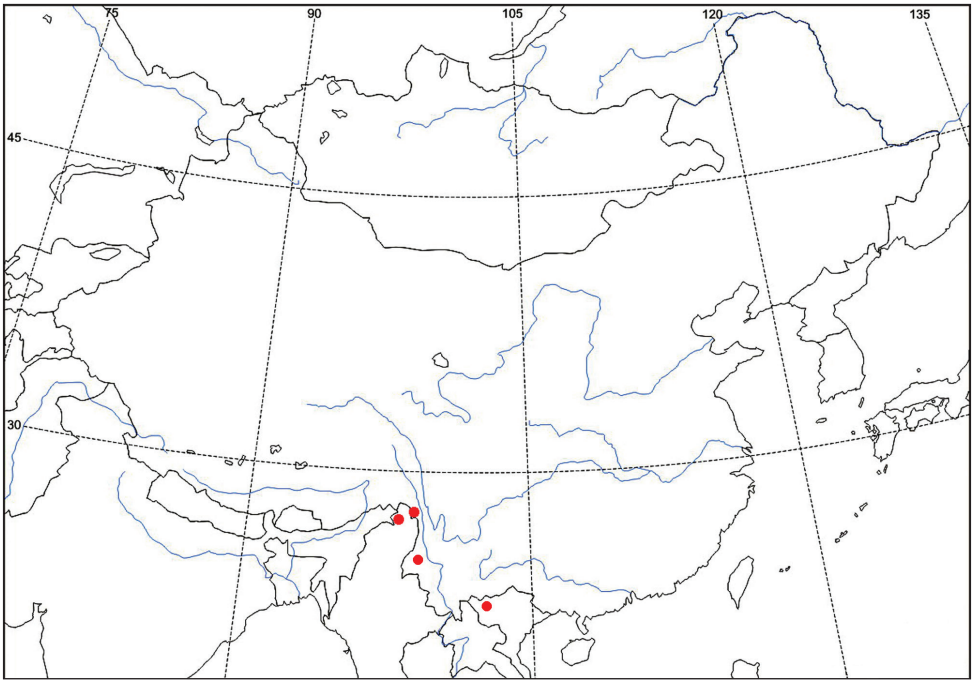


Figure 13. Distribution map for *Tsukiyamaia albimacula*, red circle.

HOLOTYPE ♂: Phutao, Kachin, N. of MYANMAR, ~1000m, 09-VI-2000, Male genitalia examined by H. Chiba, #HC030511.

PARATYPES: 1♂, the same locality as the holotype, 29-V-2000.; 1♂ ditto, 08-VI-2000.; 1♂ Panglan, ~700m, Kachin, N. of MYANMAR, 02-IX-2002. 1♂ ditto, 04-IX-2002. . 1♂ ditto, 05-IX-2002, 1♂ ditto, 29-IX-2002, 1♂ Mt. Fan Shi Pang, ~1800m, N. VIETNAM, IV-2002. 1♂ Baopo, Dulongjiang, Yunnan, CHINA, 1500m, 29-V-2011, Jia-Qi Wang leg.; 1♀ , Maku, Dulongjiang, Yunnan, CHINA, 1900m, 03-VI-2009, Jian-Qing Zhu, leg; 1♂ CHINA, Yunnan, Tengchong, Gaoligongshan National Nature Reserve, Zhengding, 2200m, 26-IV-2014, LO Yik Fui Philip coll. (YFL140055).

Voltinism. Judging from the collecting data, the species is expected to be multivoltine.

Distribution (Fig. 13)**.** China (Yunnan), Myanmar (Kachin), and Vietnam (Mt. Fan Shi Pang).

Biology. *Tsukiyamaia* prefers open habitats, such as open field on the hillside, farmland and heavily disturbed shrub land. It is active near the ground and stream under strong sunlight. The female frequents flowers and the male performs padding behavior.

Etymology. The species is named for its large white marking on underside of the hindwing.

Diagnosis. In appearance, *Tsukiyamaia* is peculiar in Baorini with a large white marking in the center of the hindwing underside. The male genitalia of *Tsukiyamaia* can be separated from those of Baorini genera by the uncus lacking a pair of basal processes, and the harpe dorsally with a long and straight elongated process and ventrally with a relatively short and small process.

Molecular information

Sequence information

The gene length used in this study included *cox1* (1531bp), tRNA-Leu (71 bp), *cox2* (141 bp), and *EF-1α* (1225 bp) genes. Pairwise distance based on mitochondrial sequences showed that the smallest one between *Tsukiyamaia albimacula* and *Polytremis matsuii* was 6.8% (Table 2). If it was compared with other *Polytremis* skippers, it ranged from 7.2 to 10.6%. Whereas comparing to other genera, it ranged from 7.6% (*Iton watsonii*) to 12.8% (*Dubiella belpa*).

Molecular phylogenies

The total of eight topologies, inferred by four partitioning datasets and by two tree-reconstructing methods, have similar phylogenetic relationships (summarized in Fig. 14, Appendix: S1–S3). All the Baorini members are grouped together and *Parnara guttata* is the most primitive taxa. Although the genus-level relationships within Baorini are still unresolved, *Tsukiyamaia* is sister to *Polytremis* members with high support value. In addition, our Baorini topology also indicated that the genus *Polytremis* might not be a monophyletic group, and more taxa-sampling is needed for further phylogenetic studies.

Discussion

Although the monophyly of the tribe Baorini is well-supported by the molecular data, no synapomorphic character in external morphology have been found (Warren et al. 2009). Characters are either shared by most but not all the members of the tribe, or shared by members of other tribes.

Evans (1949) merely gives diagnostic difference between his Gegenes- group (= Baorini) and Taractrocera-group (= Taractrocerini), which is the wing color. The former is brown while the latter is yellow or orange. As mentioned in the introduction, it is not applicable for *Prusiana*, which Evans (1949) considered a member of Taractrocera-group, nor the African *Zenonia* as well as the new genus. The outstanding

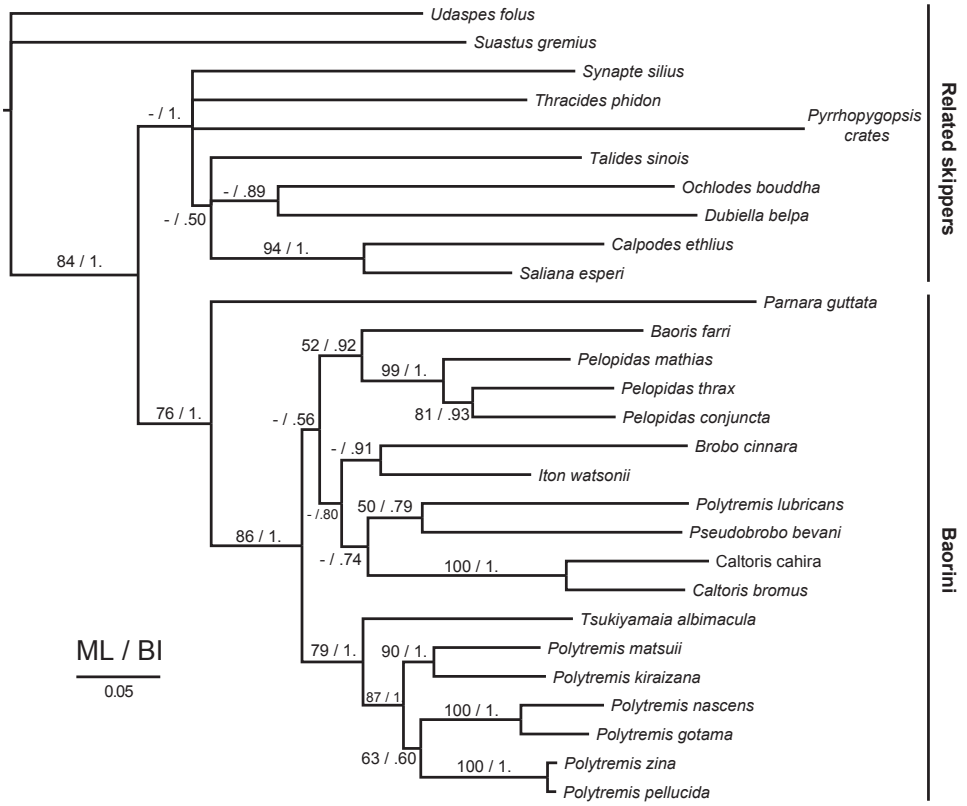


Figure 14. Bayesian phylogeny of the tribe Baorini based on four gene-partitioned dataset. The numbers above or below the branches are the ML bootstrap value / BI posterior probability.

coloration of *Tsukiyamaia* may imply that there exist some unknown adaptive advantages driving the evolution of the peculiar marking with the slightest resemblance to its allies.

Eliot (1978) claims that the "internal veinlet entering the cell from just above the origin of vein 3 on the forewing" is the character shared by members of his Pelopidas-group but not the Taractrocera-group of genera. However, he only illustrated the wing venation of *Caltoris tulsi*, which apparently shows the veinlet. Figures of wing venation in Bascombe et al. (1999) suggest that the veinlet can be observed clearly only in *Caltoris bromus*, recognizable in *Borbo cinnara*, *Pelopidas conjunctus*, and *Polytremis lubricans*, absent in *Parnara guttata* and *Baoris farri*. We could not recognize the veinlet in the wing venation of *Tsukiyamaia*.

If the key for separation of genera in Evans (1949) or Eliot (1978) is applied, *Tsukiyamaia* is assigned to *Polytremis*, which is consent to the phylogeny based on molecular data.

Table 2. Pairwise distance based on the substitution model of Kimura 2-parameter and *cox1 – cox2* sequences (aligned length 1743 bp). The dash symbol means the overlap sequence is below 50 bp, the value is excluded.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 *Udaspes folus*														
2 *Suastus gremius*	0.106													
3 *Synapte silius*	0.122	0.101												
4 *Thracides phidon*	0.123	0.116	0.113											
5 *Pyrrhopygopsis crates*	0.137	0.105	0.116	0.130										
6 *Talides sinois*	0.121	0.065	0.097	0.109	0.118									
7 *Ochlodes bouddha*	0.124	0.104	0.095	0.113	0.116	0.097								
8 *Dubiella belpa*	0.132	0.111	0.112	0.105	0.133	0.118	0.096							
9 *Calpodes ethlius*	0.120	0.067	0.094	0.097	0.107	0.087	0.096	0.107						
10 *Saliana esperi*	0.118	0.080	0.101	0.100	0.124	0.095	0.088	0.118	0.071					
11 *Parnara guttata*	0.131	0.121	-	-	-	-	0.123	-	-	-				
12 *Baoris farri*	0.134	0.124	-	-	-	-	0.113	-	-	-	0.110			
13 *Pelopidas mathias*	0.113	0.111	0.106	0.096	0.112	0.104	0.103	0.115	0.097	0.102	0.109	0.090		
14 *Pelopidas thrax*	0.121	0.091	0.115	0.112	0.118	0.100	0.100	0.121	0.098	0.096	-	-	0.060	
15 *Pelopidas conjuncta*	0.123	0.111	0.103	0.104	0.117	0.104	0.103	0.116	0.114	0.115	0.094	0.086	0.059	0.059
16 *Brobo cinnara*	0.122	0.109	-	-	-	-	0.117	-	-	-	0.112	0.096	0.092	-
17 *Iton watsonii*	0.113	0.112	0.106	0.100	0.110	0.089	0.093	0.117	0.101	0.102	-	-	0.074	0.084
18 *Caltoris cahira*	0.126	0.098	0.111	0.115	0.127	0.109	0.106	0.117	0.101	0.101	0.116	0.117	0.101	0.100
19 *Caltoris bromus*	0.125	0.113	0.116	0.113	0.129	0.105	0.114	0.119	0.096	0.103	0.127	0.137	0.110	0.095
20 *Pseudobrobo bevani*	0.126	0.099	-	-	-	-	0.123	-	-	-	0.114	0.113	0.102	-
21 *Polytremis lubricans*	0.137	0.133	0.097	0.116	0.135	0.126	0.124	0.151	0.106	0.107	0.131	0.117	0.104	0.122
22 *Polytremis matsuii*	0.127	0.129	-	-	-	-	0.105	-	-	-	0.089	0.100	0.091	-
23 *Polytremis kiraizana*	0.123	0.116	0.107	0.103	0.128	0.101	0.103	0.115	0.109	0.102	0.104	0.107	0.094	0.092
24 *Polytremis nascens*	0.116	0.115	-	-	-	-	0.113	-	-	-	0.105	0.100	0.092	0.000
25 *Polytremis gotama*	0.107	0.115	-	-	-	-	0.104	-	-	-	0.101	0.083	0.086	0.000
26 *Polytremis zina*	0.116	0.115	0.108	0.123	0.142	0.100	0.107	0.117	0.092	0.109	0.093	0.091	0.082	0.089
27 *Polytremis pellucida*	0.117	0.124	0.115	0.116	0.123	0.096	0.107	0.116	0.104	0.108	0.099	0.095	0.083	0.089
28 *Tsukiyamaia albimacula*	0.120	0.119	0.114	0.121	0.123	0.114	0.108	0.128	0.121	0.109	0.105	0.098	0.087	0.085

Table 2. Continued.

	15	16	17	18	19	20	21	22	23	24	25	26	27
1 *Udaspes folus*													
2 *Suastus gremius*													
3 *Synapte silius*													
4 *Thracides phidon*													
5 *Pyrrhopygopsis crates*													
6 *Talides sinois*													
7 *Ochlodes bouddha*													
8 *Dubiella belpa*													
9 *Calpodes ethlius*													
10 *Saliana esperi*													
11 *Parnara guttata*													
12 *Baoris farri*													
13 *Pelopidas mathias*													
14 *Pelopidas thrax*													
15 *Pelopidas conjuncta*													
16 *Brobo cinnara*	0.085												
17 *Iton watsonii*	0.078	-											
18 *Caltoris cahira*	0.106	0.122	0.089										
19 *Caltoris bromus*	0.111	0.115	0.083	0.077									
20 *Pseudobrobo bevani*	0.098	0.096	-	0.109	0.116								
21 *Polytremis lubricans*	0.111	0.121	0.088	0.116	0.133	0.095							
22 *Polytremis matsuii*	0.084	0.095	-	0.119	0.124	0.107	0.105						
23 *Polytremis kiraizana*	0.087	0.084	0.072	0.105	0.112	0.098	0.105	0.061					
24 *Polytremis nascens*	0.092	0.091	-	0.102	0.102	0.095	0.098	0.065	0.077				
25 *Polytremis gotama*	0.085	0.085	-	0.102	0.094	0.088	0.089	0.061	0.066	0.038			
26 *Polytremis zina*	0.081	0.079	0.073	0.109	0.117	0.087	0.094	0.061	0.069	0.052	0.051		
27 *Polytremis pellucida*	0.081	0.083	0.075	0.113	0.121	0.095	0.104	0.060	0.073	0.056	0.057	0.009	
28 *Tsukiyamaia albimacula*	0.089	0.093	0.076	0.104	0.115	0.110	0.106	0.068	0.077	0.079	0.074	0.072	0.078

Acknowlegments

We are extremely grateful to Mr Hiroshi Tsukiyama for providing specimens of this peculiar skipper from his collection and his assistance throughout this project. It is our honor to name the new genus after him. We thank Ms Ting-Wei Chen for some DNA sequence work and Mr Yik Fui Philip Lo for the information of the new skipper collected during a joint biodiversity survey project between Kadoorie Conservation China and Tengchong Branch of Gaoligongshan National Nature Reserve.

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Appendix

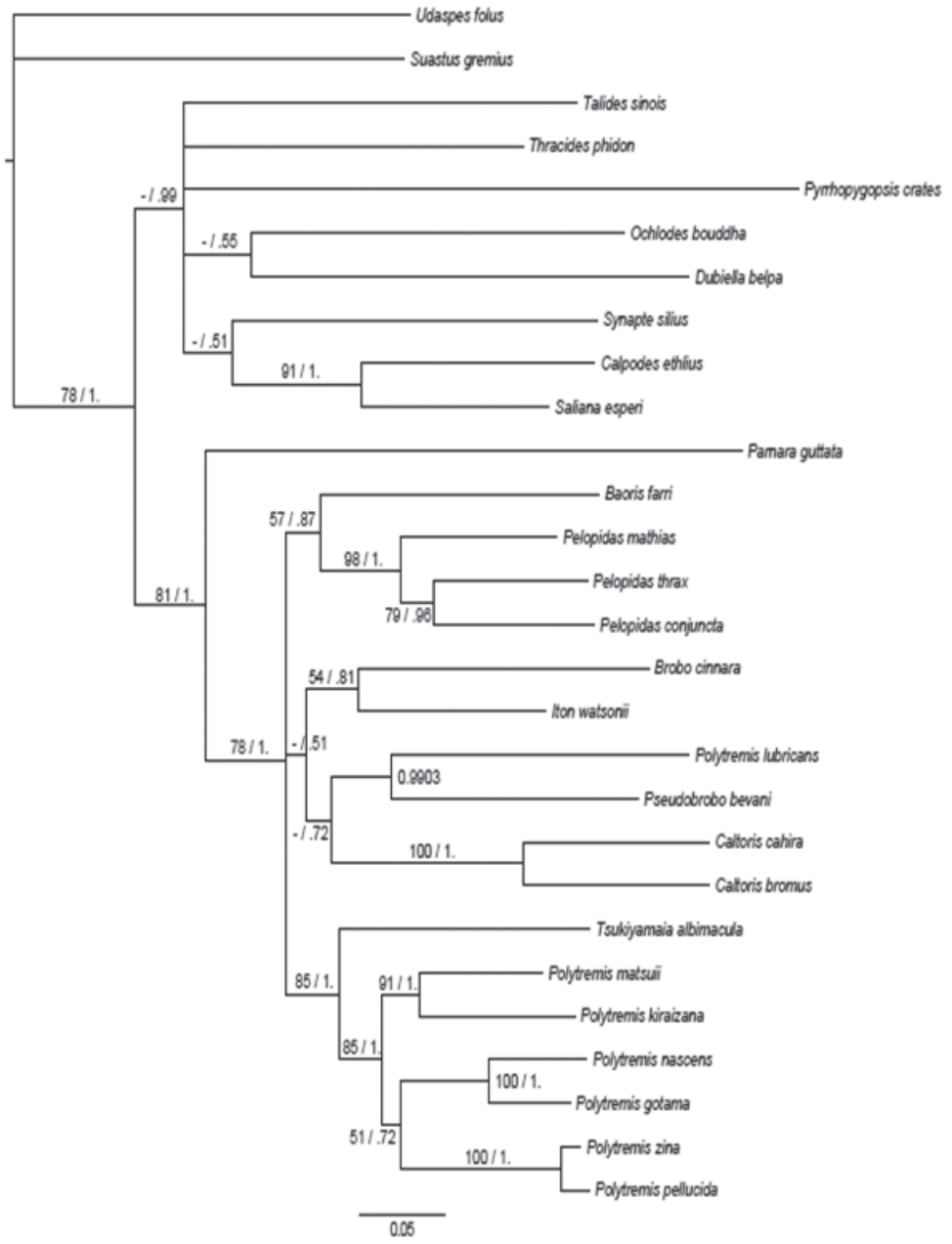

Figure S1. Bayesian phylogeny of the tribe Baorini based on no partitioned dataset. The numbers above or below the branches are the ML bootstrap value / BI posterior probability.

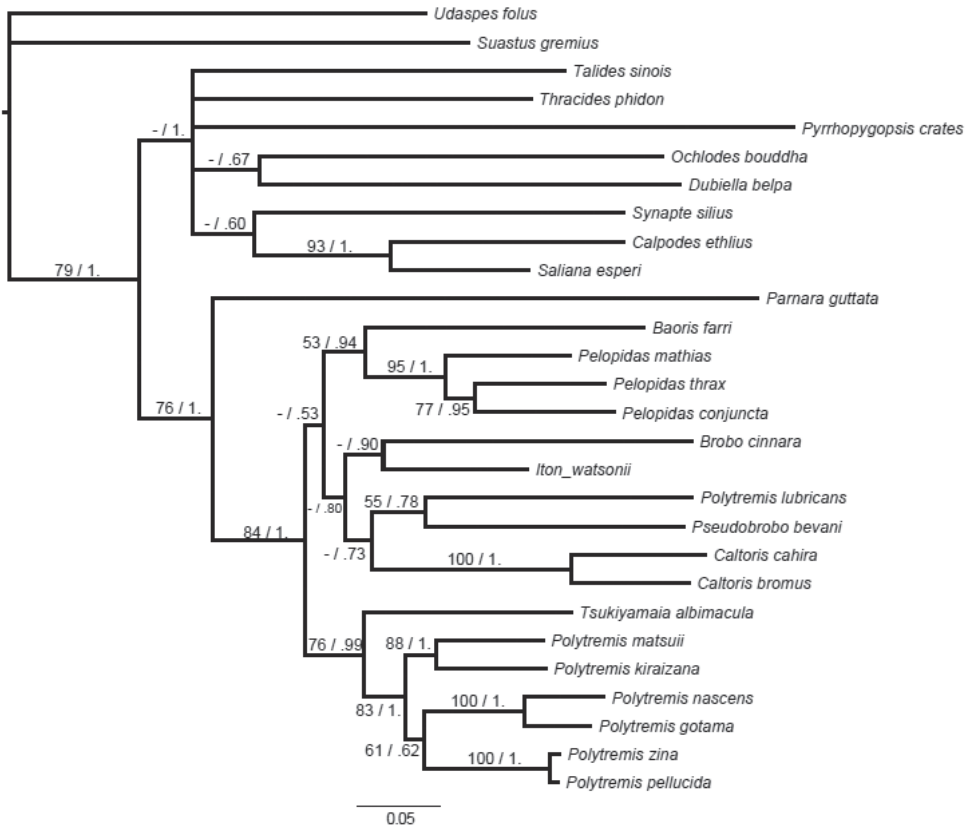

Figure S2. Bayesian phylogeny of the tribe Baorini based on two partitioned dataset. The numbers above or below the branches are the ML bootstrap value / BI posterior probability.

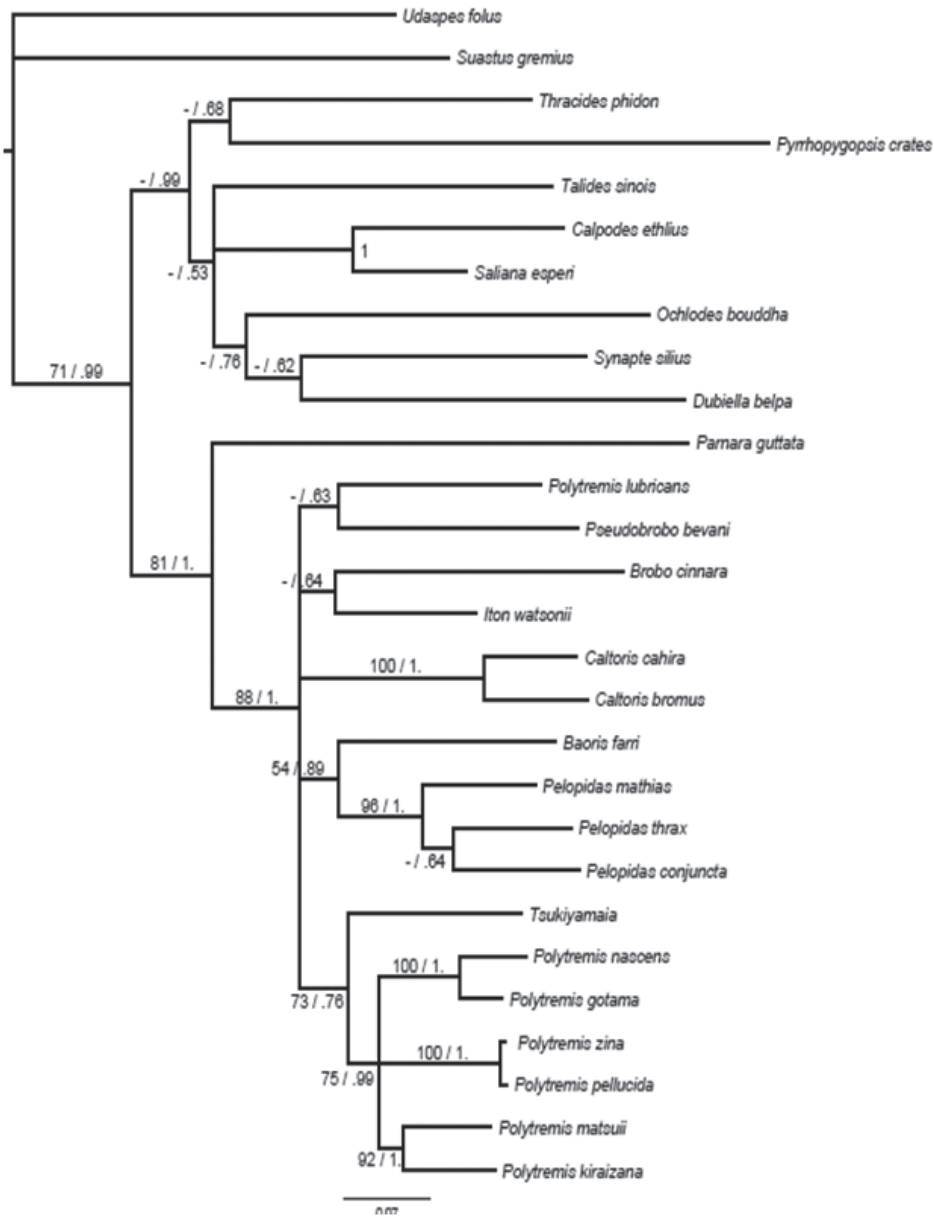

Figure S3. Bayesian phylogeny of the tribe Baorini based on codon partitioned dataset. The numbers above or below the branches are the ML bootstrap value / BI posterior probability.

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RESEARCH ARTICLE

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A new genus and species of arboreal toad with phytotelmonous larvae, from the Andaman Islands, India (Lissamphibia, Anura, Bufonidae)

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Abstract

A new bufonid amphibian, belonging to a new monotypic genus, is described from the Andaman Islands, in the Bay of Bengal, Republic of India, based on unique external morphological and skeletal characters which are compared with those of known Oriental and other relevant bufonid genera. *Blythophryne* **gen. n.** is distinguished from other bufonid genera by its small adult size (mean SVL 24.02 mm), the presence of six presacral vertebrae, an absence of coccygeal expansions, presence of an elongated pair of parotoid glands, expanded discs at digit tips and phytotelmonous tadpoles that lack oral denticles. The taxonomic and phylogenetic position of the new taxon (that we named as *Blythophryne beryet* **gen.** et **sp. n.**) was ascertained by comparing its 12S and 16S partial genes with those of Oriental and other relevant bufonid lineages. Resulting molecular phylogeny supports the erection of a novel monotypic genus for this lineage from the Andaman Islands of India.

Keywords

Amphibian, bufonid, tadpole, rRNA, molecular phylogeny, skeletal characters

* Equal contribution

Introduction

Neobatrachian anurans of the family Bufonidae Gray, 1845 are represented in the Oriental portion of Asia by 14 genera (Table 1). Recent analyses of both morphological and molecular data have revealed that several terrestrial genera such as *Adenomus*, *Duttaphrynus*, *Ingerophrynus* and *Xanthophryne* had remained obscurely hidden under the catch-all generic name '*Bufo*' Garsault, 1754 (*fide* Manamendra-Arachchi and Pethiyagoda 1998; Frost et al. 2006; Biju et al. 2009). Likewise, the arboreal forms of Oriental Asia were once considered to be members of the African genus *Nectophryne* Buchholz & Peters, 1875 (see Boulenger 1892, 1896, 1919), till Barbour (1938) recognised the morphological variations and allocated them to two different genera by revalidating Günther's (1875) *Pedostibes* and describing as new *Pelophryne*. Following this taxonomic treatment, subsequent studies on the systematics of Oriental arboreal toads have reconfirmed the distinctiveness of these genera and have led to the recognition of additional bufonid genera, based on morphological as well as molecular evidence (see Fei et al. 2003; Matsui et al. 2007).

Of all the above, *Duttaphrynus melanostictus* (Schneider, 1799) is the only bufonid reported from the Andaman Islands (Sarkar 1990; Das 1999). In the adjacent Nicobar archipelago; however, a second putative taxon, *Docidophryne spinipes* (a *nomen nudum*) was reported earlier (Fitzinger 1861), which was subsequently described erroneously as a new taxon, *Bufo camortensis* by Mansukhani and Sarkar (1980) from Camorta, in the central Nicobar Islands. Both these were later synonymised with *Bufo melanostictus* by Crombie (1986). Recent herpetological surveys conducted in the Andaman Islands resulted in the collection of a diminutive, arboreal toad species in the hill forests of Mt. Harriet National Park and on a few adjacent islands, which is described herein, allocated to a new monotypic genus and compared with other currently valid (Frost 2014) Oriental and related bufonid genera.

Materials and methods

Specimen collection and preservation

Specimens were hand-collected, euthanised and fixed in absolute ethanol for a minimum of 24 hours, and eventually transferred to 60% ethanol for preservation. Tissue samples were extracted and stored in absolute ethanol (prior to specimen fixation) for phylogenetic analyses. Tadpoles were collected and reared for preservation of samples across developmental stages in 4% formalin solution. Conspecificity between tadpoles and the adults was confirmed by rearing them to metamorphosis, as well as matching 16S ribosomal DNA sequences to those of the adults. Staging of tadpoles follow Gosner (1960). Type specimens were deposited in the collection of the Zoological Survey of India, Kolkata (ZSIC). Museum abbreviations follow Sabaj Pérez (2012) except for WII, which represents vertebrate collections at the Wildlife

Table 1. Members of the Neobatrachian anurans of the family Bufonidae Gray, 1845 represented in the Oriental portion of Asia.

	Genus	Number of species	Distribution
1	*Adenomus* Cope, 1860	2	Sri Lanka
2	*Ansonia* Stoliczka, 1870	28	Sundaland and Philippine archipelago
3	*Bufoides* Pillai & Yazdani, 1973	1	Khasi Hills, Meghalaya, India
4	*Duttaphrynus* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green & Wheeler, 2006	29	Eastern Africa to Papua New Guinea; 25 species are known from India and south east Asia
5	*Ingerophrynus* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green & Wheeler, 2006	12	Indochina and the Sundaland
6	*Leptophryne* Fitzinger, 1843	2	Sundaland
7	*Parapelophryne* Fei, Ye & Jiang, 2006	1	Indochina
8	*Pedostibes* Günther, 1876 "1875"	5	Western Ghats and Eastern Himalayas, India; Malay Peninsula, Borneo & Sumatra
9	*Pelophryne* Barbour, 1938	11	Sundaland and the Philippines Archipelago
10	*Phrynoidis* Fitzinger, 1843	2	Indochina-Sundaland
11	*Pseudobufo* Tschudi, 1838	1	Sundaland
12	*Sabahphrynus* Matsui, Yambun & Sudin, 2007	1	Borneo
13	*Xanthophryne* Biju, Van Bocxlaer, Giri, Loader & Bossuyt, 2009	2	Northern Western Ghats, India
14	*Ghatophryne* Biju, Bocxlaer, Giri, Loader & Bossuyt, 2009	2	Western Ghats, India

Institute of India, Dehradun, India. Morphometric measurements were done using Mitutoyo™ dial calipers, to the nearest 0.01 mm, between 3-4 months of preservation of the adults and tadpoles. Morphometric measurements (Table 2) were recored for all the adults, metamorphs, and tadpoles.

Skeletal characters of a paratype were examined under a microscope by clearing using trypsin and 0.5% potassium hydroxide solution and staining with alcian blue and alizarin red dye, following Hanken and Wassersug (1981). Additional information on osteology of selected genera for comparison is based on an examination of comparative material (Appendix I), as well as published literature. Webbing formulae follow Savage and Heyer (1997). Geographic coordinates of the localities were recorded using a Garmin GPSmap 78s (map datum WGS84). Calls were recorded using a digital stereo microphone and analysed using Raven™ and are archived in the Macaulay Library, Cornell Lab of Ornithology (Voucher no: ML 174095).

Tadpoles were described based on collections made in May 2011, from a phytotelm, located *ca*. 1.3 m above the ground. The clutch was monitored continuously

Table 2. Abbreviations and definitions of morphometric measurements made on adult, metamorph of frogs and tadpole. Measurements made only on tadpoles are indicated by an asterisk after the abbreviation.

AG	Distance from posterior point of the forelimb at its insertion into the body to the anterior point of the hindlimb insertion
BL*	Distance from snout tip to the point of the initiation of tail from the body
BW	Distance at the broadest point at the trunk
DFH*	Height of the fin measured at the place of the maximum height of the dorsal fin
DL fold	Length of dorso-lateral fold
ED	Horizontal diameter of the orbit
EN	Distance between anterior border of the eye to posterior edge of the nostril
ES	Distance between anterior border of the eye to the snout tip
ETY	Distance between posterior border of the eye to anterior margin of the tympanum
f1 to 4	Distance measured from the fork of the fingers to the tip of the finger disc for fingers 1 to 4
FEL	Distance measured from the cloaca to the tip of the knee
FOL	Distance measured from the anterior end of the tarsus to the tip of the fourth toe
HD	Height of the head measured at the post-orbital region before the parotoid gland
HL	Distance from the tip of the snout to the posterior edge of the mandible
HW	Width of the head measured at the jaw angle
IN	Closest distance between the nares
IND*	Distance between the external nares
IO	Distance between the anterior margins of the upper eyelids
IOL*	Distance between the two orbits
LAL	Distance measured from the elbow to the base of the outer metacarpal tubercle; palm length
MBW*	Distance measured at point of the maximum width of the body
MTH*	Distance measured at the point of the maximum height of the tail by laterally positing the tadpole
MTMW*	Distance measured on the tail at the point of initiation of the tail from the body where the tail width is maximum
NA	Not measured
NED*	Distance between nostril and eye
NSD*	Distance from the snout to the eye
ODD*	Oral disc diameter
PAL	Distance measured from the posterior border of the outer metacarpal tubercle to the tip of the third finger
PL	Length of the parotoid gland
PW	Maximum width of the parotoid gland
SS*	Distance from snout to the spiracle
SV*	Distance from snout to the vent
SVL	Distance from tip of the snout till the cloaca
t1 to 5	Distance measured from the fork of the toe to the tip of the toe disc for toes 1 to 5
TBL	Distance from the knee to the obtuse margin of the tibia
TL*	Distance from the point of initiation of tail till the tip of the tail
TMH*	Distance measured on the tail at the point where the tail muscle reaches maximum height
TYH	Horizontal diameter of the tympanum
TYV	Vertical diameter of the tympanum
UAL	Distance measured from the point of insertion of the forelimb to the trunk to elbow
UEW	Maximum width of the upper eyelid
VFH*	Ventral fin height measured at the place of the maximum height of the ventral fin
VTL*	Vent tube length

till complete transformation. The observed eggs got transformed into pale white embryos on 2 May 2011; subsequently, tadpoles at different developmental stages were collected and preserved in 5% formalin. Tail tips of these individuals were collected and preserved in absolute ethanol for DNA barcoding studies before the tadpoles were preserved in formalin.

Molecular phylogeny. Total genomic DNA was extracted from the alcohol-preserved soft tissue (muscle), taken from the holotype, following the standard procedure of SDS & proteinase-K lysis, followed by chloroform-isoamyl extraction method. The taxonomic position of the toad was ascertained by rDNA typing of both 16S and 12S rDNA genes of the mitochondrial genome broadly following the method as described earlier by Dutta et al. (2004). The parts of 16S and 12S rDNA were amplified and sequenced for both strands using the published primers (Palumbi et al. 1991), 16Sar-L [5′-CGCCTGTTTATCAAAAACAT-3′], 16Sbr-H [5′-CCGGTCTGAACTCAGATCACGT-3′] and 12saL [5′-AAACTGGGATTAGATACCCCACTAT-3′], 12sbH [5′-GAGGGTGACGGGCGGTGTGT-3′], respectively. The raw sequences from both strands were end-clipped, edited and assembled to build partial 12S (417 bp) and 16S (551bp) gene sequences of the taxon individually. The sequences were subjected to BLAST search against the NCBI database sequences in order to ascertain the gene and broad taxonomic identity. Multiple sequence alignments using CLUSTALX 2.0 (Thompson et al. 1997), along with representative Asian and African origin sequence homologs under the Bufonidae, spanning 21 genera and 43 species (Table 3), were constructed individually for both 12S and 16S partial genes. Subsequently, manually edited alignments of both 12S and 16S were concatenated to get a final single alignment, which was then used for all further phylogenetic analysis. Initially, the analysis was conducted using sequence data of 36 species of the 21 genera and *Rhaebo guttatus* as outgroup to ascertain the broad affinity of the new taxon in the Bufonidae. Subsequently, sub-trees were constructed using mainly the Asian toad species and *Ghatophryne*, *Pedostibes*, and *Adenomus* as successive outgroups, to better resolve the phylogenetic status of the new taxon.

For each of the phylogenetic analysis, the concatenated 12S+16S sequence alignment was first used to find the best fitting DNA substitution model using Akaike Information criterion (AIC), as implemented in jModelTest2 (Guindon and Gascuel 2003; Darriba et al. 2012) was found to be for both the domains. Phylogenetic analysis was then conducted using the inferred GTR+G+I base substitution model and both Maximum likelihood (ML) and Bayesian inference (BI) methods. BI was implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the following parameters: GTR+G+I model of DNA substitution, Nst as 6 (all different substitution rates subjected to GTR), flat substitution rates and the stationary nucleotide frequencies of the GTR rate matrix (as calculated using Dirichlet Process Prior; Heath et al. 2012), a uniform distribution (0,1) for both, the shape parameter of the gamma distribution of rate variation and the prior for the proportion of invariable sites; 3,000,000 MCMC iterations in two runs and four chains; with sampling every 300 iterations; minimum standard deviation of the split frequencies as 0.01; burn-in of initial 25% of stored

Table 3. Taxon sampling for phylogenetic analysis of selected Oriental members of the Bufonidae.

Taxon	Range/ Collection location	NCBI Acc. No.	Tree_7a	Subtree_7b	Subtree_7c	Reference
Blythophryne beryet gen. et sp. n.	India (A&N Islands)	KT991336, KT991347	+	+	+	This study
Adenomus kelaartii	Sri Lanka	FJ882780	+	+	+	Bocxlaer et al. 2009
Amietophrynus brauni	Tanzana	DQ158437	+			Pramuk et al. 2008
Amietophrynus gracilipes	Equatorial Guinea	DQ158456	+			Pramuk et al. 2008
Amietophrynus gutturalis	Kenya	DQ158460	+			Pramuk et al. 2008
Amietophrynus poweri	Namibia	DQ158482	+			Pramuk et al. 2008
Amietophrynus steindachneri	Kenya	DQ158488	+			Pramuk et al. 2008
Ansonia hanitschi	Malaysia	FJ882794	+			Bocxlaer et al. 2009
Ansonia longidigita	Malaysia	KT991329, KT991340	+			This study
Bufo bufo	Turkey	DQ158438	+			Pramuk et al. 2008
Bufoides meghalayanus	India	KT991331, KT991342	+	+	+	This study
Duttaphrynus atukoralei	India	FJ882835		+	+	Bocxlaer et al. 2009
Duttaphrynus brevirostris	India	FJ882786		+	+	Bocxlaer et al. 2009
Duttaphrynus crocus	India	FJ882789		+	+	Bocxlaer et al. 2009
Duttaphrynus dhufarensis	India	FJ882837		+	+	Bocxlaer et al. 2009
Duttaphrynus himalayanus	India	KT991334, KT991345	+	+	+	This study
Duttaphrynus hololius	India	FJ882781		+	+	Bocxlaer et al. 2009
Duttaphrynus melanostictus	India	KT991335, KT991346	+	+	+	This study
Duttaphrynus parietalis	India	FJ882784		+	+	Bocxlaer et al. 2009
Duttaphrynus scaber	India	KT991332, KT991343	+	+	+	This study
Duttaphrynus stomaticus	India	KT991333, KT991344	+	+	+	This study
Duttaphrynus stuarti	India	FJ882788		+	+	Bocxlaer et al. 2009
Ghatophryne ornata	India	FJ882797	+	+		Bocxlaer et al. 2009
Ingerophrynus divergens	Malaysia	KT991328, KT991339	+			This study
Ingerophrynus galeatus	Laos	DQ158452	+			Pramuk et al. 2008
Ingerophrynus macrotis	Laos	DQ158468	+			Pramuk et al. 2008
Leptophryne borbonica	Malaysia	FJ882799	+			Bocxlaer et al. 2009
Mertensophryne micranotis	Tanzania	FJ882821	+			Bocxlaer et al. 2009
Mertensophryne uzunguensis	Tanzania	FJ882819	+			Bocxlaer et al. 2009
Nectophryne afra	Cameroon	DQ283360	+			Frost et al. 2006
Nectophryne batesi	Gabon	DQ283169	+			Frost et al. 2006
Nectophrynoides minutus	Tanzania	FJ882814	+			Bocxlaer et al. 2009
Nectophrynoides tornieri	Tanzania	DQ283413	+			Frost et al. 2006

Taxon	Range/ Collection location	NCBI Acc. No.	Tree_7a	Subtree_7b	Subtree_7c	Reference
Pedostibes hosii	Malaysia	KT991330, KT991341	+			This study
Pedostibes tuberculosus	India	FJ882793	+	+		Bocxlaer et al. 2009
Pelophryne api	Malaysia	KT991326, KT991337	+			This study
Phrynoidis asper	Brunei	DQ158431	+			Pramuk et al. 2008
Phrynoidis juxtasper	Malaysia	KT991327, KT9913387	+			This study
Sabahphrynus maculatus	Malaysia	AB331718	+			Matsui et al. 2007
Schismaderma carens	Zimbabwe	DQ158424	+			Pramuk et al. 2008
Vandijkophrynus robinsoni	Namibia	GU183857	+			Bocxlaer et al. 2010
Xanthophryne koynayensis	India	FJ882782	+	+	+	Bocxlaer et al. 2009
Rhaebo guttatus	Brazil	DQ158459	+			Pramuk et al. 2008

trees and parameters. Similarly, ML analyses were implemented in RaxML (Stamatakis 2006) with 500 replicates, applying a separate GTRGAMMA model to each partition. The output tree was visualised using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). For comparison based on genetic distances, uncorrected k2p pair-wise distances were calculated both within and across genus for both 16S and 12S partial gene sequences in MEGA 6.06 (Tamura et al. 2013), using the complete deletion option.

Results

Systematics

***Blythophryne* gen. n.**
http://zoobank.org/2BAB0154-53B8-43E3-BB14-F36F12FDD8DE

Type species. *Blythophryne beryet* gen. et sp. n. by monotypy (Fig. 1, Table 4).

Content. A single species is currently known.

Type material. Holotype ♀ : ZSI_A-12521(Fig. 1), (SVL 27.4 mm) leg. S. R. Chandramouli and S. Harikrishnan on 12 December 2010 near Mt. Harriet National Park (ca. 11°42'N, 92°44'E, 175 m asl.) within evergreen forests at *ca*. 2130 hours. Paratypes (paratopotypes): ZSI_A-12522 to ZSI_A-12530 (three ♀ and six ♂; Fig. 1g); leg. S. R. Chandramouli and S. Harikrishnan during 22 - 25 June 2010 from the above location but at an altitude range of ~150–330 m asl. Other paratypes (larvae): seven tadpoles (WII-115) collected from a phytotelm on Rutland Island. Referred material: WII-113, an adult topotype with six toes on both the feet.

Etymology. The generic name is a patronym, coined in appreciation of Edward Blyth (1810–1873), the first curator of the Asiatic Society of Bengal, who initiated

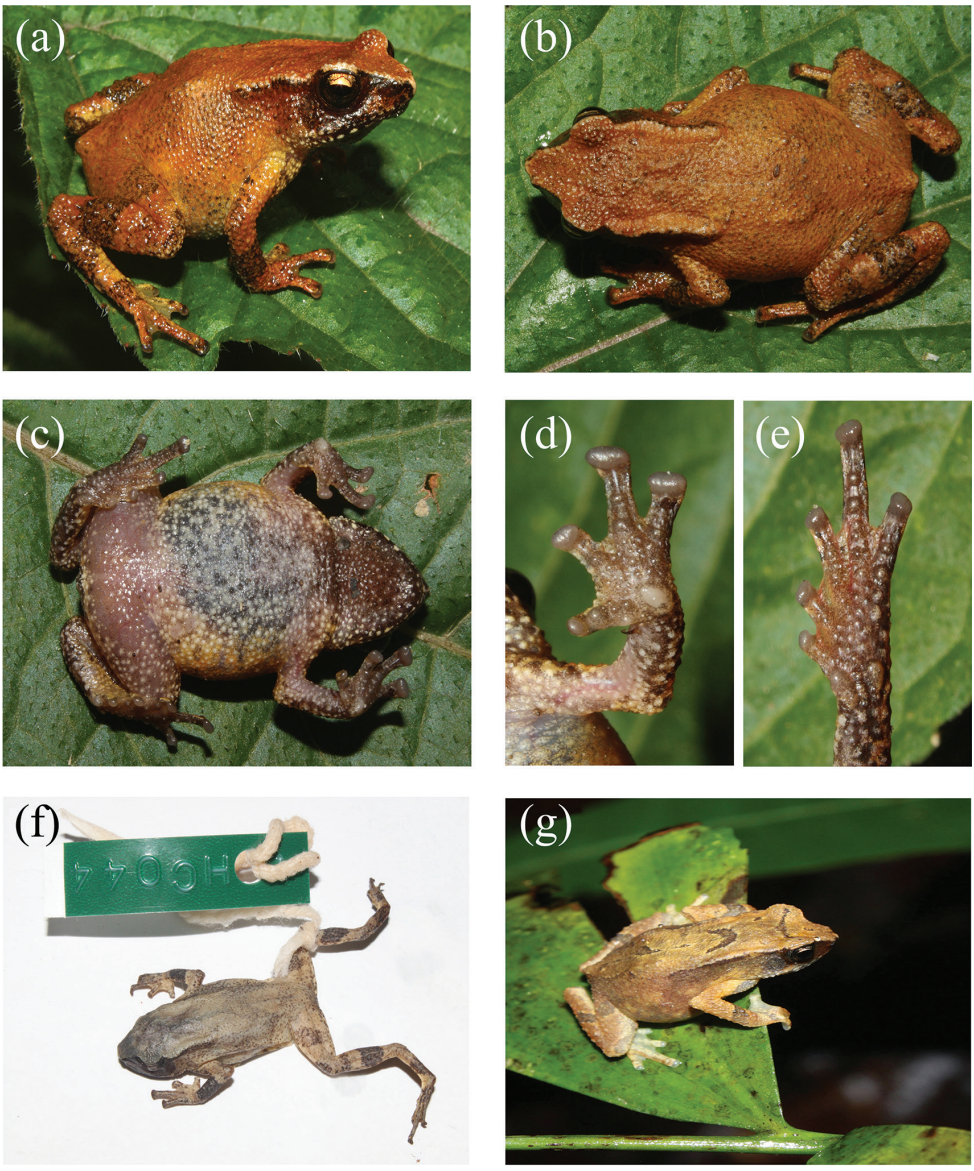

Figure 1. Morphological characters of the *Blythophryne beryet* gen. et sp. n.: **a** dorso-lateral view **b** dorsal view **c** ventral view **d** ventral view of left palm **e** ventral view of left foot of the adult female holotype (ZSI_A-12521) in life **f** adult female holotype in preservation **g** dorsal view of the male paratype (ZSI_A-12529) in life showing inverted-V shaped markings and the inter-ocular band on the dorsum.

herpetological studies in the Andaman and Nicobar Islands, through his phenomenal, pioneering paper "Notes on the fauna of the Nicobar islands" (Blyth 1846). Das (1999) remarked, "Blyth is to be credited for the description of a large number of species from the Andaman and Nicobar Islands that are still valid. Blyth (1846) wrote the

Table 4. Morphometric measurements of the holotype and paratype series of adult and two gravid (g) individuals of *Blythophryne beryet* gen. et sp. n.

	ZSI A-12521	ZSI A-12524	ZSI A-12522	ZSI A-12523	ZSI A-12526	ZSI A-12529	ZSI A-12527	ZSI A-12530	ZSI A-12528	ZSI A-12525
Sex	♀	♀(g)	♂	♀(g)	♂	♂	♀	♂	♂	♂
SVL	27.4	25.5	25.5	25.2	24.5	23.0	22.7	22.3	22.2	21.8
AG	10.6	9.2	9.8	12.5	8.0	7.3	8.0	6.7	6.5	8.5
HL	7.7	7.5	8.2	6.9	7.5	7.9	7.5	7.6	7.6	7.1
HW	7.9	7.6	8.1	6.8	8.0	7.8	7.6	7.7	7.4	7.2
HD	4.3	3.5	3.9	3.4	3.9	3.4	3.2	3.2	3.0	3.2
BW	9.9	10.3	9.1	11.8	8.3	7.1	6.1	7.5	6.3	9.8
EN	2.2	2.3	1.7	1.9	1.9	2.3	2.1	2.0	2.1	2.2
ES	3.4	3.4	3.3	3.1	3.5	3.3	3.2	3.2	3.3	3.1
ETY	0.5	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.5
UEW	1.9	1.5	2.0	1.8	1.8	1.7	1.7	1.5	1.9	1.8
IO	3.8	3.8	3.5	3.4	3.5	3.8	3.4	3.5	3.6	3.4
IN	2.2	2.1	2.2	2.2	1.8	2.1	2.1	1.9	2.0	1.9
TYH	1.6	1.6	1.9	1.6	1.6	1.4	1.5	1.4	1.8	1.5
TYV	1.8	1.7	1.9	1.6	1.6	1.6	1.6	1.5	1.8	1.5
UAL	5.1	4.7	4.4	5.2	4.3	4.3	4.3	4.6	4.3	4.1
LAL	5.8	5.4	5.6	5.5	5.6	5.5	5.4	5.3	5.4	5.3
PAL	6.2	5.7	6.2	6.8	5.8	5.9	5.9	5.8	5.9	6.1
FEL	9.2	7.5	7.7	7.2	9.3	8.2	9.5	8.2	8.6	8.5
TBL	10.6	8.0	9.4	8.4	9.1	7.9	9.0	8.1	8.3	8.5
FOL	9.6	9.7	9.4	8.3	9.4	8.0	9.2	8.3	8.7	8.3
ED	2.8	2.5	2.4	2.6	2.5	2.1	1.9	2.4	2.1	2.3
DL fold	13.3	12.2	11.3	11.9	12.0	12.3	11.7	12.0	11.4	11.9
PL	6.1	5.9	6.5	6.0	4.5	3.7	4.0	5.9	3.2	3.9
PW	1.4	1.4	1.3	1.6	1.0	1.0	0.9	1.3	0.9	0.9
f1	1.8	1.2	1.1	1.5	1.6	1.3	2.0	1.2	1.6	0.9
f2	1.9	1.4	1.6	1.7	2.2	1.8	2.2	1.4	1.9	1.6
f3	3.1	3.0	2.9	2.8	2.8	2.6	2.8	2.9	2.4	2.9
f4	2.2	1.9	1.8	2.1	2.2	1.9	2.1	1.8	1.6	1.8
t1	1.1	1.1	1.2	1.1	1.3	1.0	1.3	1.2	1.0	1.1
t2	1.4	1.7	1.4	1.4	1.7	1.4	1.5	1.1	1.5	1.5
t3	2.6	2.0	2.0	2.7	2.3	2.1	2.6	2.1	2.2	1.9
t4	4.7	4.1	3.9	4.9	4.4	2.9	4.6	3.7	3.0	4.0
t5	3.0	2.1	2.3	2.6	2.5	2.1	2.5	2.1	2.0	1.9

first account on the vertebrate fauna of these islands, and in 1863, compiled the first check-list". Further details of Edward Blyth and his contributions to studies on Indian natural history are in Das (2004) and Sridharan (2013). The specific epithet '*beryet*' (in Great Andamanese language; http://www.andamanese.net/Great_Andamanese_Lexicon_English.pdf) refers to 'small frog'. We believe that the Great Andamanese knew of the existence of this small arboreal anuran that is here described as new species to

science. We hope the name given here will also raise awareness about the dwindling, indigenous tribal populations in the Andamans, their culture and extinction of their tribal languages.

Diagnosis. This currently monotypic genus and species is diagnosed by the following suite of external morphological and osteological characters: small adult size (mean SVL 24.0 mm; range 21.8–27.4 mm); distinct tympanum, slightly smaller than eye; absence of cephalic ridges; absence of vomerine teeth; presence of a single, median, external vocal sac in males; presence of elongated pair of parotoid glands; absence of enlarged, keratinised tubercles on dorsum; presence of well developed, sheath-like webbing on fingers and on toes; digit tips dilated to discs, lacking circum-marginal grooves; presence of six presacral vertebrae; urostyle lacking lateral dilations; absence of omosternum and presence of arciferal pectoral girdle. Mature ova small (0.62 mm mean diameter), yolky and unpigmented; tadpoles lacking keratodont.

Description of the holotype. A small bufonid (mean SVL 24.2 ± 0.6 mm), with depressed, moderately robust (AG:BW 1.0) habitus (Fig. 1a–c). Head almost as long as broad (HL:HW 0.97), devoid of cephalic ridges, with a single, median internal vocal sac in males. Snout obtusely pointed in dorsal view, projecting beyond mandibles; nostrils oriented laterally, situated on lateral fold closer to tip of snout than to eye (EN:ES 0.7), loreal region mildly concave, canthal ridge well defined between nostril and the eye, distance between orbit and nostril greater than internarial distance (IN:EN 0.96), upper eyelid rough, densely covered with minute warts, eyes large (ED:HL 0.4), about twice length of tympanum (TYH:ED 0.6), separated from each other by twice internarial distance (IN:IO 0.6), and over twice width of upper eyelid (IO:UEW 1.9), pineal ocellus absent; vomerine teeth absent, tongue elongate, slender and oval, free posteriorly, not bifid, lacking lingual papilla; dorsolateral fold conspicuous, almost up to 48% SVL, beyond which it becomes indistinct and disappears; parotoid glands slender and elongate (PL:PW 4.3), as well-defined postorbital ridge. Limbs slender, upper arm short, 18.7% of SVL, lower arm longer than the upper arm (21% SVL), fingers basally webbed, webbing between Fingers II and III not exceeding penultimate subarticular tubercle (webbing formula $I_{0-1}II_{1-2}III_{2-1}IV$; Fig. 1d); an enlarged, prominent outer metacarpal tubercle at palmar base (subequal to disc on Finger I), nuptial pad absent , subarticular tubercles prominent on fingers and toes, finger tips dilated to discs lacking circummarginal grooves that are much broader than long, and are less discernible in the first and second fingers; relative length of fingers 3 > 4 > 2 > 1; thigh 33.7% SVL, subequal to shank (38.6% SVL); toes partially webbed, webbing between Toes III and IV extending to penultimate subarticular tubercle (webbing formula $I_{0-1}II_{0-1}III_{1-2}IV_{2½-½}V$; Fig. 1e); tarsal ridge absent, inner meta-tarsal tubercle larger than outer. Relative length of toes 4 > 5 > 3 > 2 > 1. Skin rough dorsally and granular ventrally; lower abdomen with free, loose skin flap. Tubercles or granules absent on dorsum, scattered over venter, under surface of thighs less granular; throat and limb-insertions with dense granules, tibia with enlarged granular tubercles.

Colouration in life. Dorsum reddish-brown, with two feeble dark brown inverted 'V' shaped markings which fail to reach flanks, interorbital band indistinct, canthus

dark chocolate brown, colour extending a little beyond tympanum, subequal to half-length of parotoid gland; forearm and hind limbs barred, one each on thigh, shank and tarsus. Venter heavily speckled with dark brown spots, throat dark brown, lower lip spotted with white and brown, pupil large, horizontally elliptical.

Colouration in alcohol. Dorsum drab brown with indistinct 'inverted-V' shaped pattern, darker bands on limbs, venter cream, with black mottled pattern, throat black throughout (Fig. 1f).

Osteology (based on paratype ZSI_A12527). Axial and appendicular skeleton composed primarily of bony elements; cartilaginous elements not observed. Atlas (the first vertebra) with rudimentary hypapophysis and not fused to axis, presacral vertebrae six in number, Vertebrae II–V bearing horizontally elongate hypapophyses, those on Vertebrae II and V oriented anteriorly; Vertebrae III–IV oriented horizontally; sacral diapophysis laterally dilated; coccyx not fused to sacrum; articulating with former by a double condyle and lacking lateral expansions, omosternum absent, pectoral girdle arciferal, with epicoracoids united to each other anteriorly and overlapping posteriorly (Fig. 2). Phalangeal formula of fingers 2-2-3-3; toes 2-2-3-4-3, terminal phalange obtusely curved, not truncate. Nasal bones of the skull large, about 1/3rd of frontoparietals and 1.25 times as large as orbital cavity. Maxillary and vomerine teeth absent.

Morphological variations. Adult females and males range between 25.2–27.4 mm and 21.8–25.5 mm, respectively. Measurements of paratypes are provided in Table 4. Dorsal colour in different shades of brown or reddish-brown. Intensity of inverted 'V'-shaped pattern on dorsum variable. On one occasion, an abnormal specimen (WII-113) with a deformity was observed, with six digits, the first toe being preceded by a small additional toe on both feet. Fingers showed no such anomalies.

Description of calls. (Macaulay Library, Cornell Lab of Ornithology; voucher no: ML 174095). A calling male was observed on 24 November 2010 on the surface of leaves within bushes. Calls were composed of continuous syllables of "pip-pip-pip-pip-pip-" at a constant frequency of 8 kHz, without pause, lasting for 23 seconds, with mean amplitude of -3 db / 20 kU (Fig. 3). The call was composed of 198 pulses uttered within duration of 23 s, at a rate of 8 to 9 (mean = 8.6) pulses per second. Each pulse lasted for duration of 0.3 s (n = 198) with an interval of 8.5 s (n = 197) between two consecutive pulses.

Distribution. This species has been documented from five islands of the Andaman archipelago, namely, the South Andaman (Mt. Harriet), Rutland, Little Andaman, Havelock Island in the Ritchie's Archipelago and North Andaman (Saddle Peak) (Fig. 4).

Vernacular name. 'Andaman bush toad' is proposed as the common English name for this new species, indicating its arboreal habit and restricted distribution as understood currently.

Ecological notes. The new species is often seen on surface of leaves of herbaceous bushes. It is nocturnal and regularly seen year round. It was the third most common anuran in the islands (Harikrishnan and Vasudevan 2015). The high abundance of this species seems to be the result of it occupying a narrow range of distribution and a

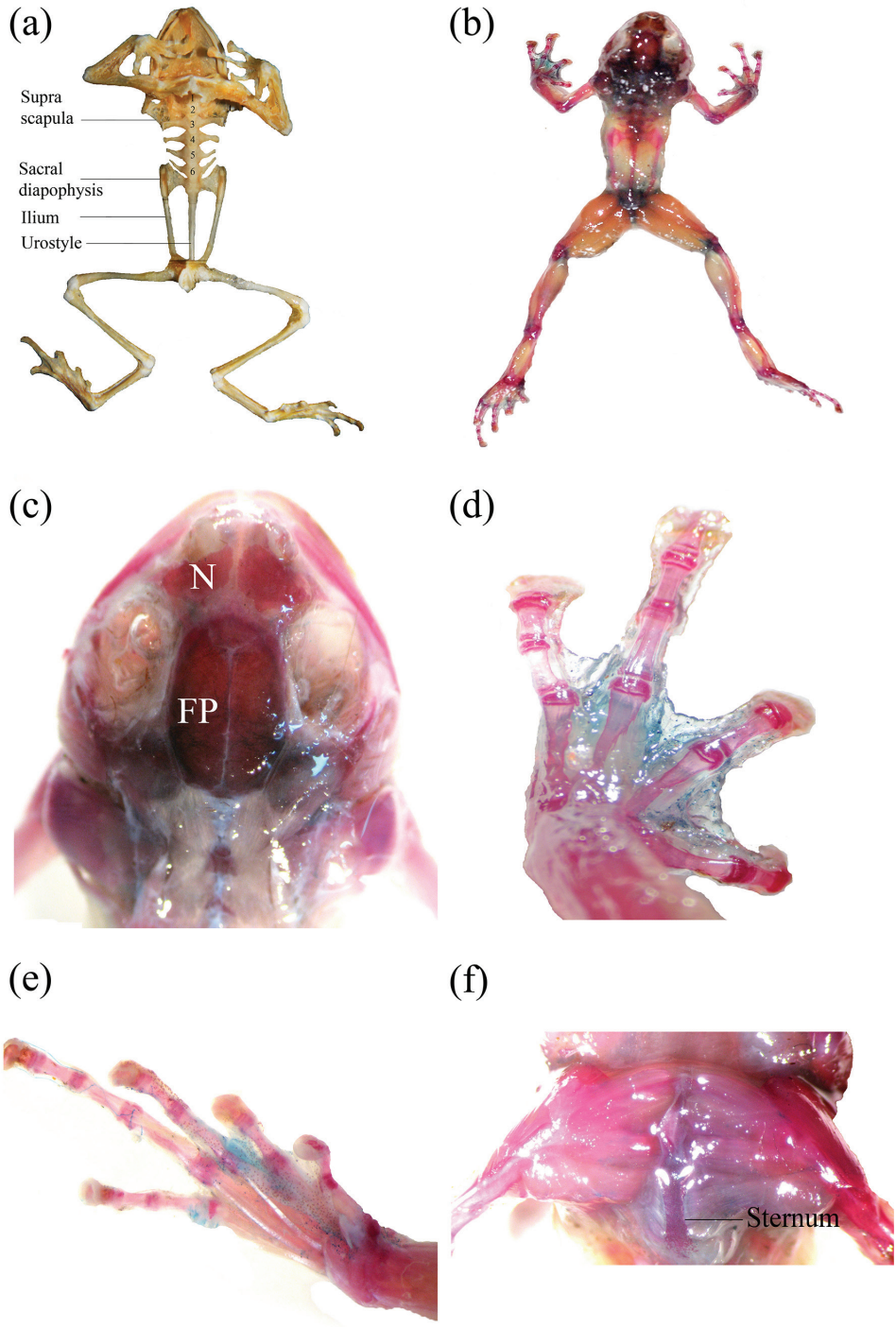

Figure 2. Skeletal characters of paratypes (ZSI_A-12527) of *Blythophryne beryet* gen. et sp. n. **a** complete dry structure **b–f** various characters visible after staining/clearing of the skeleton. FP – frontoparietal; N – nasal.

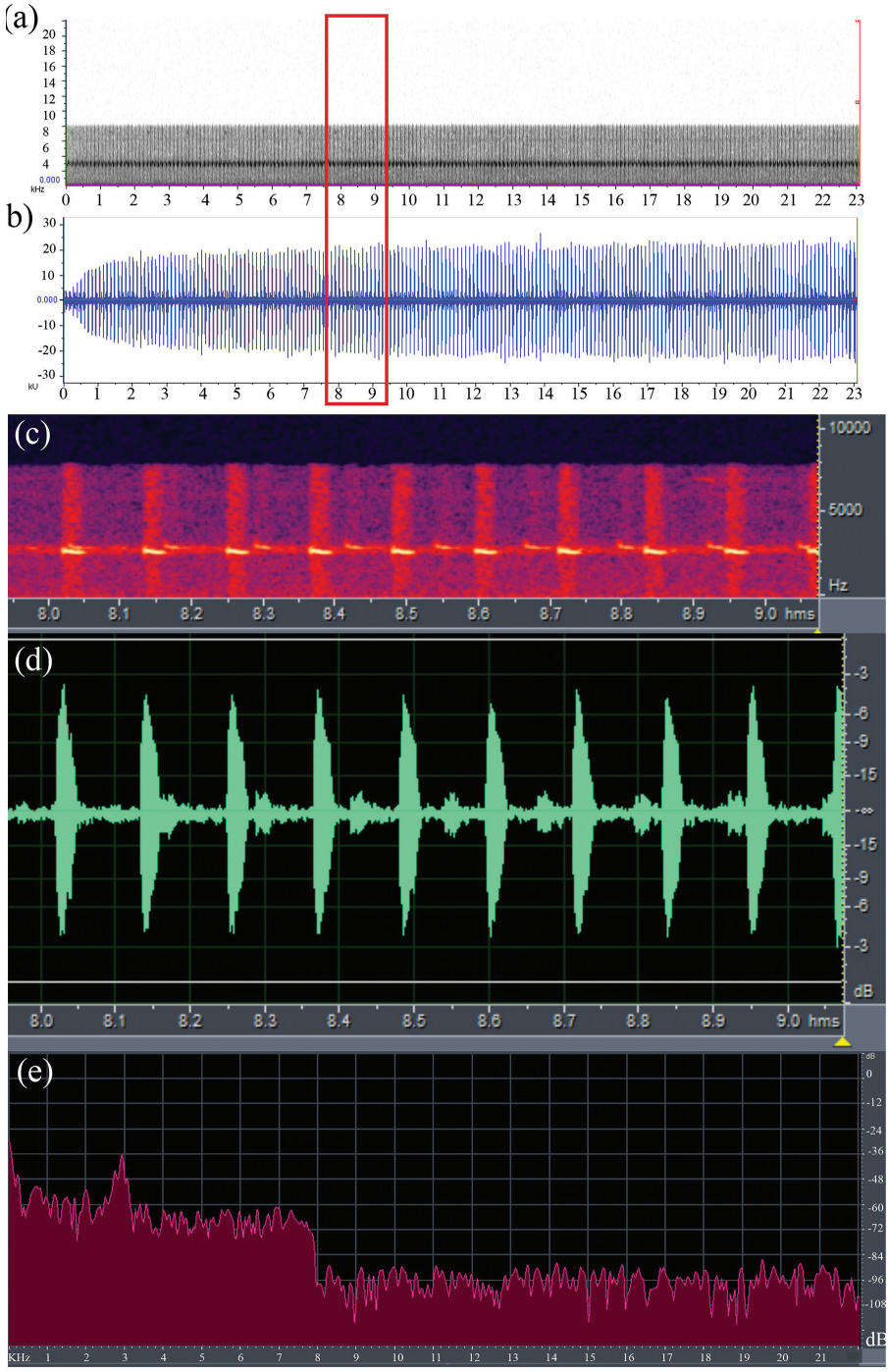

Figure 3. Sound spectrogram **a** and oscillogram **b** of a 23 second clip of a call of *Blythophryne beryet* gen. et sp. n. . Detailed view of **c** frequency and **d** amplitude modulations of a one second long clip of the call **e** power spectrum of the call of *Blythophryne beryet* gen. et sp. n.

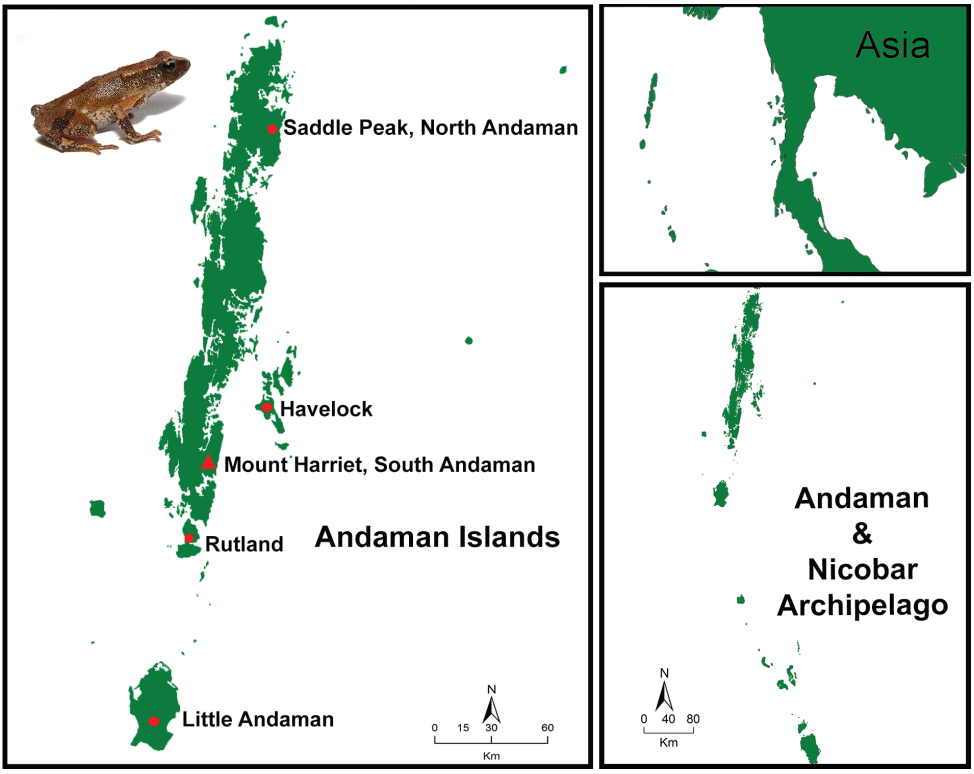

Figure 4. Map showing distribution of *Blythophryne beryet* gen. et sp. n. in the Andaman Islands, Bay of Bengal, India. Holotype collected from Mt Harriet (indicated with a red triangle).

unique niche of frogs belonging to the Old World tree frog family (Rhacophoridae), which are not known to occur on the Andaman Islands. All other anuran amphibians recorded from these islands are ground-dwelling, with the exception of *Kaloula baleata ghoshi,* which is semi-arboreal, and *Ingerana charlesdarwini*, which is known to use phytotelms for breeding and oviposition (Das 1998). During day time, bush toads were found under leaf litter on the forest floor.

The Andaman bush toad emits a white, viscous, pungent smelling secretion from the parotoid glands when handled (Fig. 5a); the secretion seems to be toxic, as other frogs kept within the same bag as one of these toads suffered mortality. Breeding commences in June with the onset of the Southwest Monsoon. Males were observed to call from heights of ca. 1–1.5 m above ground while sitting on leaves of bushes. Amplexus is axillary (Fig. 5b), and females deposit ova in phytotelms, which are tree-holes at a height of about 1–1.5 m above the ground filled with rainwater. Tadpoles develop in these phytotelms. The shrub from which the tadpoles described here were collected, measured 19 cm diameter at breast height, and eggs were found in a depression of 6 cm depth, filled with water up to 3 cm. The tree hole was oval, measuring 5 × 3 cm across (Fig. 6a).

The Andaman bush toad is widely distributed in islands where it occurs, and occupies forested habitats from 29–250 m asl, more common above 100 m asl and rarer at lower altitudes. The forest types in this elevation range include littoral, moist-deciduous, giant evergreen and montane stunted evergreen forests (Champion and Seth 1968).

Conservation status. The Andaman bush toad is known from five islands: North Andaman (Saddle Peak National Park only), South Andaman, Rutland, Havelock (only in a small patch of wet forest towards the south of the island) and Little Andaman. Based on searches carried out using 21 bounded quadrats of 100 m^2 each in these islands, the new species occurs at densities of 1.1 ± 0.37 toads per 100 m^2 of forest floor (unpublished data). It is considered 'Endangered' based on IUCN Ver. 3.1. Second Edition (IUCN 2014): criteria B.1 - extent of occurrence < 20000 km^2 and B.1.a - severely fragmented population and known to exist at no more than 10 locations. A large array of invasive fauna in these Islands threatens the population of this toad. Additionally, stochastic events and anthropogenic pressures are potential threats to the species and its habitat.

Notes on larval development. (Fig. 6b–f) The clutch of ova in the phytotelm located in May 2011 at Rutland Island was monitored continuously until complete tadpole transformation. Unpigmented, early-stage larvae were observed on 2nd May 2011. A total of 73 hatchlings presumably from a single clutch could be counted in the phytotelm. Following subsequent rain showers four days later on 6th May, only 25 tadpoles of Stage 20 could be observed, the rest presumably washed out by overflow. At this stage, the tadpoles were translucent and colourless, but speckled with black, with white abdominal yolk region, dorsally positioned eyes and labia visible. On 19th May, i.e., 13 days later, two samples of Stages 30 and 35 were collected and preserved in formalin. Tadpoles of these stages had exposed hind limbs, lacking forelimb buds and were dull purplish-brown in colour, without a dorsal pattern. A week later, on 25th May, the tadpoles that developed into Stages of 41 and 43, were preserved. At these advanced stages, the tadpoles showed developed forelimbs, with expanded discs of fingers, more intense pigmentation on skin, and feeble barred pattern on limbs. The Stage 43 larva is briefly described: mouth positioned anteriorly, with prominent, keratinised pair of jaw sheaths; keratodont absent, eyes and nostrils positioned dorsolaterally (IO 1.46 mm), nostrils much closer to eyes than snout tip. Body depressed, head-body 1.5 times as long as broad (HBL: HBW 1.53), tail almost twice as long as head-body (tL/HBL 1.95) with well-developed caudal musculature. Measurements of the tadpoles are in Table 5.

Description of Tadpole (Stage 35). Body tubular in dorsal and ovoid in lateral views, respectively (Fig. 6c). When viewed laterally, body dorsum is flattened and depressed medially; ventrally body slightly flattened at anterior end and convex towards posterior; body length 35% of total length; body attains maximum diameter in region immediately behind eyes. Snout broad and truncate in dorsal and pointed in lateral views, respectively. Eyes large; located and oriented dorso-laterally. Nostrils rounded with elevated rim, located almost midway but closer to eyes than snout, placed linear to eye in dorsal view; internarial distance subequal to interorbital distance. Spiracle

Figure 5. a A live, uncollected specimen of *Blythophryne beryet* gen. et sp. n. showing milky white secretion from the parotoid gland **b** Amplecting pair (live, uncollected) of *Blythophryne beryet* gen. et sp. n. showing axillary amplexus.

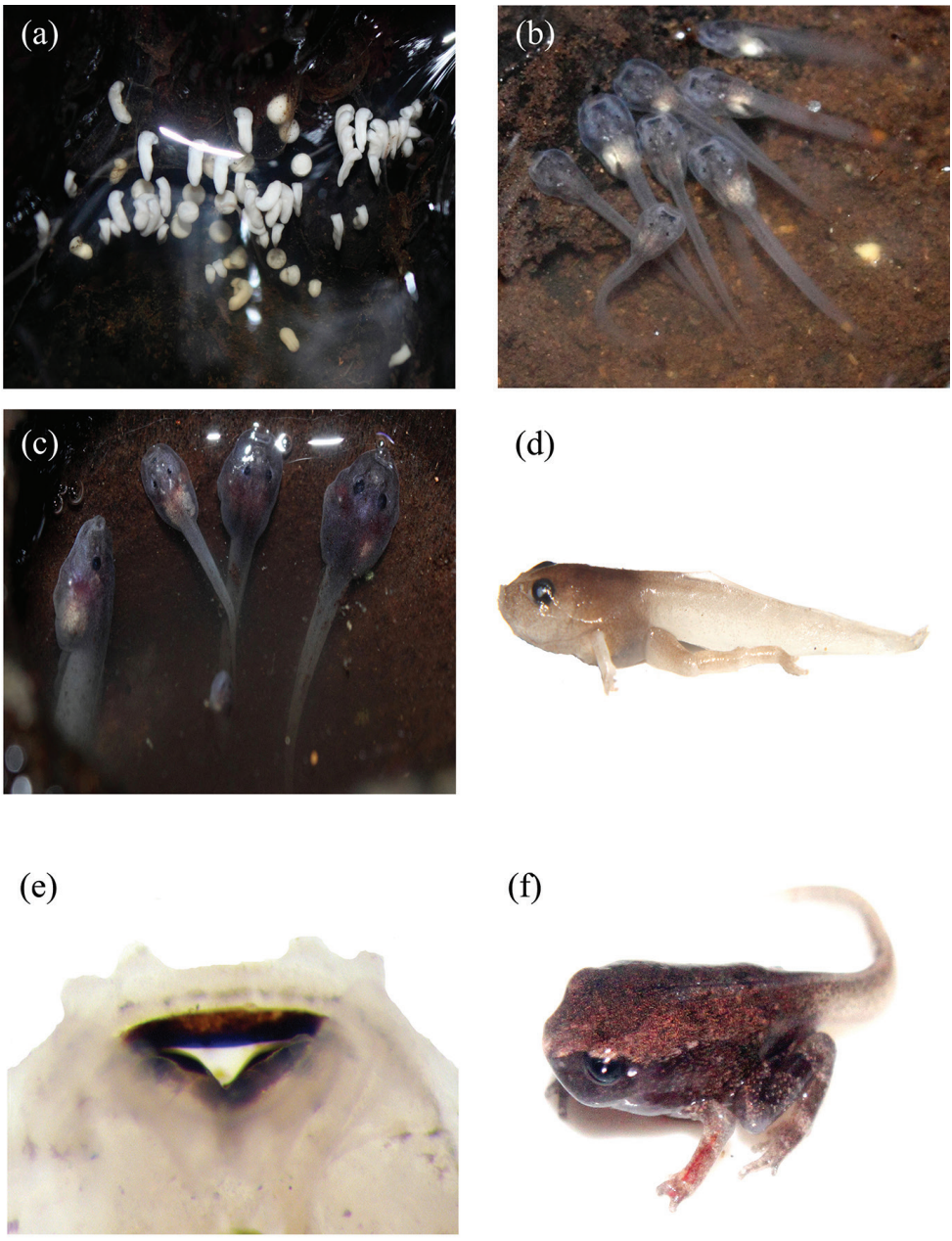

Figure 6. a Eggs and hatchling tadpoles of *Blythophryne beryet* gen. et sp. n. **b, c** endotrophic larvae of *Blythophryne beryet* gen. et sp. n. showing pale white abdominal yolk **d** Lateral view of a Stage 43 tadpole of *Blythophryne beryet* gen. et sp. n. **e** Oral disc of a Stage 35 larva of *Blythophryne beryet* gen. et sp. n., showing absence of keratodont and the presence of keratinised jaw sheaths **f** a metamorph of *Blythophryne beryet* gen. et sp. n. showing initiation of tail absorption.

Table 5. Morphometric measurements of tadpoles of *Blythophryne beryet* gen. et sp. n.

Stage	IOL	IND	NED	NSD	SS	SV	BL	TL	MBW	MTH	MTMW	TMH	ODD	VTL	DFH	VFH
30	1	0.8	0.4	0.7	3.6	1.6	5	11.1	2.8	2.5	1.1	1.3	1.2	0.7	0.6	0.4
35	1.1	1.1	0.4	0.8	5	3.3	6.8	12.4	3.7	3.5	1.3	2	1	1.7	0.9	0.7
41	1	1	0.4	0.6	3.9	2.4	6.1	11.8	3.8	2.8	1.3	1.8	1.5	0.9	0.7	0.7
42	1.3 (±.20)	1.1	0.4 (±.05)	0.7 (±.10)	NA	NA	7.3 (±.05)	13.6 (±.05)	3.5 (±.05)	3.2 (±.10)	1.8 (±.20)	1.9 (±.15)	1.3 (±.25)	NA	0.9 (±.05)	0.7 (±.05)
43	1.5 (±.30)	1.1	0.8 (±.20)	NA	NA	NA	7.0 (±.10)	9.4 (±1.05)	3.8 (±.20)	1.9 (±.35)	1.2 (±.10)	1.6	1.7 (±.10)	NA	0.4 (±.05)	0.3

sinistral and long with no inner wall; spiracle opening large; tube orientated posterolaterally, opening located approximately at midbody. Distance between spiracle and snout about 60% of body length. Intestinal coils not visible through the belly wall; vent tube medial. Tail tip broadly rounded; musculature linear till 1/3rd length of tail, after which it tapers. Dorsal fin slightly wider than ventral fin, originates posterior to body – tail junction and ventral fin at ventral terminus; both fins run parallel to tail muscle parallel through entire length of tail. Maximum tail height attained at about mid-length. Lateral line faintly visible. No glands observed on outer integument.

Oral disc positioned at terminal portion of body opening antero-ventrally (Fig. 6e); Rostral width of oral disc 27% body width, non-emarginate; entire oral disc visible dorsally; single row of seven to eight large marginal papillae present on lower labium and two to three on lateral corners; none present on upper labium; a single submarginal papilla located at each lateral corners; lower labium larger than upper labium. Denticle rows absent. Jaw sheaths well developed, heavily keratinised. Jaw sheaths completely serrated with minute serrations on lower jaw than upper jaw; suprarostrodont convex medially, longer than wide and lateral process of subequal height through length; infrarostrodont U-shaped.

Measurements (in mm; mean shown without parentheses and standard errors are shown in parentheses): Measurements of the seven tadpoles of various stage of development (Stages 30, 35, 41, 42 and 43) are presented in Table 5.

Colour. In life, dorsally, outer integument brown, with no melanopores. Ventrally, integument translucent but the gut was not visible; throat speckled. Both tail fins transparent with few melanophores. Laterally, tail muscle white with a few brown spots spread mainly at anterior region of tail. A completely transformed metamorph (SVL 10.6 mm; HL 4.23 mm) resembles adult in morphology, with an evident inverted 'V' mark on dorsum and transverse crossbars on limbs.

Morphological comparisons. Morphological and osteological characteristics of this new taxon are compared with members of other known Oriental bufonid genera below. The new taxon described here differs from the following known genera thus (only opposing character states in the genera being compared are mentioned):

Parapelophryne Fei, Ye & Jiang, 2003: type species– *Nectophryne scalptus* [current name combination: *Parapelophryne scalpta* (Liu and Hu 1973)]: Presence of eight presacral vertebrae and absence of parotoid glands (Fei et al. 2003). The phylogenetic position of this taxon was assessed by Matsui et al. (2015), who found it to be sister taxon to *Bufo japonicus*, thereby providing additional evidence for its distinctness from *Blythophryne* gen. n. described here. Distribution: Hainan, eastern China.

Pedostibes Günther, 1875: type species – *Pedostibes tuberculosus* Günther, 1875: Larger adult size (SVL 36.6–38.5 mm), presence of eight presacral vertebrae; short, rounded parotoid glands; tips of fingers dilated into truncated discs; small, numerous pigmented ova laid in strings, as in members of the genus *Duttaphrynus* and exotrophic larvae (Günther 1875, Inger et al. 1984, Fei et al. 2003, Matsui et al. 2007). Currently, the genus *Pedostibes* is represented by five nominal species, which show a disjointed distribution pattern. The westernmost of all, *P. tuberculosus*, is the type species associated

to the generic name (Günther 1875). *Pedostibes kempi* is known from the Garo Hills in Meghalaya, north-east India. Presently, *Pedostibes kempi* is considered congeneric, but differs in having a concealed tympanum. The remaining species, namely, *P. rugosus*, *P. hosii* and *P. everetti* occur in the Indo-Chinese and Indo-Malayan regions (Frost 2014). Bocxlaer et al. (2009), and more recently Ron et al. (2015), in their phylogenetic studies, showed that the genus *Pedostibes*, as currently defined, does not constitute a monophyletic group. According to their study, the type species, *P. tuberculosus* does not show a close relationship with the south-east Asian *P. hosii*. On the other hand, they demonstrated that *P. hosii* is the sister taxon to *Phrynoidis juxtasper*. In addition, the generic placement of *P. kempi* is also uncertain owing to the inconsistencies in morphological characters associated with this taxon. Hence, resolving the higher level systematic status of the south-east Asian taxa currently allocated to the genus *Pedostibes* will require further study. Distribution: Western Ghats, Indochina, Malay Peninsula.

Bufoides Pillai & Yazdani, 1973: type species– *Ansonia meghalayana* [current name combination: *Bufoides meghalayanus* (Yazdani & Chanda, 1971); currently monotypic, but additional, unnamed species recognised; Das et al. 2009]: Larger adult size (mean 42.9 mm, range 37–47 mm), absence of webbing and expanded discs in fingers, hidden tympanum, presence of cranial ridges and large, pigmented ova laid in strings, as in *Duttaphrynus* (Yazdni and Chanda 1972, Pillai and Yazdani 1973, Fei et al. 2003), presence of seven presacral vertebrae, distinguish this taxon from the newly described genus. Distribution: Khasi Hills, Meghalaya, north-east India (Frost 2014). *Pelophryne* Barbour, 1938: type species– *Pelophryne albotaeniata* Barbour, 1938: Presence of coccygeal expansions, absence of parotoid glands; fleshy manus with one phalange free of web and presence of seven (occasionally six) presacral vertebrae, urostyle fused to the sacrum and less number ($n \leq 30$) of larger sized yolky eggs (Barbour 1938, Inger 1954, 1966; Matsui et al. 2007). Distribution: eastern Asia, Sundaland and the Philippines (Frost 2011).

Sabahphrynus Matsui, Yambun & Sudin, 2007: type species– *Nectophryne maculata* [current name combination: *Sabahphrynus maculatus* (Mocquard, 1890)]: Larger adult size (41.21 ± 2.5, 30.4–52.6), presence of eight presacral vertebrae, absence of tympanum and parotoid glands, absence of webbing between the fingers, over 50 eggs/ovary and absence of an external vocal sac in males (Matsui et al. 2007). Distribution: endemic to Borneo (Frost 2014).

Duttaphrynus Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green & Wheeler, 2006: type species– *Bufo melanostictus* [current name combination: *Duttaphrynus melanostictus* (Schneider, 1799)]: Large adult size (mean SVL 43.7 mm), presence of eight presacral vertebrae, presence of keratinised cephalic ridges in some species, presence of large, keratinised warts on the dorsum, absence of expanded discs in finger and toe tips, absence of webbing between the fingers, numerous black pigmented ova laid in long, continuous strings, exotrophic larvae and terrestrial habit (Dubois and Ohler 1999, Manamendra-Arachchi and Pethiyagoda 1998). Particularly, the nomen *Bufo camortensis* (holotype – ZSI A 6955) erected for a species that is currently considered to represent *Duttaphrynus melanostictus* differs from the new taxon described here by its

considerably large adult size [SVL – 67 mm (*vs*. much smaller mean adult size of 24 mm in *Blythophryne* gen. n.), presence of keratinised cephalic ridges and glandular tubercles on the body (vs. absent in *Blythophryne* gen. n.), absence of webbing between the fingers and dilated terminal discs in the digits (vs. present in *Blythophryne* gen. n.). Distribution: East Africa through the Middle East, India, Indochina, east to the Sundas till Bali (Frost 2014).

Ansonia Stoliczka, 1870: type species – *Ansonia penangensis* Stoliczka, 1870: small to medium adult size (35–40 mm), absence of (or rudimentary) webbing between the fingers, presence of eight presacral vertebrae, absence of dilations in finger and toe tips, absence of parotoid glands, exotropic larvae with prominent oral discs and torrential stream dwelling habit (Inger 1960, Matsui et al. 2010). Distribution: Indo-Malayan region and the Philippines (Frost 2014).

Adenomus Cope, 1861: type species– *Adenomus badioflavus* Cope, 1861, a junior synonym of *Bufo kelaartii* [current name combination: *Adenomus kelaarti* (Günther, 1858)]: The genus *Adenomus* was resurrected from the synonymy of '*Bufo*' by Manamendra-Arachchi and Pethiyagoda (1998) to accommodate members of the '*Bufo*' *kelaarti* group, characterised by smooth finger edges; differing from the new taxon described here by its larger adult size (mean SVL 38.4 mm), presence of seven presacral vertebrae, absence of sheath-like webbing between fingers, absence of expanded discs at digit tips, presence of cranial ridges and indistinct tympanum (in *A. kelaarti*), terrestrial habit, pronounced sexual size dimorphism and unpigmented ova laid in long, continuous strings as in *Duttaphrynus* (Manamendra-Arachchi and Pethiyagoda 1998; Haas 1999; Meegaskumbura et al. 2015). Distribution: endemic to Sri Lanka (Frost 2014).

Ghatophryne Biju, Bocxlaer, Giri, Loader & Bossuyt, 2009: type species– *Ansonia ornata* [current name combination: *Ghatophryne ornata* (Günther, 1876)]: larger adult size (up to 35 mm SVL), characteristic reddish dorsal and ventral colouration, absence of parotoid glands, absence of webbing between the fingers, finger tips not dilated to discs and torrential stream dwelling habit (Biju et al. 2009). Distribution: Central Western Ghats in the states of Kerala and Karnataka (Frost 2014).

Xanthophryne Biju Bocxlaer, Giri, Loader & Bossuyt, 2009: type species– *Bufo koynaensis* [current name combination: *Xanthophryne koynaensis* (Soman, 1963)]: Larger adult size (up to 35.3 mm SVL), presence of characteristic chrome yellow patches along the flanks and sides of the abdomen, indistinct tympanum, weak, rounded parotoid glands, absence of webbing in fingers and discs in toes and fingers; large, pigmented ova laid in stagnant puddles on the ground (Biju et al. 2009). Distribution: Known only from Northern Western Ghats in Maharashtra, India (Frost 2014).

Leptophryne Fitzinger, 1843: type species – *Bufo cruentatus* [current name combination: *Leptophryne cruentata* (Tschudi, 1838)]: Dubois (1982) resurrected the genus *Leptophryne* Fitzinger, 1843 as the senior synonym of *Cacophryne* Davis, 1935, which currently comprises two species – *Leptophryne borbonica* (Tschudi, 1838) and *L. cruentata* (Tschudi, 1838). Presence of eight presacral vertebrae; firmisternal pectoral girdle; elongate subarticular tubercles near the base of each toe, numerous pigmented eggs and exotrophic larvae (Fei et al. 2003) distinguish it from *Blythophryne beryet* gen. et sp. n. Distribution: Sundaland (Frost 2014).

Pseudobufo Tschudi, 1838: type species – *Pseudobufo subasper* Tschudi, 1838: Large body size, stout habitus; presence of seven presacral vertebrae (vs. six in *Blythophryne* gen. n.) completely (to the tip of Toe IV) webbed feet (vs. incomplete toe webbing in *Blythophryne beryet* gen. et sp. n.), fingers basally webbed; parotoid glands absent; dorsal, lateral and ventral skin surfaces with fine spinules, dorsoventrally depressed body with large, round warts and dorsally positioned nostrils (vs. lateral) distinguish it from the new genus described here (Fei et al. 2003; Inger and Stuebing 2005). Distribution: Sundaland.

Ingerophrynus Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green & Wheeler, 2006: type species– *Bufo biporcatus* [current name combination: *Ingerophrynus biporcatus* (Gravenhorst, 1829)]: Presence of seven presacral vertebrae (vs. six); absence of lateral dilations in the digit tips (vs. present); absence of webbing between the fingers (vs. present) and endotrophic (vs. exotrophic) larvae distinguish *Blythophryne beryet* gen. et sp. n. from *Ingerophrynus*. Distribution: Southern Yunnan, Indochina, the Malay Peninsula, the islands of Indo-Malaya, and Philippines.

Phrynoidis Fitzinger, 1843: type species – *Bufo asper* [current name combination: *Phrynoidis asper* (Gravenhorst, 1829)]: Large adult size (up to 100 mm SVL) presence of an omosternum, (vs. absent); presence of seven presacral vertebrae (vs. six); absence of lateral dilations of digit tips (vs. present) and exotrophic (vs. endotrophic) larvae distinguish this genus from the new genus *Blythophryne* gen. n. Distribution: Myanmar through western and peninsular Thailand, the Malay Peninsula, Sumatra, Borneo, and Java.

Apart from the above bufonid genera known from Oriental Asia, the new taxon described herein differs from the following central-west African genera:

Nectophryne Buchholz & Peters, 1875: type species – *Nectophryne afra* Buchholz & Peters, 1875 by the presence of eight presacral vertebrae (vs. six in *Blythophryne beryet* gen. et sp. n.); presence of lamelliform subdigital pads – a character unique to *Nectophryne* which is absent in the new taxon described here. Oriental forms including members of the genera *Pedostibes* and *Pelophryne* were attributed to *Nectophryne* earlier (Boulenger 1892, 1896, 1919), until Barbour (1938) redefined these genera.

Nectophrynoides Noble, 1926: type species – *Nectophryne tornieri* [current name combination: *Nectophrynoides tornieri* (Roux, 1906)]: The comparisons made here are restricted to the type species of *Nectophrynoides* because the genus is poorly defined and is composed of representatives with a broad spectrum of morphological and developmental characteristics. Though unique among bufonids in possessing an omosternum and a direct developmental mode (in *N. viviparus*), members of this genus are poorly diagnosed with respect to other genera (Menegon et al. 2004). Larger adult size (SVL 21–30 mm), presence of expanded, truncate fingertips (vs. expanded and curved in *Blythophryne beryet* gen. et sp. n.), presence of eight presacral vertebrae (vs. 6 in *Blythophryne beryet* gen. et sp. n.) however, distinguish *Nectophrynoides* from the new taxon described here (see Tihen 1960; Menegon et al. 2004; Harper et al. 2010).

Molecular phylogeny. Multiple sequence alignment of the 16S homologous regions resulted in 498 conserved sites and 246 parsimoniously informative sites. In the

phylogenetic analysis using both Maximum likelihood and Bayesian inference, the focal taxon showed a unique taxonomic position. The phylograms of both inference methods were similar (Fig. 7). *Bufoides meghalayanus* was found to be the closest taxon to the focal species, *Blythophryne beryet* gen. et sp. n. in the tree generated using 36 species from Asia and Africa but with relatively low support (Fig. 7a). However, when analysed with more of the Asian toads, it clearly separates out from species of *Duttaphrynus*, as well as, those of *Xanthophryne* and Bufoides (Fig. 7b, c). The average within-genus pairwise K2p distances at the partial 16S rRNA gene for all the described genera considered under this study was 0.0642, with 99% confidence interval (CI) of 0.0512–0.0687 (Table 6). The average pairwise k2p distance of the focal species with all other taxa at partial 16S rRNA gene considered here was 0.103, with a 99% CI of 0.096–0.113, strongly supporting its distinctiveness and unique phylogenetic position within the Bufonids. Similarly, for partial 12S rRNA gene, the average within-genus pairwise K2p distances for all described genera was 0.0495, with the 99% CI of 0.0387–0.0603. The average pairwise k2p distance of the focal species with all other taxa at partial 12S rRNA gene was 0.0783, with a 99% CI of 0.072–0.085. Both tree-based and distance-based analyses clearly indicate the uniqueness of its phylogenetic position. Thus, the rDNA typing strongly suggest the new taxon as a candidate to be named as a new genus/species.

Discussion

The small-sized bush toad described here is an interesting new find from the Andaman Islands, in the Bay of Bengal, Republic of India. It has a number of unique external morphological and skeletal characters, in comparison to known Oriental and other relevant bufonid genera. Its distinctiveness and unique taxonomic position (warranting the erection of a monotypic genus), is also robustly supported by phylogenetic reconstruction carried out using partial 16S and 12S gene sequences and showing its position relative to other Asian and African bufonids (Pramuk et al. 2008; Van Bocxlaer et al. 2009, 2010; Matsui et al. 2007). Much of the rapid radiation and diversification of toads happened during the Paleogene, and show short intermodal distances (Pramuk et al. 2008). The phylogenetic inference obtained in the present study is concordant with those of the earlier studies.

Biogeographic remarks. Bufonidae is a species-rich family, with nearly cosmopolitan distribution around the globe (Frost 2014). Pramuk et al. (2008) suggests a post-Gondwanan, South American origin of the family, and a rapid diversification and dispersal across the globe, and a return to South America within a short span of 80 million years. They hypothesised overland dispersal routes for both out-of and into-South America. While this explains the possible routes of dispersal and diversification of bufonids across the continental mainland, the routes of diversification of the Bufonidae on islands is unclear, including evolution of endemic bufonid lineages on Sri Lanka, insular south-east Asia and the Andaman Islands.

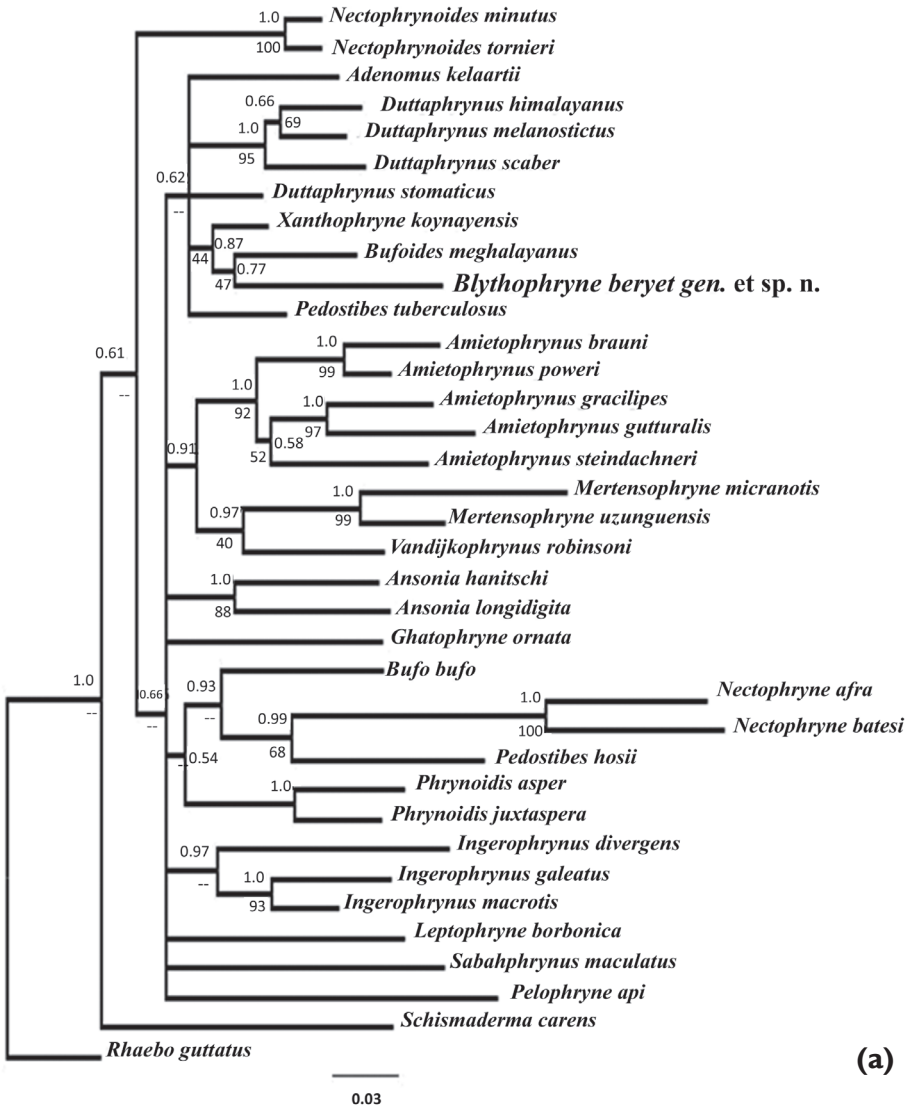

Figure 7. Phylogenetic position of *Blythophryne beryet* gen. et sp. n., inferred from concatenated partial 12S and 16S rDNA sequences. The posterior probabilities for Bayesian Inference (BI) and the bootstrap support values for the ML are given as (BI /ML) above and below the branch nodes. **a** The tree was generated using 36 species related to 21 genera, and was rooted using *Rhabeo gutattus* as outgroup **b** the subclade containing the Indian and Sri Lankan toads (7 genera, 17 species) rooted using *Ghatophryne ornata* as outgroup; and **c** the subclade containing the Indian and Sri Lankan toads (5 genera, 15 species) rooted using *Adenomus kelaartii* as outgroup.

The herpetofauna of Andaman and Nicobar Islands are considered to be of either Indo-Chinese or Indo-Malayan affinities (Das 1999). While it is hypothesised that the Nicobar Islands are of volcanic origin, most of the Andaman Islands are uplift of sub-

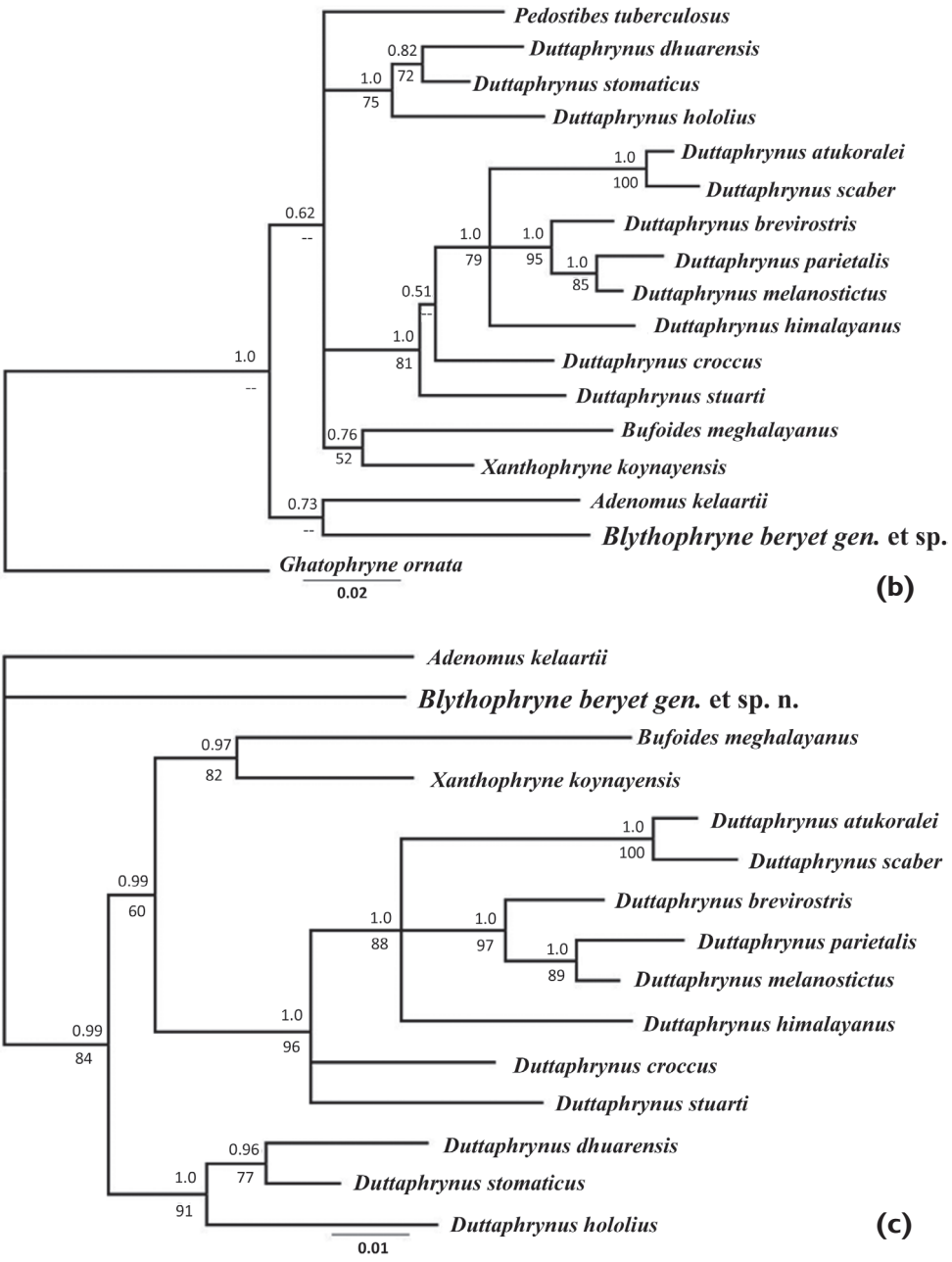

Figure 7. b and **c** continued.

merged landmass (Krishnan 1961). Exchange of biota would have been facilitated via either a physical connection of the islands to the mainland during lowering of sea level (Rodolfo 1969) or through trans-oceanic or other forms of across-water dispersal, espe-

Table 6. The average uncorrected K2p distance estimates across genera of Bufonidae based on partial 16S (lower triangular matrix) and 12S (upper triangular matrix) rDNA sequences, calculated using MEGA6.06; within genus K2p distances are given along the diagonal (left value for 16S and right value for 12S).

		16S/12S k2p uncorrected pair-wise distance estimates																				
Taxa (Genus*/Species)		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1	*Amietophrynus*	**0.068/ 0.044**	0.060	0.049	0.072	0.067	0.094	0.066	0.059	0.074	0.071	0.066	0.049	0.077	0.043	0.100	0.103	0.067	0.063	0.043	0.037	0.071
2	*Ansonia*	0.106	**0.066/ 0.060**	0.061	0.086	0.083	0.115	0.079	0.066	0.081	0.073	0.065	0.071	0.104	0.061	0.114	0.097	0.067	0.067	0.055	0.063	0.085
3	*Duttaphrynus*	0.092	0.089	**0.049/ 0.038**	0.070	0.063	0.099	0.067	0.060	0.065	0.076	0.065	0.054	0.084	0.045	0.098	0.099	0.074	0.054	0.045	0.049	0.077
4	*Ingerophrynus*	0.090	0.084	0.074	**0.056 /0.077**	0.077	0.102	0.075	0.082	0.073	0.085	0.071	0.081	0.093	0.065	0.113	0.111	0.078	0.071	0.062	0.075	0.085
5	*Mertensophryne*	0.095	0.100	0.099	0.090	**0.059/ 0.061**	0.098	0.079	0.066	0.075	0.083	0.075	0.079	0.094	0.067	0.108	0.114	0.083	0.079	0.059	0.065	0.073
6	*Nectophryne*	0.143	0.141	0.127	0.139	0.140	**0.089 /0.049**	0.092	0.095	0.098	0.107	0.093	0.098	0.108	0.073	0.146	0.136	0.102	0.083	0.090	0.102	0.081
7	*Nectophrynoides*	0.097	0.091	0.077	0.082	0.099	0.132	**0.021/ 0.015**	0.068	0.084	0.069	0.069	0.069	0.085	0.051	0.119	0.089	0.061	0.077	0.034	0.077	0.069
8	*Pedostibes*	0.091	0.086	0.066	0.083	0.104	0.115	0.074	**0.076 /0.065**	0.075	0.071	0.067	0.063	0.085	0.051	0.130	0.107	0.068	0.069	0.048	0.063	0.071
9	*Phrynoidis*	0.086	0.084	0.073	0.082	0.102	0.135	0.080	0.080	**0.039 /0.085**	0.089	0.079	0.091	0.108	0.063	0.123	0.119	0.085	0.069	0.071	0.085	0.081
10	*Ghatophryne ornata*	0.092	0.104	0.075	0.086	0.103	0.148	0.088	0.083	0.087	**n/a**	0.061	0.081	0.102	0.053	0.124	0.097	0.081	0.077	0.057	0.065	0.086
11	*Leptophryne borbonica*	0.103	0.107	0.095	0.096	0.117	0.121	0.099	0.082	0.099	0.092	**n/a**	0.073	0.085	0.045	0.106	0.085	0.069	0.053	0.061	0.061	0.069
12	*Vandijkophrynus robinsoni*	0.083	0.102	0.063	0.071	0.082	0.132	0.077	0.077	0.073	0.064	0.102	**n/a**	0.072	0.053	0.110	0.097	0.073	0.072	0.037	0.049	0.085
13	*Schismaderma carens*	0.096	0.099	0.076	0.088	0.086	0.132	0.082	0.079	0.095	0.097	0.107	0.092	**n/a**	0.089	0.110	0.110	0.089	0.069	0.077	0.081	0.094
14	*Bufo bufo*	0.102	0.113	0.078	0.107	0.116	0.134	0.085	0.088	0.082	0.087	0.105	0.076	0.105	**n/a**	0.110	0.085	0.057	0.057	0.034	0.045	0.057
15	*Sabahphrynus maculatus*	0.092	0.085	0.077	0.071	0.097	0.124	0.093	0.079	0.081	0.082	0.093	0.087	0.082	0.104	**n/a**	0.145	0.114	0.110	0.102	0.106	0.123
16	*Pelophryne api*	0.108	0.105	0.102	0.092	0.100	0.155	0.106	0.097	0.106	0.100	0.113	0.100	0.122	0.126	0.112	**n/a**	0.089	0.093	0.068	0.101	0.093
17	*Bufoides meghalayanus*	0.088	0.091	0.055	0.080	0.109	0.133	0.073	0.064	0.069	0.078	0.095	0.062	0.087	0.071	0.087	0.105	**n/a**	0.073	0.041	0.069	0.069
18	*Adenomus kelaartii*	0.091	0.091	0.065	0.084	0.107	0.136	0.070	0.071	0.070	0.066	0.098	0.059	0.090	0.083	0.085	0.103	0.062	**n/a**	0.061	0.069	0.065
19	*Xanthophryne koynayensis*	0.084	0.078	0.054	0.070	0.098	0.129	0.072	0.056	0.077	0.073	0.087	0.059	0.082	0.078	0.078	0.083	0.039	0.057	**n/a**	0.049	0.049

		16S/12S k2p uncorrected pair-wise distance estimates																			
Taxa (Genus*/Species)		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
20	*Rhaebo guttatus*	0.111	0.085	0.092	0.088	0.104	0.136	0.089	0.083	0.086	0.087	0.105	0.092	0.088	0.114	0.094	0.112	0.092	0.085	0.075	**n/a**	0.086
21	***Blythophryne beryet* gen. et sp. n.**	0.103	0.119	0.089	0.098	0.118	0.165	0.098	0.102	0.088	0.099	0.112	0.092	0.101	0.105	0.106	0.112	0.080	0.082	0.075	0.109	**n/a**

#: The data in the first nine rows for samples '1' to '9'are average k2p estimates (Intra-/inter species) for all the species of the indicated genus considered in the study; these are as follow:

Taxa-1: Amietophrynus : *A. brauni, A. poweri, A. gracilipes, A. gutturalis, A. steindachneri*
Taxa-2: Ansonia : *A. hanitschi, A. longidigita*
Taxa-3: Duttaphrynus : *D. himalayanus, D. melanostictus, D. scaber, D. stomaticus*
Taxa-4: Ingerophrynus : *I. divergens, I. macrotis, I. galeatus*
Taxa-5: Mertensophryne : *M. micranotis, M. uzunguensis*
Taxa-6: Nectophryne : *N. afra, N. batesi*
Taxa-7: Nectophrynoides : *N. minutus, N. tornieri*
Taxa-8: Pedostibes : *P. hosii, P. tuberculosus*
Taxa-9: Phrynoidis : *P. asper, P. juxtasper*

cially for the Nicobar archipelago. There are records of long-distance overseas dispersal routes, which could be the only possible route for certain endemic taxa of the archipelago, such as the Andaman Day Gecko, *Phelsuma andamanense* (see Austin et al. 2004). Amphibians, although generally considered intolerant to salinity, have also been known to show long-distance, overseas dispersal (e.g., Vences et al. 2003).

The submerged chain of mountains referred to as the "Burma arc" was formed at the same time as the main Himalayan chain, during the late Cretaceous (Krishnan 1961). The occurrence of a distinct lineage prompts us to propose the following explanations: (i) overland dispersal when the Islands were connected to the mainland due to lowering of sea level; (ii) trans-oceanic dispersal; (iii) relic lineage surviving in the Islands due to a vicariant event that might have occurred during Cretaceous by isolation in on mountain tops on the "Burma arc". While there are also records of long-distance overseas dispersal into the Islands, such as Andaman day Gecko, *Phelsuma andamanense* (see Austin et al. 2004) and in frogs (e.g. Vences et al. 2003), evidence for the other hypotheses are clearly not available at present. Scanty geological data and poor sampling of toad lineages in the mountains of Myanmar that precludes unambiguous molecular dating of sister lineages, also make it difficult to infer the biogeographic affinities of the Andaman bush toad at present.

The new taxon described here is characterised with a small adult body size, semi-arboreality high specificity for larval microhabitat niche, absence of inguinal fat bodies, relatively low number of mid-sized ova and a narrow distributional range. Further, it seems to be an exception in possessing parotoid glands, which was a character associated with widely distributed bufonid species (Bocxlaer et al. 2010), and presumably relate to reduction of predation via development of specialised glands for storage of dietary-sequestered toxins.

Likewise, the larvae of this new taxon with a moderate, intermediate clutch size and a high specificity towards the site of oviposition (i.e., phytotelms) explain its limited range of distribution as currently understood. Further studies in the Andaman archipelago are needed to understand the identity and origins of its fauna.

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Appendix 1

List of comparative material of Oriental and other relevant members of the Bufonidae examined.

Adenomus 'dasi' WHT 2267–69; *Adenomus kandianus* BMNH 1947.2.62–63; *Adenomus kelaartii* MNHN 140.0; WHT 1447; WHT 1451; *Ansonia guibei* UNIMAS 7746; *Ansonia hanitschi* UNIMAS 8050; *Ansonia longidigita* UNIMAS 7925–26; *Ansonia minuta* UNIMAS 7427; *Ansonia muleleri* FMNH 96125 (cleared and stained); *Ansonia penangensis* USNM 216034 (radiograph), ZSIC 2717–18; 3585–61; *Ansonia spinulifer* UNIMAS 7580; *Bufoides meghalayanus* WII uncatalogued (cleared and stained) ZSIC A6969–70; *Duttaphrynus melanostictus* WII 38.6.92 (cleared and stained); WHT 2276; UNIMAS 9313, UNIMAS 9349; *Duttaphrynus olivaceus* ZSIC 3523–25; *Duttaphrynus silentvalleyensis* ZSIM/SRS VA/77; *Duttaphrynus stuarti* ZSIC 19958; *Ghatophryne rubigina* ZSIM/SRS VA/775; *Ingerophrynus divergens* FMNH 138867 (cleared and stained); UNIMAS 7943; *Ingerophrynus kumquat* ZRC 1.3137–42; 1.3584; *Ingerophrynus parvus* ZSI 15196–97; *Ingerophrynus quadriporcatus* UNIMAS 9433; *Leptophryne borbonica* FMNH 185792 (cleared and stained); UNIMAS 9055; *Nectophryne afra* ZMB8472 (holotype); MCZ A2607 (radiographs); *Pedostibes hosii* FMNH 77369 (cleared and stained); UNIMAS 8434; UNIMAS 8972; *Pedostibes tuberculosus* WII 38.6.91 (cleared and stained); *Pedostibes kempi* ZSI 18481 (syntype); *Pelophryne albotaineata* MCZ A–23291 (holotype; radiograph); *Pelophryne linanitensis* ZRC 1.11906–10; *Pelophryne misera* UNIMAS 8053; *Pelophryne murudensis* ZRC 1.11902–905; *Pelophryne signata* UNIMAS 7589, UNIMAS 7930, UNIMAS 7931; *Phrynoidis asper* FMNH 219718 (cleared and stained); UNIMAS 7874; UNIMAS 9432; *Pseudobufo subasper* FRIM uncat., USNM 313624, MCZ A 19579; *Sabahphrynus maculatus* MNHN P1899–267 (lectotype; radiograph); *Xanthophryne koynayensis* BNHM 377; ZSIC A1784; ZSIM/SRS VA/775.

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A new species of *Liolaemus* related to *L. nigroviridis* from the Andean highlands of Central Chile (Iguania, Liolaemidae)

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Abstract

The *Liolaemus nigroviridis* group is a clade of highland lizards endemic to Chile. These species are distributed from northern to central Chile, and currently there are no cases of sympatric distribution. This study describes a new species, *Liolaemus uniformis* **sp. n.**, from this group, and provides a detailed morphological characterization and mitochondrial phylogeny using cytochrome-b. *Liolaemus uniformis* was found in sympatry with *L. nigroviridis* but noticeably differed in size, scalation, and markedly in the color pattern, without sexual dichromatism. This new species has probably been confused with *L. monticola* and *L. bellii*, both of which do not belong to the *nigroviridis* group. The taxonomic issues of this group that remain uncertain are also discussed.

Keywords

Liolaemus nigroviridis, *L. uniformis* sp n., lizard, *Cyt-b*, mtDNA

Introduction

The *Liolaemus nigroviridis* group is a clade of highland lizards endemic to central and northern Chile, the species of which are allopatrically distributed (Pincheira-Donoso and Núñez 2005). Almost all species of this group have a complicated taxonomic history with several cases of synonymies (*e.g*. Núñez and Jaksic 1992, Pincheira-Donoso and Núñez 2007, Troncoso-Palacios 2013). In his book on lizards from northwest, northeast, and eastern Argentina, Cei (1993) proposed the *nigroviridis* group and included within it *L. constanzae* Donoso-Barros, 1961. Lobo (2001) performed the first cladistic analysis of this group and, based on morphological characteristics, included the following species: *L. campanae* Hellmich, 1950, *L. lorenzmuelleri* Hellmich, 1950, *L. maldonadae* Núñez, Navarro & Loyola, 1991, and *L. nigroviridis* Müller & Hellmich, 1932. Later, Lobo (2005), updating the morphological phylogeny, added to the *nigroviridis* group *L. nigroroseus* Donoso-Barros, 1966 and *L. isabelae* Navarro & Núñez, 1993, but excluded *L. lorenzmuelleri*.

Pincheira-Donoso and Núñez (2005), through phenetic analysis recovered all of the species listed by Lobo (2005) within the *nigroviridis* group and also reincorporated *L. lorenzmuelleri* and *L. constanzae*. Furthermore, *L. constanzae* was listed with two subspecies, *L. c. constanzae* and *L. c. donosoi* Ortiz, 1975. These authors also incorporated *L. juanortizi* Young-Downey & Moreno, 1991 and *L. melanopleurus* (Philippi, 1860), the latter of which was included as *incertae sedis*. Moreover, *L. nigroroseus* was considered a junior synonym of *L. constanzae*, as has also been proposed by other authors (Núñez and Jaksic 1992, Troncoso-Palacios 2013), while *L. campanae* was regarded as a junior synonym of *L. nigroviridis*. In fact, *L. campanae* was previously described as a subspecies of *L. nigroviridis* (Hellmich 1950) and later proposed to be a synonym of *L. nigroviridis* (Núñez and Jaksic 1992, Valencia et al. 1979).

Lobo et al. (2010) accepted all of the species listed by Pincheira-Donoso and Núñez (2005) as members of the *nigroviridis* group, except for *L. donosoi* which they placed into the *nigromaculatus* group. Finally, Troncoso-Palacios (2013) indicated that *L. donosoi* is a junior synonym of *L. constanzae*, as previously suggested (Núñez and Jaksic 1992, Veloso et al. 1982), and recognized the seven species listed by Lobo et al. (2010) as members of the *nigroviridis* group – *L. constanzae*, *L. isabelae*, *L. juanortizi*, *L. lorenzmuelleri*, *L. maldonadae*, *L. melanopleurus*, and *L. nigroviridis*.

Very few studies have used molecular data within this group. Schulte and Moreno-Roark (2010) constructed a mitochondrial phylogeny of 733 Iguanian lizards. The authors concluded that *L. n. nigroviridis*, and *L. n. campanae* are sister taxa and that *L. isabelae* does not belong to the *nigroviridis* group. Cianferoni et al. (2013) performed a cytochrome-b (*Cyt-b*) phylogeographic study in *L. nigroviridis* populations and proposed that this species could contain at least two different species-level lineages.

In a field trip to the vicinity of Piuquenes (Valparaíso Region, Chile), we believe we found some populations probably previously assigned to *Liolaemus monticola* Müller & Hellmich, 1932 by Núñez et al. (2010:57). Subsequent *Cyt-b* phylogenetic analysis

and morphological comparisons determined that this population represents a new species that belongs to the *nigroviridis* group. This new species occurred in sympatry with *L. nigroviridis*, constituting the first case of sympatry within this group of lizards.

The current study describes this new species and provides a full diagnosis in regards to other species of the *nigroviridis* group. Although the color pattern of this new species resembles *L. juanortizi* and *L. lorenzmuelleri*, the scalation is markedly different and the distribution is allopatric (> 240 km of separation). Moreover, various taxonomical aspects of the *nigroviridis* group that require attention are discussed.

Materials and methods

Morphological data and analyses

Specimens of all species currently considered within the *nigroviridis* group were examined. Morphological characteristics were examined according to Etheridge (1995) and Lobo (2005). Body measurements were taken with a digital Vernier caliper (0.02 mm precision) and given as the mean ± standard deviation (x ± SD). We applied a Kolmogorov-Smirnov test to verify data normality, a subsequent t-test or Mann-Whitney U test was used if data passed or failed the normality test, respectively, to compare scale count (midbody, dorsal and ventral) and size (snout vent length, SVL) of the new species against some related species (*Liolaemus constanzae*, *L. juanortizi*, *L. lorenzmuelleri* and *L. nigroviridis*). Only significant results are presented. Scales were observed with different magnifying lenses. Scalation and measurements were recorded on the right side of the specimen. Dorsal scales were counted between the occiput and the anterior border of the hind limbs. Ventral scales were counted from the mental scale to the anterior margin of the cloacal opening. Stomach and intestinal contents were analyzed under a binocular stereoscope for one specimen of the new species. Data for the midbody scales of *Liolaemus juanortizi* were taken from one revised specimen and six reported in Young-Downey and Moreno (1991). Classification was carried out considering species currently assigned to the *nigroviridis* group (Troncoso-Palacios 2013). *Liolaemus isabelae* is included in the comparison but the relationship of this species with the *nigroviridis* group is uncertain (see Discussion). The examined specimens are listed in Appendix I. Some mapping data were taken from existing literature or field observations without specimen collection: 1) *L. nigroviridis* from Manque (Mella 2005), El Arpa and El Roble (Cianferoni et al. 2013), Riecillo (Núñez et al. 2010), Campana (Hellmich 1050), Chepical and Juncal (field observations, 32°16'S - 70°30'W and 32°53'S - 70°07'W respectively); 2) *L. maldonadae* from Los Molles (Núñez et al. 1991). Acronyms used are: Museo Nacional de Historia Natural de Chile (MNHNCL), Museo de Zoología de la Universidad de Concepción (MZUC) and Colección de Flora y Fauna, Profesor Patricio Sánchez Reyes of the Pontificia Universidad Católica de Chile (SSUC).

DNA purification, PCR amplification, and sequencing

Samples from liver and thigh muscle were obtained from ethanol-fixed lizards which were subject to a rehydration process according to Coura (2005). Samples were washed twice in distilled water for 5 min at 55 °C to remove the fixative and then rehydrated with 1x Tris/EDTA for 5 min at 55 °C and then 1M Tris pH 7.5, at 55 °C overnight. Right after, samples were digested with proteinase K (20 mg/ml) at 55 °C overnight. Genomic DNA isolation (mitochondrial and nuclear) was done with the Wizard® Genomic DNA Purification kit (Cat # A1120, Promega, USA) following manufacturer´s instructions. The mitochondrial gene *Cyt-b* was amplified from total DNA through two phase conventional PCR with the primers GLUDGL (5´-TGA CTT GAA RAA CCA YCG TTG-3´) and CB3 (5´-GGC AAA TAG GAA RTA TCA TTC-3´), reported in Torres-Pérez et al. (2009), to generate a 700bp amplicon. PCR reactions were performed with the SapphireAmp® Fast PCR Master Mix (Cat # RR350A, Takara Clontech, USA) using 100 ng of total genomic DNA as a template and following the instruction manual. Two-phase PCR cycling was as follows: Phase 1, initial 98 °C denaturation for 3 min, then 5 cycles of 98 °C denaturation for 30 s, 47 °C annealing for 45 s and 72 °C extension for 45 s. The Phase 2, next 40 cycles of 98 °C denaturation for 30 s, 58 °C annealing for 45 s and 72 °C extension for 45 s. A final 72 °C extension step for 5 min was added to finish the PCR. The 700 bp PCR amplicon was checked by DNA electrophoresis on a 1% agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer. The amplicons were purified with the E.Z.N.A.® Cycle-Pure Kit (Cat # D6492-02, Omega Biotek, USA) and sent for capillary sequencing to Macrogen, Korea.

Phylogenetic reconstruction

The accession numbers of the *Cyt-b* mitochondrial loci sequences generated in this study and the sequences obtained from GenBank are indicated in Appendix II. Forty three nucleotide sequences involved in the analysis were aligned using MUSCLE (Edgar 2004). We used the JModelTest v2.1.7 (Darriba et al. 2012, Guidon and Gascuel 2003) to select an appropriate substitution model (HKY + G + I), with a BIC index. We performed a Bayesian inference (BI) analyses with MrBayes v3.1.5 (Ronquist and Huelsenbeck 2003). Two independent analyses, each consisting of two groups of four chains that run independently, that were run for 15.0×10^6 generation and a at sample frequency = 1000. Priors were let by default. *Phymaturus vociferator* Pincheira-Donoso 2004, was selected as the outgroup. The 25% of samples were discarded as burnin when calculating the convergence diagnostic, assessed examining values of average standard deviation of the Potential Scale Reduction Factor (PSRF) for all parameters.

Results

The genetic tree constructed from mitochondrial DNA (mtDNA) (Fig. 1) placed the newly identified *Liolaemus* species as a sister taxon of *L. nigroviridis* (posterior probability pp = 1). However, no data are available for most of the species in the *nigroviridis* group as sample collection is hampered by the high altitudes where these species inhabit. Therefore, the discovered topology should be considered preliminary (see Discussion). *Liolaemus monticola* is nested with strong support (pp = 1) in the *monticola* group, the sister clade of the *nigroviridis* group. *Liolaemus bellii* is not closely related to the new *Liolaemus* or *L. monticola*, and is nested in a node with polytomy.

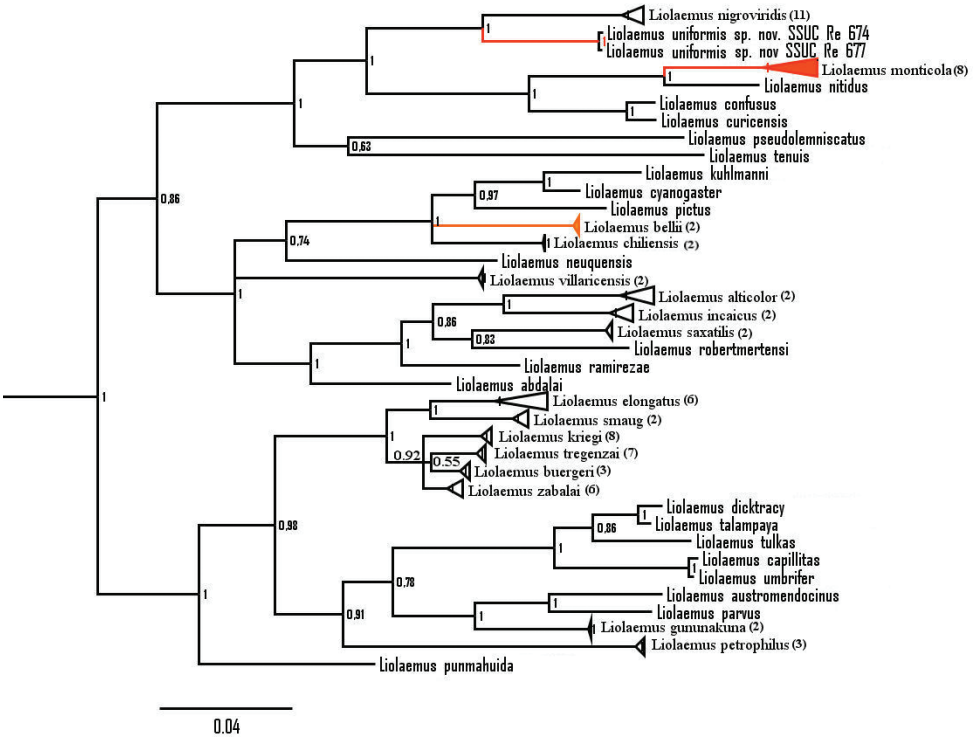

Figure 1. Bayesian inference of phylogeny tree using *Cyt-b* showing phylogenetic relationships of *Liolaemus uniformis* sp. n. (red) and related species (HKY+G+I model). *Liolaemus bellii* and *L. monticola*, probably confused with the new species, are also in red. Posterior probability is indicated at each node. Scale shows the number of substitutions per site. Number between parentheses indicates the number of sequences for the collapsed nodes.

***Liolaemus uniformis* sp. n.**
http://zoobank.org/B412BEF2-C337-4472-A4CE-9AFD73876B07
Fig. 2A, B

Liolaemus altissimus altissimus (in part?), Mella. 2005, Guía Camp. Rep. Chil. Zon. Cent., p. 38.
Liolaemus monticola?, Núñez et al. 2010, Bol. Mus. Nac. Hist. Nat., p. 57.

Holotype. SSUC Re 674. Adult male. Collected in the west shore of the Chepical Lagoon (32°15'S – 70°30'W), approximately 30 km NE Alicahue, San Felipe de Aconcagua Province, Valparaíso Region, Chile. Collectors: J. Troncoso-Palacios and E. Alfaro. December, 2012.

Paratypes (Fig. 2C, D, E, F). SSUC Re 675, male. SSUC Re 676–78, three females. SSUC Re 679, juvenile. The same data as the holotype.

Etymology. The species name "*uniformis*" (Latin) refers to the lack of dorsal pattern and uniform color found for both males and females.

Diagnosis. *Liolaemus uniformis* is larger than *L. constanzae* (Mann–Whitney U = 0.5, P < 0.01, Table 1). *Liolaemus constanzae* has sexual dichromatism, a feature absent in *L. uniformis*. Males of *L. constanzae* have a black vertebral line and black spots on the paravertebral fields (Fig. 3A), whereas *L. uniformis* has no dorsal pattern. Additionally, the southern distributional limit of *L. constanzae* in Agua Verde, Antofagasta Region, Chile (Ortiz 1975), is more than 750 km north of the type locality recorded for *L. uniformis*.

Liolaemus uniformis differs from *L. isabelae* (Fig. 3C), because in the latter the nasal and the rostral scales are in contact only in 25% of specimens, whereas in *L. uniformis,* these scales are always in contact. Males of *L. isabelae* have black ventral coloration, a yellow dorsal color with a black vertebral line, black bars in the paravertebral fields, and a black lateral band, or some males have a completely black dorsal color; all traits that are absent in *L. uniformis*. Additionally, the southern distributional limit of *L. isabelae* in Salar de Pedernales, Atacama Region, Chile (Pincheira-Donoso and Núñez 2005) is more than 650 km north of the type locality recorded for *L. uniformis.*

Liolaemus uniformis resembles *L. lorenzmuelleri* (Fig. 3E) and *L. juanortizi* (Fig. 3D), species suggested as conspecific (Pincheira-Donoso and Núñez 2005). However, the dorsal scales in *L. lorenzmuelleri* and *L. juanortizi* are noticeably larger than those of *L. uniformis*, and have a distinct "ovoid" shape. *Liolaemus uniformis* has more dorsal scales (60.0 ± 2.9) than *L. lorenzmuelleri* (48.4 ± 4.2) (t = -5.4, P < 0.01). On the other hand, while only one specimen of *L. juanortizi* was examined, this one has 52 dorsal scales, which is below of the range for *L. uniformis* (Table 1). *Liolaemus uniformis* has more midbody scales (60.4 ± 1.7) than *L. lorenzmuelleri* (54.9 ± 4.5) (t = 2.6, P < 0.05) and *L. juanortizi* (56.7 ± 2.1) (t = 3.2, P < 0.05). *Liolaemus lorenzmuelleri* has a dark vertebral line and dark transversal lines running from the paravertebral fields to the flanks, whereas *L. uniformis* has no dorsal pattern. The dorsal pattern of *L. juanortizi* is very similar to *L. lorenzmuelleri*, but some specimens have a black ventral coloration, a black lateral band, and the lack of a dark vertebral line, whereas *L. uniformis* has no

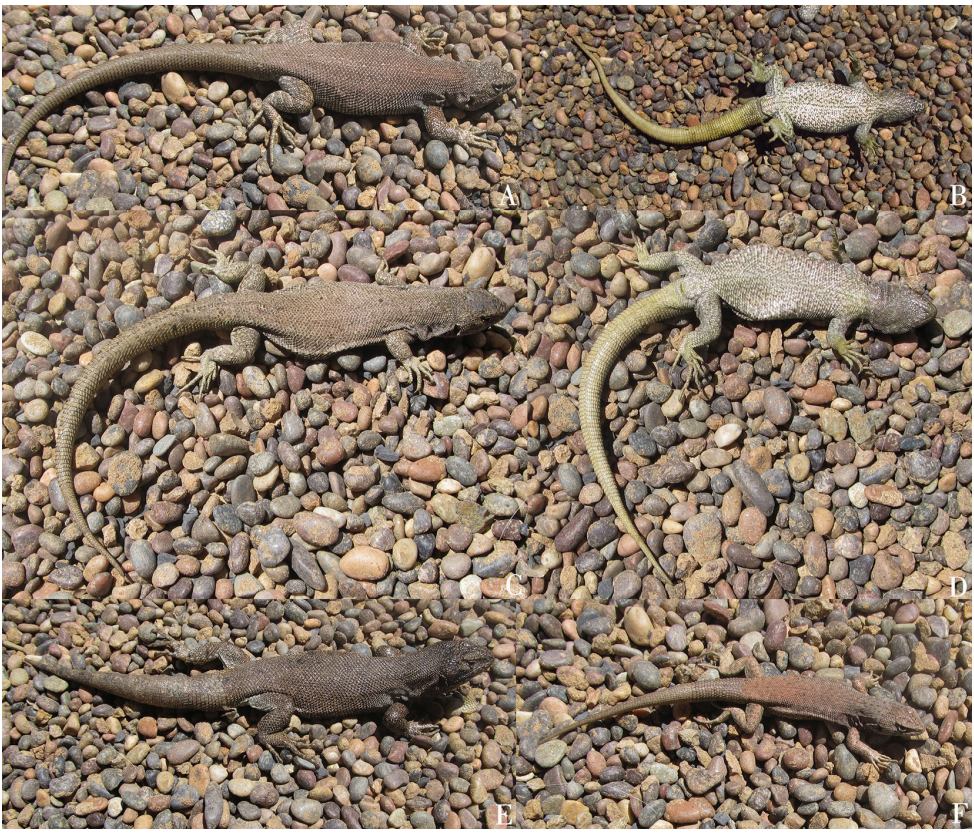

Figure 2. *Liolaemus uniformis* sp. n. **A, B** Holotype, male **C, D** Paratype, female **E** Paratype, male **F** Paratype, juvenile (unknown sex). All from the type locality.

black ventral color or black lateral band. Additionally, the southern distributional limit of *L. lorenzmuelleri* (Embalse La Laguna, Coquimbo Region, Chile) is more than 240 km north of the type locality recorded for *L. uniformis*; and the southern distributional limit of *L. juanortizi* in Quebrada Contrabando, Atacama Region, Chile (MNHNCL collection catalog, unpublished) is more than 520 km north of the type locality recorded for *L. uniformis*.

Liolaemus uniformis differs from *L. melanopleurus* (a species with only three known specimens from an undetermined location, Fig. 3B) in that the latter has a blue-gray dorsal coloration (Philippi 1860) and a black lateral band running from the axilla to the midbody, features absent in *L. uniformis*. Although the type locality of *L. melanopleurus* is undetermined, the syntypes were collected by Philippi in his journey through the Atacama Desert, between the vicinities of Copiapó (27°23'S) and San Pedro de Atacama (22°54'S), more than 530 km north of the type locality recorded for *L. uniformis*.

Liolaemus uniformis differs from *L. maldonadae* (Fig. 3F), because males of the latter have a yellowish or reddish dorsal color with black transverse dorsal and ventral bars and black lateral band, whereas *L. uniformis* has no dorsal pattern or black trans-

Figure 3. Chilean species of the *nigroviridis* group (with the exception of *Liolaemus nigroviridis*), ordered from north to south. **A** *Liolaemus constanzae*, male from vicinity of San Pedro (picture by JTP) **B** *L. melanopleurus*, male from Atacama (picture by JTP) **C** *L. isabelae*, male from Montandón (picture by JTP) **D** *L. juanortizi*, unknown sex specimen from road to Negro Francisco (picture by F. de Grotee) **E** *L. lorenzmuelleri*, unknown sex specimen from Embalse La Laguna (picture by A. Labra) **F** *L. maldonadae*, male from vicinity of Alcohuaz (picture by JTP).

verse ventral bars. Dorsal scales in *L. maldonadae* are noticeably larger than found in *L. uniformis*, and they have an "ovoid" shape. Dorsal and ventral scale counts in *L. maldonadae* do not overlap with the same scale counts in *L. uniformis* (Table 1). Finally, the southern distributional limit of *L. maldonadae* in Los Molles (Núñez et al. 1991) is more than 150 km north of the type locality of *L. uniformis*.

Liolaemus uniformis is found in sympatry with *L. nigroviridis* (Fig. 4), but is larger than *L. nigroviridis* (Mann–Whitney U = 8.0, $P < 0.05$, Table 1). *Liolaemus uniformis* also has more dorsal scales (60.0 ± 2.9) than *L. nigroviridis* (49.4 ± 2.7) ($t = 7.4$, $P < 0.01$). *Liolaemus nigroviridis* has strongly mucronated dorsal scales, whereas *L. uniformis* has no mucrons (Fig. 5). *Liolaemus nigroviridis* has sexual dichromatism, absent in *L. uniformis*. Males of *L. nigroviridis* have a bluish or yellowish green dorsal color

Table 1. Scalation and morphological characteristics for the species of the *nigroviridis* group. Juvenile specimens examined are excluded. M = males; F = females. (*) Taken from Navarro and Núñez (1993). (**) Examined specimen plus Young-Downey and Moreno (1991) data. (***) Taken from Young-Downey and Moreno (1991). (****) Counted only for one specimen.

	L. constanzae **(M = 14, F = 13)**	***L. isabelae*** **(M = 4)**	***L. juanortizi*** **(M = 1)**	***L. lorenzmuelleri*** **(M = 3, F = 5)**	***L. maldonadae*** **(M = 3)**	***L. melanopleurus*** **(M = 2)**	***L. nigroviridis*** **(M = 9, F = 4)**	***L. uniformis*** **sp. n. (M = 2, F = 3)**
Midbody scales	54–64	54–60	54–59**	50–62	58–64	42–56	55–64	58–62
Dorsal scales	56–67	56–67	52	44–55	48–50	40–51	45–53	56–63
Vental scales	86–96	86–97	88	86–96	83–91	91****	85–97	91–102
Nasal-rostral contact	92.6%	25%	100%	100%	100%	100%	100%	100%
Sexual dichromatism	Present	Present*	Absent***	Absent	?	?	Present	Absent
Vertebral line (males)	Present	Present/ absent	Present/ absent	Present	Absent/ inconspicuous	Absent	Absent/ inconspicuous	Absent
Maximum SVL (mm)	75,3	82,8	94.4***	88.8	85.6	70.6	73,8	89.1

Figure 4. Variation in *Liolaemus nigroviridis*. **A** Male from Farellones (picture by H. Díaz) **B** Male from Carpa Mountain (picture by JTP) **C** Male from Provincia Mountain (picture by JTP) **D** Female from Juncal (picture by JTP).

with black reticulation, and females have a brown dorsal color with a black lateral band, black vertebral line, and black paravertebral spots. In contrast, *L. uniformis* has a brown dorsal color without any pattern.

Molecular data show that *Liolaemus uniformis* is not closely related to *L. monticola* (Fig. 1). Moreover, *L. monticola* is smaller (maximum SVL = 65.6 mm) than *L. uniformis* (max. SVL = 89.1 mm) ($t = 3.9$, $P < 0.01$) according to our samples, and although Pincheira-Donoso and Núñez (2005) recorded a max. SVL = 67.3 mm for *L. monticola*, the difference between both species is marked. Moreover, *L. monticola* exhibit a characteristic black lateral band between the axilla and midbody (diffuse in females), and males have white dots dispersed on the dorsum and a series of small black spots on the dorsum (Fig. 6). All these traits are absent in *L. uniformis*. The upper altitudinal limit of *Liolaemus monticola* distributions is 2000 m a.s.l. (Espinoza et al. 2004, Fuentes and Ipinza 1979), whereas *L. uniformis* has a lower altitudinal distribution limit of 2820 m a.s.l.

Molecular data show that *Liolaemus uniformis* is not closely related to *L. bellii* (Fig. 1). Moreover, *L. bellii* is smaller (maximum SVL = 80.8 mm) than *L. uniformis* (max. SVL = 89.1 mm) ($t = 2.7$, $P < 0.05$). *Liolaemus uniformis* has more midbody scales (60.4 ± 1.7) than *L. bellii* (52.9 ± 2.6) ($t = 6.1$, $P < 0.01$); more dorsal scales (60.0 ±2.9) than *L. bellii* (43.3 ±3.1) ($t = 10.2$, $P < 0.01$); and more ventral scales (96.2 ±4.8) than *L. bellii* (89.7 ±4.6) (Mann–Whitney $U = 10.5$, $P < 0.05$). Dorsal scales in *L. bellii* are strongly keeled and mucronated, whereas there are no mucrons in *L. uniformis*.

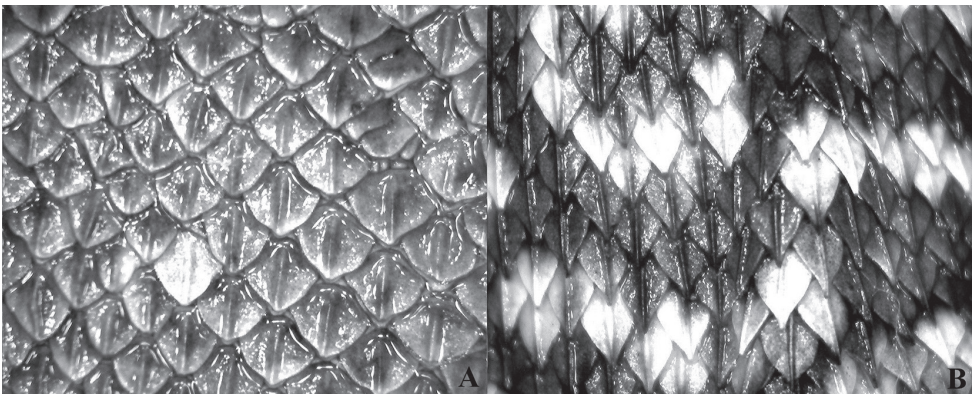

Figure 5. Dorsal scales, 8 mm width of view. **A** Male of *Liolaemus uniformis* sp. n. **B** *Liolaemus nigroviridis*.

Moreover, *L. bellii* exhibit a characteristic series of black dorsal "W" o "V" shaped spots (Fig. 6), whereas *L. uniformis* has no dorsal pattern.

Description of the holotype. Adult male. SVL = 84.7 mm. Horizontal diameter of the eye: 4.3 mm. Subocular length: 4.5 mm. Length of the fourth supralabial: 4.1 mm. Head length (from the posterior border of the auditory meatus to the tip of the snout): 22.1 mm. Head height (distance between the two ear openings): 10.4 mm. Head width (at the level of ear openings): 15.8 mm. Neck width: 12.4 mm. Interorbital distance: 6.3 mm. Ear-eye distance: 7.5 mm. Internarine distance: 3.8 mm. Ear width: 2.5 mm. Ear height: 3.5 mm. Axillary-groin distance: 34.9 mm. Body width: 24.7 mm. Forelimb length: 25.7 mm. Hindlimb length: 46.1 mm. Length of the right hand: 10.4 mm. Length of the right foot: 22.4 mm. Tail length (not autotomized): 132.4 mm, with relation tail length/SVL = 1.56. Pentagonal rostral scale, wider (4.2 mm) than high (1.4 mm).

Two postrostrals. Four internasals. Heptagonal interparietal, with a central, small, and whitish central spot marking the position of the parietal eye. Interparietal smaller than the parietals, surrounded by seven scales. Seven scales between the interparietal and rostral. Thirteen scales between the occiput and the rostral. Orbital semicircle incomplete on the right side and complete on the left (formed by thirteen scales). Three supraoculars on the left side and four on the right. Six superciliary scales. Frontal area divided into three scales (1 posterior and 2 anterior). Preocular separated from the lorilabials by one loreal scale. Two scales between nasal and canthal. Nasal in contact with the rostral, surrounded by six scales. One row of lorilabials between the supralabials and subocular. Four lorilabials in contact with the subocular. Six supralabials, the fourth is curved upward without contacting the subocular. Four infralabials scales. Pentagonal mental scale, in contact with four scales. Four pairs of post-mental shields, the second is separated by two scales. Temporal scales smooth or slightly keeled, imbricated. Six temporal scales between the level of superciliary scales and the rictal level. Four scales on the anterior edge of the ear, which do not cover the auditory meatus. Poorly differ-

Figure 6. Variation in species probably confused with *Liolaemus uniformis* sp. n. **A** *L. monticola* from Salto de Apoquindo (picture by JTP) **B** *L. monticola* from La Cruz Mountain (picture by J. Abarca-Díaz) **C** *L. monticola* from Provincia Mountain (picture by JTP) **D** *L. bellii* from La Parva (JR Martini) **E** *L. bellii* from Lagunillas (picture by JTP) **F** *L. bellii* from San Ramón Mountain (picture by JTP).

entiated auricular scale, pentagonal and located at the upper part of the meatus. Thirty gulars between the auditory meatus. Lateral neck fold is "Y" shaped. Ventrolateral fold running from the neck to the groin. Dorsolateral fold slightly developed, running from the ear to the base of the tail. Midbody scales: 60. Dorsal scales are lanceolated, imbricated, keeled (without mucrons), with few interstitial granules. Dorsal smaller than the ventrals. Dorsal scales: 58. Ventrals scales are polymorphic (rounded, rhomboidal, pentagonal or hexagonal) smooth, imbricated, without interstitial granules. Ventrals: 91. Three precloacal pores. Supra-femoral scales lanceolate, imbricated, smooth or keeled. Infra-femoral scales lanceolate or rounded, smooth and imbricated. Supra-antebrachials scales are rounded or lanceolated, imbricated and smooth or keeled. Infra-antebrachials are rounded, imbricated and smooth. Dorsal scales of tail are pentagonal or rhomboidal, imbricated and keeled. Ventral tail scales are rounded or rhomboidal, smooth and imbricated. Lamellae of the fingers: I: 9, II: 13, III: 20, IV: 20 and V: 13. Lamellae of the toes: I: 11, II: 15, III: 21, VI: 27 and V: 17.

Color of the holotype in life. The specimen is notable for its lack of pattern and uniform color. The head is brown and darker than the body. There are several white dots dispersed over the head and cheeks. The dorsum is coppery brown and has a few white-spotted scales that did not form a pattern. The subocular is brown and crossed by three white, vertical lines. The dorsal surface of the tail is light brown and without a pattern. The limbs are a dorsal-brown, similar to the dorsal surface, with white dots

Figure 7. Distributional map for *Liolaemus uniformis* sp. n. along with geographically proximate species of the *nigroviridis* group. Red star: *L. uniformis* sp. n., Chepical Lagoon, type locality. Green circles: *L. nigroviridis* (1 = Manque, 2 = El Arpa, 3 = Juncal, 4 = Riecillo, 5 = La Campana, 6 = El Roble, without number = near Chepical Lagoon). Pink squares: *L. maldonadae* (1 = near Alcohuaz, 2 = Los Molles). Yellow triangles: *L. lorenzmuelleri* (1 = Baños del Toro, 2 = Embalse La Laguna).

dispersed on the forelimbs and white transversal lines on the hindlimbs. The flanks are whitish with abundant dark brown scales. Ventrally, the hands, feet, thighs, vent, and tail are yellowish. The belly is whitish with dark dispersed spots and a dark ventral stripe. The throat is whitish with a dark thick reticulation. The precloacal pores are orange.

Variation in the type series. Males are larger and more corpulent than females. In two males: SVL: 84.7–89.1 mm. Axilla-groin distance: 34.9–37.8 mm. Head length: 21.9–22.1 mm. Head width: 15.8–16.3 mm. Head height: 10.4–11.2 mm. Leg length: 45.4–46.1 mm. Arm length: 25.0–25.8 mm. Tail length: 132.4 mm in one specimen, with relation tail length/SVL = 1.56 (autotomized in the other). In three females: SVL: 67.7–73.1 mm. Axilla-groin distance: 33.1–35.7 mm. Head length: 17.8–20.0 mm. Head width: 11.8–13.3 mm. Head height: 7.5–8.3 mm. Leg length: 32.0–34.8 mm. Arm length: 19.2–21.3 mm. Tail length: 98.1 mm in one specimen, with relation tail length/SVL = 1.45 (autotomized in other).

The variation of the scalation in *Liolaemus uniformis* is as follows. Midbody scales: 58–62 (60.4 ±1.7). Dorsal scales: 56–63 (60.0 ±2.9). Ventral scales 91–102 (96.2 ±4.8). Fourth finger lamellae: 17–20 (19.0 ±1.4). Fourth toe lamellae: 25–27 (26.4 ±0.9). Supralabial scales: 6. Infralabial scales: 4–5 (4.4 ±0.6). Interparietal scale pentagonal, hexagonal or heptagonal, bordered by 5–7 scales (6.0 ±0.7). Nasal and rostral always in contact. Precloacal pores in males: 3. Precloacal pores are absent in females.

In general, all specimens have the pattern and color described for the holotype, with slight variations in shade. The male paratype has a dark brown throat. Two females have inconspicuous dark rings and an inconspicuous vertebral stripe on the dorsal surface of the tail. Also, two females have an olive hue on the snout. One female has a very inconspicuous series of dark crossbars on the paravertebral fields, which, while difficult to count, approximated eight. The juvenile has a similar pattern and color as the holotype, but it has an inconspicuous and fragmented dark vertebral line and inconspicuous dark spots on the paravertebral fields.

Distribution and natural history. This species is currently only known from the type locality in the surroundings of the Chepical Lagoon, approximately 30 km NE of Alicahue, in the San Felipe de Aconcagua Province, Valparaíso Region, Chile (Fig. 7). Specimens were collected on the west shore of the Chepical Lagoon (32°15'S – 70°30'W, 3050 m a.s.l.). This new species was found inhabiting rocky areas with little shrubby vegetation composed mainly of high-Andean forbs, such as *Chuquiraga oppositifolia* and *Azorella* sp. (Fig. 8). This lizard was found in abundance and was observed to have saxicolous habits. It was active between 9:00 h and 18:00 h and took refuge under rocks. Moreover, this species was found in syntopy with *Phymaturus alicahuense* Núñez, Veloso, Espejo, Veloso, Cortés & Araya 2010. Specimens were also observed at lower altitudes (32°16'S - 70°30'W, 2820 m a.s.l.) in similar environments, altitudes at which this species was found in sympatry with a few specimens of *L. nigroviridis*.

One of the collected specimens had a yellow flower inside of its mouth. An analysis of intestinal contents showed that *L. uniformis* is omnivorous; plant and Hymenoptera remains were found. A large quantity of nematodes from an unidentified species was

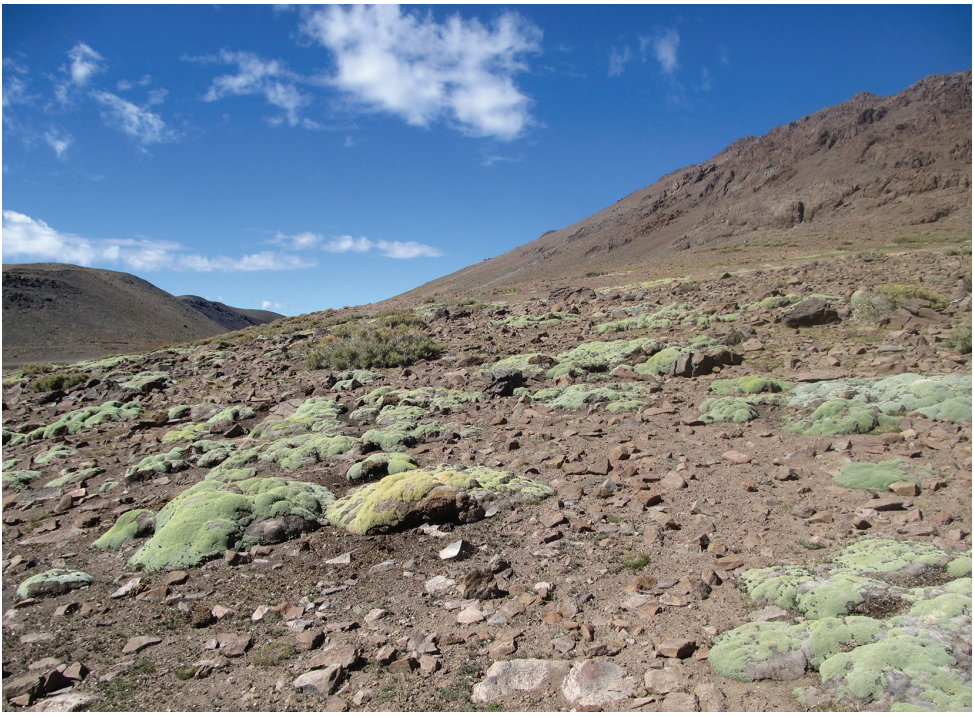

Figure 8. View of the type locality of *Liolaemus uniformis* sp. n., a high Andean environment.

found in the intestines. While the reproductive mode is yet unknown, at the time of sampling (December) no evidence of embryos was found but one female had several small oocytes. Comparisons with the reproductive modes of other species in the *nigroviridis* group would not be helpful as there is little available data. It is known that *L. nigroviridis* is viviparous (Donoso-Barros 1966) and *L. lorenzmuelleri* is oviparous (Cortés et al. 1995). Pincheira-Donoso and Núñez (2005) reported that *L. maldonadae* and *L. isabelae* are viviparous, but the source of this information is unclear (see Lobo et al. 2010:4) since the reproductive mode was not mentioned in the original descriptions (Navarro and Núñez 1993, Núñez et al. 1991).

Discussion

Almost no molecular data are currently available for the *nigroviridis* group, probably due to the great difficulties of obtaining samples since all of these species inhabit high altitude mountainous areas (Pincheira-Donoso and Núñez 2005), with only *L. constanzae* (Ortiz 1975) and *L. nigroviridis* (Espinoza et al. 2004) recorded below 2000 m a.s.l. (1400 m a.s.l. and 500 m a.s.l., respectively). Moreover, most specimens from the MNHNCL and MZUC collections (the two major herpetological collections in Chile) are fixed with formaldehyde, making DNA extraction and amplification challenging (Lin et al. 2009). In

regards to previous works, Torres-Pérez et al. (2009) performed three phylogenetic analysis (Bayesian inference, ML and maximum parsimony) and found that *L. nigroviridis* is the basalmost species of a clade also composed of *L. pseudolemniscatus* + *L. nigromaculatus* + *L. platei* and that this clade is closely related to *L. monticola* + *L. nitidus* clade. Our results are very similar with the *nigroviridis* and *monticola* clades as sister groups, but we did not want to include "*L. nigromaculatus*" from GenBank (Torres-Pérez et al. 2009) because the true identity of this species was only recently clarified (Troncoso-Palacios and Garín 2013) and although a specimen voucher is indicated (CUCH-3143), no locality data is provided. Since we have not seen this specimen we are not sure if it belongs to the true *L. nigromaculatus* or to *L. atacamensis*. We also did not include "*L. platei*" from GenBank (Torres-Pérez et al. 2009) because the specimen voucher (MZUC-30556) was collected in Laja Lagoon, Chile (according to MZUC Book catalog, unpublished) out of the known range for *L. platei* (Troncoso-Palacios and Marambio-Alfaro 2011), so it could be misidentified. In a recently mitochondrial ML phylogeny performed for a region spanning ND1-COI, Troncoso-Palacios et al. (2015b) found that the *L. nigroviridis* + *L. fuscus* clade is the sister group of the *monticola* clade (*L. monticola* + *L. nitidus* + *L. confusus*). This is also very similar to our result, but since there are not *Cyt-b* data for *L. fuscus,* it could not be included in the present analysis.

We recognize that one limitation to our work is that it is based in a phylogenetic analysis of only one mtDNA gene and that a wider phylogenetic DNA analysis (including nuclear genes) should be conducted in the future. This is also true for most of the 21 species of *Liolaemus* (*sensu stricto*) described in the last five years, which have been classified through different methodologies in regards to DNA comparisons. For example, three species (*L. chavin*, *L. pachacutec* and *L. wari*) include data from two mtDNA genes and shared data in GenBank (Aguilar et al. 2013). As our work, five species (*L. antumalguen*, *L. burmeisteri*, *L. cyaneinotatus*, *L. lonquimayensis* and *L. ubaghsi*) have been described with only *Cyt-b* data, and one species has been described with two mtDNA genes (*L. crandalli*). However, DNA data from all these have not been shared in GenBank or other online databases (Avila et al. 2010, 2012, 2015, Escobar-Huerta et al. 2015, Esquerré et al. 2014, Martínez et al. 2011) which does not allow the replication of the provided phylogenies or genetic distances. Two described species (Quinteros 2012, Troncoso-Palacios et al. 2015a), *L. abdalai* and *L. zabalai*, are supported in regards to DNA features by previously published phylogenetic works. Nine species (*L. aparicioi*, *L. carlosgarini*, *L. choique*, *L. chungara*, *L. nigrocoeruleus*, *L. pyriphlogos*, *L. riodamas*, *L. scorialis* and *L. smaug*) have been described without the support of molecular data (Abdala et al. 2010, Esquerré et al. 2013, Marambio-Alfaro and Troncoso-Palacios 2014, Ocampo et al. 2012, Quinteros 2012, Quinteros et al. 2014, Troncoso-Palacios et al. 2015a). Finally, one species, *L. shitan*, was described (Abdala et al. 2010) despite that no molecular differentiation was previously noted (Morando et al. 2003). No description in the last five year had included nuclear genes or more than two mtDNA genes and in most cases when DNA phylogeny is provided no data are shared in GenBank or other online databases. It is evident that *Liolaemus* researchers should put emphasis on trying to improve this situation in the future.

Although *L. uniformis* is strongly supported as a sister species of *L. nigroviridis* (pp = 1), a comprehensive phylogenetic study with more species of this group is needed. For example, *L. isabelae* was not placed within the *nigroviridis* group in a mitochondrial phylogenetic study that included one specimen (Schulte and Moreno-Roark 2010), despite that this species has been determined to be a member of this group in cladistic (Lobo 2005) and phenetic studies (Pincheira-Donoso and Núñez 2005) based on morphology. We included this species in our comparisons but for the time being, this should not be considered part of the *nigroviridis* group. Although the morphological cladistic analysis (Lobo 2005) found five apomorphies for the *nigroviridis* group (range of scale organs on postrostral scales, fourth supralabial - subocular not in contact, range of lamellae on the fourth finger, intraspecific female pattern and the relationship between the subocular length and the eye diameter), this study does not include all species currently accepted as part of the *nigroviridis* group and does not indicate the specific variation ranges of variation for these features in this group. On the other hand, the phenetic analysis of Pincheira-Donoso and Núñez (2005) does not provide supporting data for the features that were included in the matrix, so it cannot be replicated (see Lobo et al. 2010).

Liolaemus uniformis resembles *L. lorenzmuelleri* and *L. juanortizi* in that the three species share a similar background dorsal coloration. Although no molecular data exists to compare *L. uniformis* with these two species, we propose that the marked differences in scalation and the strongly allopatric distribution (> 240 km of separation), which is quiet considerable for lizards, support classifying *L. uniformis* as a new taxon. *Liolaemus uniformis* has probably been misidentified as *L. monticola* by Núñez et al. (2010), who noted *L. monticola* as the only lizard species to inhabit in syntopy with *Phymaturus alicahuense* (no specimen collection indicated). However, the present study found *P. alicahuense* residing at over 2900 m a.s.l, whereas the upper altitude limit for *L. monticola* is 2000 m a.s.l. (Espinoza et al. 2004, Fuentes and Ipinza 1979). Therefore, the present data indicates that the only lizards occurring in syntopy with *P. alicahuense* are *L. uniformis* and *L. nigroviridis*. Moreover, *L. uniformis* and *L. monticola* shows deep morphological and molecular differences. *Liolaemus uniformis* has probably also been confused with *L. bellii* (formerly *L. altissimus altissimus*) by Mella (2005), who found presence of the latter species in the highlands of Putaendo (no specimen collection indicated). However, a field expedition to the highlands of Putaendo by the authors of the present study found no specimens of *L. bellii*, and no additional records of *L. bellii* in this zone are known. Taking into account these details, in addition to both species having a similar background dorsal color, we think that *L. uniformis* might have been confused with *L. bellii*.

Several aspects of the *nigroviridis* group remain uncertain. For example, *L. nigroviridis* possibly contains at least two species, the nominal species from the Andean highlands and populations from Coastal highlands, formerly *L. n. campanae* (Cianferoni et al. 2013). *Liolaemus juanortizi* might be a junior synonym of *L. lorenzmuelleri* (Pincheira-Donoso and Núñez 2005), and although both are certainly very similar, it is difficult to carry out a study on this matter because the type series of *L. juanortizi* is lost (Valladares 2011) and there are very few samples of this species (Pincheira-Donoso and Núñez 2005). On the other hand, *L. melanopleurus* remains a problematic species

in terms of identification as the type locality is imprecise and no additional specimens have been found in more than 100 years (Troncoso-Palacios 2012).

The present work contributes to the existing taxonomical knowledge, but the *nigroviridis* group of *Liolaemus* lizards remains poorly studied, and new samples are required to better investigate its challenging taxonomy.

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Appendix I

Specimens examined. Acronyms are: Field Museum of Natural History (FMNH), Museo Nacional de Historia Natural de Chile (MNHNCL), Museo de Zoología de la Universidad de Concepción (MZUC) and Colección de Flora y Fauna, Profesor Patricio Sánchez Reyes de la Pontificia Universidad Católica de Chile (SSUC).

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Liolaemus constanzae. MZUC 29247, 29250–51. Toconao, Antofagasta Region. Unknown collector and date. MZUC 28763–65, 28767–69. Agua Verde (Quebrada de Taltal), Antofagasta Region. J.C. Ortiz, S. Zunino & M. Riveros colls. 10/02/1975. SSUC Re 338–347. Cuesta Barros Arana, Antofagasta Region. Ferri F. coll. 22/10/2011. SSUC Re 348. Southern Salar de Atacama, Antofagasta Region. Ferri F. coll. 24/10/2011. SSUC RE 482–83, 485, 488. El Abra, Antofagasta Region. G. Lobos & F. Torres colls. 22/11/2003. MNHNCL 1499–1500. Quebrada de Taltal, Agua Verde, 1400 m, Antofagasta Region. S. Zunino & M. Riveros colls. 10/02/1975. MNHNCL 1516–1520. Quebrada de Taltal, Agua Verde, 1480 m, Antofagasta Region. 27/09/1982. Núñez, Yáñez & Contreras colls.

Liolaemus isabelae. SSUC Re 157, 159, 160. El Cerrito, Salar de Pedernales, Atacama Region. F. Ferri & J. Troncoso-Palacios colls. 22/02/2012. SSUC Re 158. Montandón, Salar de Pedernales, Atacama Region. F. Ferri & J. Troncoso-Palacios colls. 22/02/2012.

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Liolaemus maldonadae. SSUC Re 304, 305, 560. Quebrada Los Piuquenes, Interior de Alcohuaz, Paihuano, Río Claro, Coquimbo Region. Troncoso-Palacios, J., F. Lobo, A. Laspiur & J.C. Acosta Colls. 10/02/2011.

Liolaemus melanopleurus. MNHNCL 1646 (2 specimens). Atacama. R.A. Philippi col. FMNH 9969 (only digital photographs). Atacama. R.A. Philippi coll.

Liolaemus monticola. SSUC Re 372–79. Camino a Farellones, Curva 20, Metropolitan Region, Chile. Ferri F. coll. 15/03/2012.

Liolaemus nigroviridis. MNHNCL 214–215. San Ramón, 3000 m, Metropolitan Region. H. Núñez coll. February, 1979. SSUC Re 016. El Yeso, Metropolitan Region. C. Garín coll. 01/04/2004. SSUC Re 190–200. Farellones, Casa de Piedra, Camino a Valle Nevado, Metropolitan Region. F. Ferri coll. 12/10/2010.

Liolaemus uniformis. SSUC Re 674–79. West shore of the Chepical Lagoon, approximately 30 km NE Alicahue, San Felipe de Aconcagua Province, Valparaíso Region, Chile. J. Troncoso-Palacios & E. Alfaro. December, 2012.

Appendix II

Specimens used for phylogenetic analysis.

mtDNA sequences obtained in this study. *Liolaemus uniformis* sp. n.: SSUC Re 674, KU095836. SSUC Re 677 KU095837. *L. nitidus*: SSUC Re 298, Dunas de Ritoqui, Valparaíso Region, Chile. KU095835. *L. confusus*: SSUC Re 356, Cerro Robles Riscos de Jote, O`Higgins Region, Chile. KU095832. *L. curicensis*: SSUC Re 253, Termas del Flaco, O`Higgins, Chile. KU095833. *L. kuhlmanni*: SSUC Re 285, Termas del Flaco, O`Higgins, Chile. KU095834. *L. bellii*: SSUC Re 208, El Colorado, Farellones, Metropolitan Region. KU095830. *L. bellii*: SSUC Re 209, El Colorado, Farellones, Metropolitan Region. KU095831.

mtDNA sequences obtained from GenBank. *Liolaemus nigroviridis*: Farellones. KC313199, KC313202, KC313203, KC313204, KC313205, KC313206, KC313208, KC313211, KC313207, KC313210, KC313209. *L. monticola*: Yerba Loca AY850619, Alfalfal AY850616, Maipú AY851724, Cuesta Chacabuco AY851718, Quebrada Alvarado AY851726, Colorado Norte AY851713, Cabrería AY851708, Rocín AY851710. *L. tenuis*: Termas de Chillán DQ989790. *L. abdalai*: Valle Chimehuin JN410525. *L. alticolor*: Huancarani KF923660. Santa Ana KF923659. *L. austromendocinus*: Nihuil AY173838. *L. buergeri*: El Planchón KJ494079, KJ494070, KJ494080. *L. capillitas*: Ruta Provincial AY173844. *L. chiliensis*: Termas de Chillan DQ989785, Las Trancas EU649245. *L. cyanogaster*: Tucapel DQ989786. *L. dicktracy*: Alto del Carrizal AY367816. *L. elongatus*: Esquel AY173801, Gobernador Costa AY173818, Los Manantiales AY173826, Laguna Blanca AY173855, Pampa de Lonco Luan AY173827, Las Ardillas AY173852. *L. gununakuna*: La Amarga AY367807, AY173859. *L. incaicus*: Urco KF923658, Lucre KF923657. *L. kriegi*: all from Río Negro Province AY173802, KJ494012, KJ494150, KJ494190, AY173814, KJ494155, KJ494191, KJ494188. *L. neuquensis*: Primeros Pinos AY173828. *L. parvus*: Quebrada Honda AY173836. *L. petrophilus*: El Cuy AY173796, Los Menucos JN847211, Ingeniero Jacobacci JN847103. *L. pictus*: San Carlos de Bariloche AY173795. *L. punmahuida*: Volcán Tromen AY173824. *L. ramirezae*: E Amaicha del Valle JN410520. *L. robertmertensi*: Tinogasta DQ989769. *L. saxatilis*: Achiras JN410553, Río Cuarto

JN410527. *L. smaug*: Las Leñas AY173832, Mallines Colgados AY173830. *L. talampaya*: Las Yeguas River AY173797. *L. tregenzai*: all from Termas de Copahue AY367817, KJ494036, KJ494230, KJ494040, KJ494039, KJ494037, KJ494038. *L. tulkas*: Quebrada Las Angosturas AY367813. *L. umbrifer*: Quebrada de Randolfo AY367814. *L. villaricensis*: Volcán Villarrica AY850629, AY730671. *L. zabalai*: all from Biobío Region KJ494059, KJ494056, KJ494057, KJ494086, KJ494074, KJ494085. *Phymaturus vociferator*: Laguna del Laja JX969016. *Phymaturus felixi*: Paso de Indios JX969044.

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DATA PAPER

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GPS tracking data of Lesser Black-backed Gulls and Herring Gulls breeding at the southern North Sea coast

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This paper describes version 5.5 of this resource: http://dataset.inbo.be/bird-tracking-gull-occurrences&v=5.5

Abstract

In this data paper, *Bird tracking - GPS tracking of Lesser Black-backed Gulls and Herring Gulls breeding at the southern North Sea coast* is described, a species occurrence dataset published by the Research Institute for Nature and Forest (INBO). The dataset (version 5.5) contains close to 2.5 million occurrences, recorded by 101 GPS trackers mounted on 75 Lesser Black-backed Gulls and 26 Herring Gulls breeding at the Belgian and Dutch coast. The trackers were developed by the University of Amsterdam Bird Tracking System (UvA-BiTS, http://www.uva-bits.nl). These automatically record and transmit bird movements, which allows us and others to study their habitat use and migration behaviour in great detail. Our bird tracking network is operational since 2013. It is funded for LifeWatch by the Hercules Foundation and maintained in collaboration with UvA-BiTS and the Flanders Marine Institute (VLIZ). The recorded data are periodically released in bulk as open data (http://dataset.inbo.be/bird-tracking-gull-occurrences), and are also accessible through CartoDB and the Global Biodiversity Information Facility (GBIF).

Keywords

Animal movement, bird tracking, GPS tracking, habitat use, migration, Lesser Black-backed Gull, *Larus fuscus*, Herring Gull, *Larus argentatus*, UvA-BiTS, LifeWatch, open data, MachineObservation, occurrence, observation

Data published through

http://doi.org/10.15468/02omly

Rationale

As part of our terrestrial and marine observatory for LifeWatch (http://lifewatch.inbo.be), the Research Institute for Nature and Forest (INBO), the Flanders Marine Institute (VLIZ), Ghent University (UGent), and University of Antwerp (UA) are tracking large gull species with lightweight, solar powered GPS trackers. The project builds upon the extensive knowledge the INBO has acquired over the last 15 years when studying in particular postnuptial migration, as well as mate and site fidelity of large gulls, by means of sightings of colour-marked individuals ringed in Belgium and via individual-based life-history studies by UGent and UA. The data collected through this bird tracking network allows to study the migration patterns and habitat use of the gulls in more detail. Furthermore, they are no longer biased towards locations where observers can see colour-ringed birds. To allow greater use of the data beyond our research questions, all data are published as open data.

Taxonomic coverage

The dataset contains tracking data from 75 Lesser Black-Backed Gulls (*Larus fuscus*, Figure 1) and 26 Herring Gulls (*Larus argentatus*, Figure 3) breeding at the Belgian and Dutch coast.

Figure 1. One of the tracked Lesser Black-backed Gulls ("Hans", ring code: L906682), photographed near its nest in Zeebrugge on May 29, 2013 shortly after he was equipped with a tracker (device info serial: 861). Photo by Misjel Decleer, VLIZ photo gallery.

Taxonomic ranks

Kingdom: *Animalia*
Phylum: *Chordata*
Class: *Aves*
Order: *Charadriiformes*
Family: *Laridae*
Genus: *Larus*
Species: *Larus fuscus* (Lesser Black-backed Gull), *Larus argentatus* (Herring Gull)

Geographic coverage

The tracked birds breed at the southern North Sea coast in three colonies, located in the ports of Zeebrugge (Belgium), Ostend (Belgium) and Vlissingen-Oost (the Netherlands). During the breeding season, their foraging range includes the west of Belgium and the Netherlands, northern France, the North Sea, and the English Channel. The Lesser Black-backed Gulls migrate south in winter, mainly hibernating in the south of Spain, Portugal, and North Africa (Figure 2).

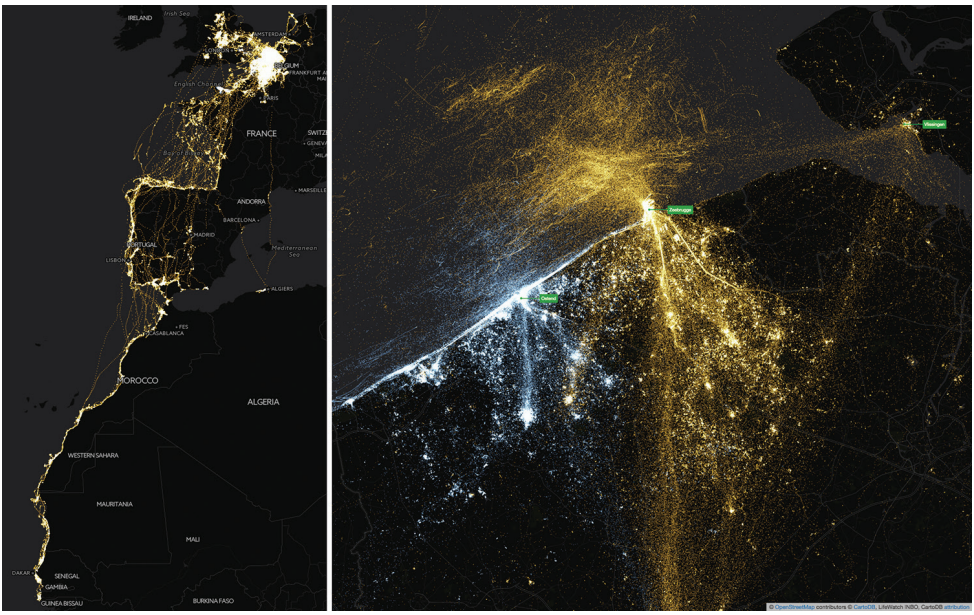

Figure 2. Left: map of western Europe and northwest Africa, showing the full extent of the gull tracking data, including two migration/wintering seasons. Right: map of the southern North Sea coast, showing mainly breeding season data. Each point represents a recorded occurrence, LBBG are indicated in orange, HG in blue. Overlapping points are brighter in colour. Maps created with CartoDB, basemap based on OpenStreetMap data. https://inbo.cartodb.com/u/lifewatch/viz/da04f120-ea70-11e4-a3f2-0e853d047bba/public_map

Bounding box

10° to 60° latitude, -25° to 10° longitude

Temporal coverage

Date range: 2013-05-17 to 2015-09-02
Formation periods: breeding season 2013
Formation periods: migration/wintering season 2013-2014
Formation periods: breeding season 2014
Formation periods: migration/wintering season 2014-2015
Formation periods: breeding season 2015

Methodology

Study extent description

The birds were trapped and tagged at or near their breeding colony at the southern North Sea coast.

The colony of Zeebrugge is situated in the western part of the port (51.341 latitude, 3.182 longitude) at sites that are not used for port activities and on rooftops. The first Herring Gulls (HG) nested here in 1987, followed by the first breeding record of Lesser Black-backed Gull (LBBG) in 1991. In the 1990s, the number of breeding pairs strongly increased, with a maximum of 2,336 pairs of HG and 4,760 pairs of LBBG in 2011 (Stienen et al. 2015). Maximum numbers amounted to 2.6% and 1.2% of the biogeographic populations of LBBG and HG (Wetlands International 2015). After 2011 the number of gulls strongly declined due to habitat loss and the presence of foxes (*Vulpes vulpes*). In the period 2000–2010, Zeebrugge hosted on average 91% of all large gulls in Belgium. This proportion decreased to 33% in 2015 (Stienen et al. 2015).

In the colony of Ostend (51.233 latitude, 2.931 longitude), breeding started in 1993. Here the the numbers of breeding pairs are still increasing with a maximum of 505 pairs of HG and 551 pairs of LBBG in 2015 (data INBO). In Ostend most gulls breed on rooftops both in industrial areas and in the town itself.

The colony of Vlissingen-Oost also know as "Sloegebied" (51.450 latitude, 3.689 longitude) is located in the industrial port area near Vlissingen. Here the gulls nest on the grassy grounds that are not yet in use for port activities. LBBG started breeding in 1984, and the area is now the second biggest colony of LBBG in the southwestern part of the Netherlands. The numbers of breeding pairs increased from a few hundred in the second part of the nineties to 5,220 pairs in 2011. HG started breeding in 1977 (5–10 pairs) with a maximum of 4,353 pairs in 2008 (Strucker et al. 2013). In 2014 the colony hosted 4,460 pairs of LBBG and 2,276 pairs of HG (Strucker et al. 2015).

Most birds were trapped on their nest using a walk-in cage. We took biometrics of all captured gulls (bill length, bill depth, tarsus length, wing length, and body mass) and a feather sample to determine the sex. The UvA-BiTS GPS trackers were attached to the back of the gull using a harness of Teflon tape (Figure 3).

The number of tagged birds and their trap location per year are:

- 2013: 5 HG nesting on the roof of the Vismijn in Ostend and 22 LBBG nesting in the port of Zeebrugge.
- 2014: 8 HB nesting on the roof of the Vismijn in Ostend, 1 HG and 24 LBBG nesting in the port of Zeebrugge, and 3 HG feeding on the Visserskaai in Ostend (using a small cannon net).
- 2015: 9 HG nesting on the roof of the Vismijn in Ostend, 13 LBBG nesting in the port of Zeebrugge, and 16 LBBG nesting in Vlissingen-Oost.

Sampling description

The birds are tracked with the University of Amsterdam Bird Tracking System (UvA-BiTS, http://www.uva-bits.nl). The system has been described in detail in Bouten et al. 2013. The lightweight, solar powered GPS trackers periodically record the 3D position and air temperature, and can be configured to collect body movements with the

Figure 3. Researchers equipping a Herring Gull with a UvA-BiTS GPS tracker on the roof of the Vismijn in Ostend on May 24, 2013. Photo by Misjel Decleer, VLIZ photo gallery.

built-in tri-axial accelerometer as well. The system allows us to remotely set or change a measurement interval per tracker: the actual interval between measurements is provided in *samplingEffort* as *secondsSinceLastOccurrence*.

The data are stored on the tracker, until these can be transmitted automatically and wireless to a base station using the built-in ZigBee transceiver with whip antenna. This receiver is also used to receive new measurement settings. The spatial range for this communication is restricted to the location of the base station (or antenna network), which is placed near the colony. Data cannot be retrieved from birds that do not return to the colony with the base station. For 3 of the 101 birds fitted with trackers no data were obtained and their *organismID*s (L909374, 5331094 ARNHEM, L909202) will thus not be found in the dataset. At the time of publication most individuals (88%) were tracked for more than 10 days and 41% for more than 100 days (Figure 4). The longest tracking period is 838 days (a HG with *organismID* H903185).

Data received by the base stations are automatically harvested, post-processed, and stored in a central PostgreSQL database at UvA-BiTS (http://www.uva-bits.nl/virtuallab), which is accessible to the involved researchers only. We periodically export the tracking data to CartoDB for visualization purposes (see the External datasets section), removing test records and flagging outliers.

To create the Darwin Core Archive, we extract the data from the database and standardize these to Darwin Core using an SQL query (https://github.com/LifeWatchINBO/data-publication/blob/master/datasets/bird-tracking-gull-

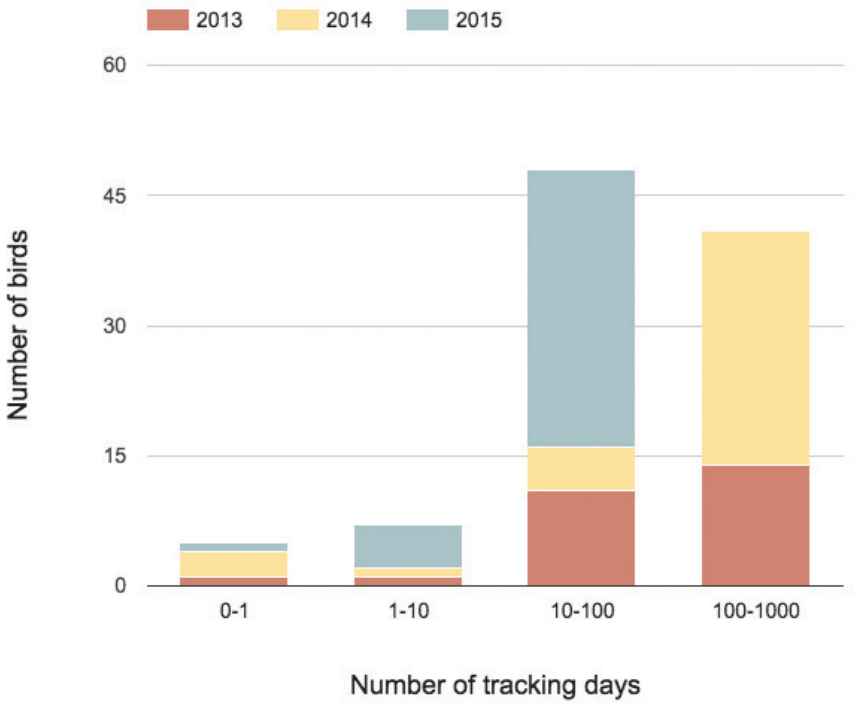

Figure 4. Number of birds grouped by number of tracking days and tracking start year.

occurrences/mapping/dwc-occurrence.sql). The dataset is documented, published via our IPT (http://dataset.inbo.be/bird-tracking-gull-occurrences), and registered with the Global Biodiversity Information System (http://www.gbif.org/dataset/83e20573-f7dd-4852-9159-21566e1e691e). Issues or remarks regarding the data or this procedure can be reported at https://github.com/LifeWatchINBO/data-publication/tree/master/datasets/bird-tracking-gull-occurrences

To extract data from one individual, one can use *organismID*, which contains the unique metal leg ring code of each bird. Tracker IDs are provided in *dynamicProperties* as *device_info_serial*. For an overview of all GPS trackers and the individual birds these are mounted on, see https://inbo.cartodb.com/u/lifewatch/tables/bird_tracking_devices/public.

Quality control description

See the section *Sampling description* for more details: our import procedure (https://github.com/LifeWatchINBO/bird-tracking/blob/master/cartodb/import-procedure.md) and standardization to Darwin Core (https://github.com/LifeWatchINBO/data-publication/blob/master/datasets/bird-tracking-gull-occurrences/mapping/dwc-occurrence.sql) are publicly documented.

Method step description

1. Researcher captures bird, takes biometrics, attaches GPS tracker, and releases bird.
2. Researcher sets a measurement scheme, which can be updated anytime.
3. GPS tracker records data.
4. GPS tracker automatically receives new measurement settings and transmits recorded data when a connection can be established with the base station at the colony.
5. Recorded data are automatically harvested, post-processed, and stored in a central PostgreSQL database at UvA-BiTS.
6. LifeWatch INBO team periodically exports tracking data to CartoDB and makes these publicly accessible.
7. LifeWatch INBO team periodically (re)publishes data as a Darwin Core Archive, registered with GBIF.
8. Data stream stops when bird no longer returns to colony or if GPS tracker no longer functions (typical tracker lifespan: 2-3 years).

Datasets

Dataset description

- **Object name:** Bird tracking - GPS tracking of Lesser Black-backed Gulls and Herring Gulls breeding at the southern North Sea coast
- **Format name:** Darwin Core Archive format
- **Format version:** 1.0
- **Character encoding:** UTF-8
- **Language:** English
- **License:** http://creativecommons.org/publicdomain/zero/1.0/
- **Usage norms:** http://www.inbo.be/en/norms-for-data-use
- **Publication date:** 2014-06-18
- **Distribution:** http://dataset.inbo.be/bird-tracking-gull-occurrences
- **DOI:** http://doi.org/10.15468/02omly

Usage norms

To allow anyone to use this dataset, we have released the data to the public domain under a Creative Commons Zero waiver (http://creativecommons.org/publicdomain/zero/1.0/). We would appreciate however, if you read and follow these norms for data use (http://www.inbo.be/en/norms-for-data-use) and provide a link to the original dataset (http://doi.org/10.15468/02omly) whenever possible. If you use these data for a scientific paper, please cite the dataset following the applicable citation norms and/

or consider us for co-authorship. We are always interested to know how you have used or visualized the data, or to provide more information, so please contact us via the contact information provided in the metadata, opendata@inbo.be or https://twitter.com/LifeWatchINBO.

External datasets

All our public bird tracking data are also available through CartoDB (https://inbo.cartodb.com/u/lifewatch), where users can query the data using SQL via the CartoDB API or download these in various formats (*csv*, *shp*, *kml*, *svg*, and *geosjon*). Two tables are of use: *bird_tracking*, containing all occurrence data and *bird_tracking_devices*, containing information on the GPS trackers and individual birds. Note that these tables are not standardized to Darwin Core, contain flagged outliers (omitted from the standardized dataset) and include data from other bird species. For more info, see https://github.com/LifeWatchINBO/bird-tracking/blob/master/cartodb/README.md

bird_tracking

- **Object name:** bird_tracking
- **Format name:** CartoDB table
- **Character encoding:** UTF-8
- **Distribution:** https://inbo.cartodb.com/u/lifewatch/tables/bird_tracking/public

bird_tracking_devices

- **Object name:** bird_tracking_devices
- **Format name:** CartoDB table
- **Character encoding:** UTF-8
- **Distribution:** https://inbo.cartodb.com/u/lifewatch/tables/bird_tracking_devices/public

Additional information

The following information is not included in this dataset and available upon request: outliers, temperature, speed, accelerometer data, GPS metadata (fix time, number of satellites used, vertical accuracy), bird biometrics data measured during tagging (bill length, bill depth, tarsus length, wing length, body mass), life history data (day of ringing, age, resightings by volunteers), as well as growth data of chicks.

Project data

Project title

Bird tracking network

Funding

This bird tracking network is funded for LifeWatch by the Hercules Foundation (http://www.herculesstichting.be/in_English/), with additional funding from the Research Foundation Flanders (FWO) to Wendt Müller and Luc Lens and Interreg Natura People (EFRO) through the Province of West Flanders.

Project website

http://www.lifewatch.be/en/gps-tracking-network-large-birds

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RESEARCH ARTICLE

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Multi-locus analysis supports the taxonomic validity of *Arborophila gingica guangxiensis* Fang Zhou & Aiwu Jiang, 2008

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Abstract

The taxonomic status of subspecies has long been debated, especially in conservation biology. Some proposed subspecies must be evolutionarily distinct to be considered conservation units. White-necklaced Partridge (*Arborophila gingica*) comprises two subspecies, *A. g. gingica* and *A. g. guangxiensis*. *A. g. guangxiensis*, restricted to three isolated small areas in Guangxi, China, with limited population sizes, is a newly discovered subspecies based on recently identified geographic and phenotypic differences between *A. g. gingica*; however, evidence is lacking that can effectively identify whether the subspecies is evolutionarily distinct. Here, three mitochondrial DNA segments and four nuclear introns were used to test whether the two subspecies are reciprocally monophyletic, which has been proposed as an objective method to evaluate evolutionary distinctiveness. The results indicate that the two subspecies are genetically divergent and form reciprocal monophyletic groups. Therefore, this study further supports the taxonomic validity and distinctiveness of *A. g. guangxiensis* and suggests that this subspecies be considered as a conservation unit.

Keywords

Conservation unit, evolutionary distinctiveness, hill partridge, mitochondrial DNA, nuclear introns, monophyletic groups

Introduction

The taxonomic status of subspecies has long been debated (Wilson and Brown 1953; Mayr 1982), especially in resource-limited conservation biology (Zink 2004; Phillimore and Owens 2006). Zink (2004) proposed that subspecies must be evolutionarily distinct to be considered conservation units. However, a recent global analysis showed that only 36% of traditional avian subspecies can be distinguishable by mitochondrial DNA (mtDNA) (Phillimore and Owens 2006). Therefore, assessing the validity of subspecies before proposing conservation efforts may be a practical way to effectively protect biodiversity with limited resources.

The near threatened (NT) White-necklaced Partridge (*Arborophila gingica*) (BirdLife International 2012), also known as the Collared or Rickett's hill partridge, is a small partridge endemic to the southern Chinese forests of Hunan, Jiangxi, Zhejiang, Fujian, Guangdong and Guangxi (Fig. 1, Cheng 1978; Zheng 2011). Although the distribution area appears extensive, populations are severely fragmented and continuously declining (Zhou 1996; BirdLife International 2012), except for the Fujian population (He et al. 2007). This species was believed to be monotypic (Johnsgard 1988; Madge and McGowan 2002) until Zhou and Jiang (2008) discovered that the populations in north and central Guangxi differed from other southeastern populations in the coloration of the forehead, which is chestnut instead of white. Zhou and Jiang (2008) described these populations as a new subspecies, *A. g. guangxiensis*, a proposal subsequently accepted by several avian checklists (Zheng 2011; Dickinson and Remsen 2013; del Hoyo and Collar 2014; Gill and Donsker 2015). However, except for its diagnostic forehead coloration, no other plumage differences are known, and body weight, body length, wing, culmen, tarsus and tail show no obvious differences between the two subspecies (Zhou and Jiang 2008). *A. g. guangxiensis* is only found in three isolated small areas of north (Jiuwanshan Mountain and Sijianshan Mountain) and central (Damingshan Mountain) Guangxi (Fig. 1, Zhou and Jiang 2008). The estimated population is about 600 to 1000 individuals, thus undoubtedly *A. g. guangxiensis* suffers more threats than *A. g. gingica* (Taylor 2011). However, the subspecies status of *A. g. guangxiensis* is solely dependent on geographic and phenotypic differences, lacking molecular evidence that can effectively identify whether this isolated subspecies is evolutionarily distinct and can be considered a conservation unit (Zink 2004). Indeed, some operational criteria for subspecies recognition require that subspecies are both phenotypically distinct and correlate with evolutionary independence according to population genetic structure (Braby et al. 2012).

Recently, molecular systematics has become one of the most vigorous disciplines to assist in avian taxonomy (Fjeldså 2013). Specifically, mtDNA has been extensively used at various taxonomic levels (Zink and Barrowclough 2008). Zink (2004) advocated that subspecies should be reciprocally monophyletic in mtDNA gene trees to document the evolutionary distinctiveness of subspecies. However, studies that are based solely on mtDNA have been debated because population differentiation relies on the accumulated signals from many genes and mtDNA only represents a single locus (Rubinoff

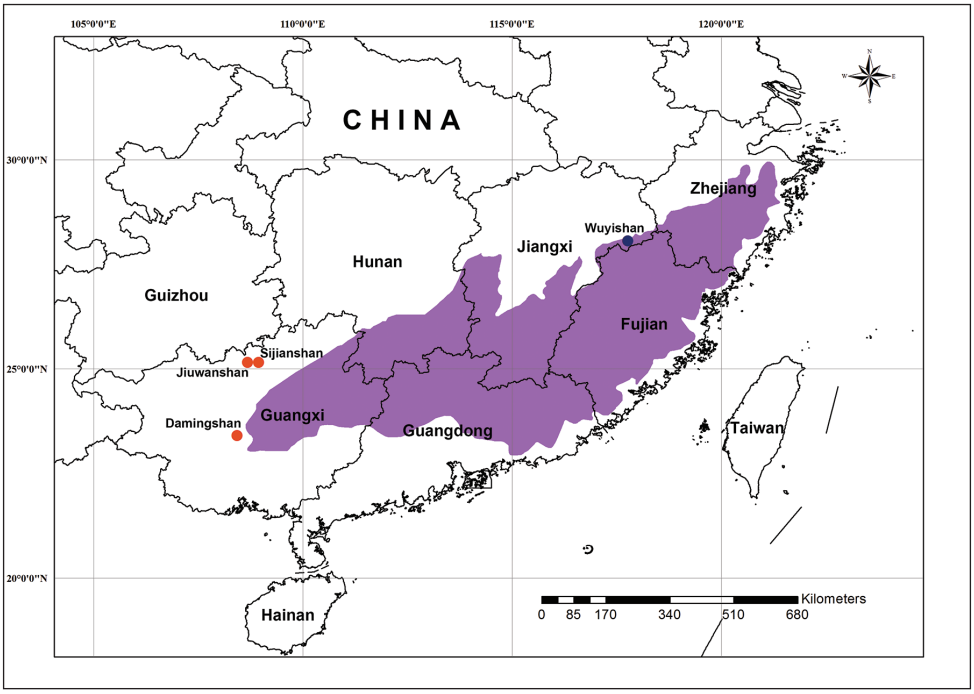

Figure 1. Map of southeast China showing the distribution area of *A. gingica*. The purple area represents the distribution of the nominate subspecies *A. g. gingica* according to del Hoyo and Collar (2014), with the dark blue dot indicating the sampling site in Wuyishan Mountain. The orange dots represent the three isolated populations of *A. g. guangxiensis* (Zhou and Jiang 2008), with the sampling site in Jiuwanshan Mountain.

and Holland 2005; Edwards and Bensch 2009). Therefore, a reasonable strategy for phylogenetic analysis is to combine mtDNA with nuclear DNA (nuDNA) sequences (Corl and Ellegren 2013).

Here, three mtDNA segments and four nuclear introns of White-necklaced Partridge were combined to conduct a series of phylogenetic analyses and test whether *A. g. guangxiensis* and *A. g. gingica* form reciprocally monophyletic groups. Furthermore, times of divergence within *A. gingica*, between *A. gingica* and its closest relative, were investigated, and attempts to identify possible drivers of the diversification process were made.

Methods

Sampling, DNA extraction, PCR and sequencing

Seven individuals of *A. g. guangxiensis* were sampled from Jiuwanshan National Nature Reserve, Guangxi, and three individuals of *A. g. gingica* from Wuyishan National Nature

Reserve, Jiangxi (Fig. 1). Previous studies indicated that the sister species of *A. gingica* was *A. rufogularis* (Wang et al. 2013); therefore, we used one individual of *A. rufogularis* from Tongbiguan National Nature Reserve in Yunnan as an outgroup. All samples were taken from live birds (blood or feather). Permissions for blood or feather sampling were granted by the regional forestry departments. Total DNA was extracted using a TIANamp Blood Genomic DNA Extraction Kit (TIANGEN BIOTECH CO, BEIJING, CHINA).

We amplified three mtDNA segments, cytochrome oxidase subunit 1 (COI), cytochrome *b* (CYTB) and NADH dehydrogenase subunit 2 (ND2); and four nuclear introns, aldolase b intron 6 (ALDOB), fibrinogen intron 5 (FGB), glyceraldehyde 3-phosphate dehydrogenase intron 11 (G3PDH) and ovomucoid intron G (OVOG) using the primers listed in Suppl. material 1. Both strands of each PCR product were sequenced by BGI-BEIJING. The sequences were visually proofread to the original chromatograms and were also checked against published DNA sequences. Each sequence was then assembled using MEGA v6 (Tamura et al. 2013). Then, we aligned the sequences using MUSCLE (Edgar 2004) implemented in MEGA v6 (Tamura et al. 2013) to obtain seven partitions, all sequences obtained from this study were submitted to GenBank (KU057820–KU057877). Each nuclear partition was then phased (Stephens and Donnelly 2003) in DNASP v5.10 (Librado and Rozas 2009) to resolve the haplotypes of diploid nuclear sequences. Finally, we assembled the seven partitions into a complete matrix, an mtDNA matrix and a nuDNA matrix.

Phylogenetic analysis

The best-fitting nucleotide substitution model for each partition was selected using the Akaike Information Criterion with JMODELTEST v2.1.7 (Darriba et al. 2012). The mean genetic distances between and within subspecies were calculated in MEGA v6 (Tamura et al. 2013) using the Kimura two-parameter (K2P) model (with *A. rufogularis* removed); and standard error estimates were obtained by a bootstrap procedure (1000 replicates). Partitioned maximum likelihood (ML) analyses were conducted in GARLI v2.0 (Bazinet et al. 2014) using the best-fitting nucleotide substitution model for each partition. The subtree pruning and regrafting tree-searching method was used, and bootstrap values (BS) were calculated with 1,000 replicates. Partitioned Bayesian Inference (BI) was performed in BEAST v1.8.0 (Drummond and Rambaut 2007) with the best-fitting nucleotide substitution model for each partition (similar to Divergence time estimates, see below for details).

Divergence time estimates

First, we performed molecular clock tests in MEGA v6 (Tamura et al. 2013). The results showed that each partition was clock-like. Therefore we used the strict clock model for each partition. It is believed that a species tree analysis using combined

mtDNA, Z-linked (ALDOB) and autosomal (FGB, G3PDH and OVOG) loci can substantially improve the resolution of the tree (Corl and Ellegren 2013). Therefore, we performed a species tree analysis using the complete matrix in *BEAST (Heled and Drummond 2010) implemented in BEAST v1.8.0 (Drummond and Rambaut 2007), with a fixed molecular rate of 2.38% for CYTB (average molecular rate for Galliform birds, Weir and Schluter (2008)) to estimate the molecular rates of the other loci. The ESS value was verified to be greater than 200 in TRACER v1.5 (Rambaut and Drummond 2009) to confirm that the chains had reached apparent stationarity. The final analysis was run for 100 million generations with trees sampled every 1,000 generations. TREEANNOTATOR v1.8.0 was then used to discard the first 20% of trees and to generate the consensus tree with Bayesian posterior probability.

Results

The complete matrix was 4750 base pairs (bp) in length, including 2861 bp of mtDNA sequence data, and 1889 bp of nuclear intron sequence data. Exclude outgroup, there were 18 variable and 13 informative sites in mtDNA, and 24 variable and 19 informative sites in nuDNA (after phasing). The genetic distance between the two subspecies was higher in mtDNA (0.0038) than in nuDNA (0.0028), and in nuclear introns the genetic distance within subspecies partially overlapped with that between subspecies (Table 1) due to some shared haplotypes (data not shown).

Phylogenetic analyses of the complete matrix and mtDNA matrix showed that *A. g. guangxiensis* and *A. g. gingica* formed monophyletic groups, with relatively high support (Fig. 2). However, analyses of the nuDNA matrix and separate analyses of each of the nuclear introns failed to recover the monophyletic relationships between *A. g. guangxiensis* and *A. g. gingica* (Suppl. material 2), and the support values are extremely low (data not shown).

Divergence time estimates from the species tree showed that the two subspecies *A. g. guangxiensis* and *A. g. gingica* diverged approximately 0.11 (0.05–0.19) mya (million years ago), whereas the divergence between *A. gingica* and *A. rufogularis* occurred 2.02 (0.91–2.91) mya (Fig. 2).

Discussion

This study documents genetic differentiation between *A. g. guangxiensis* and *A. g. gingica*. The phylogenetic analyses based on mtDNA indicate that *A. g. guangxiensis* and *A. g. gingica* form reciprocal monophyletic groups (Fig. 2), which meets the criterion that subspecies should be monophyletic in mtDNA to demonstrate evolutionary distinctiveness (Zink 2004). Monophyly was also supported by the multi-locus tree (Fig. 2).

However, although the *A. g. guangxiensis* clade received high support in the mtDNA tree (Fig. 2), nuDNA trees failed to recover the two subspecies as monophyletic

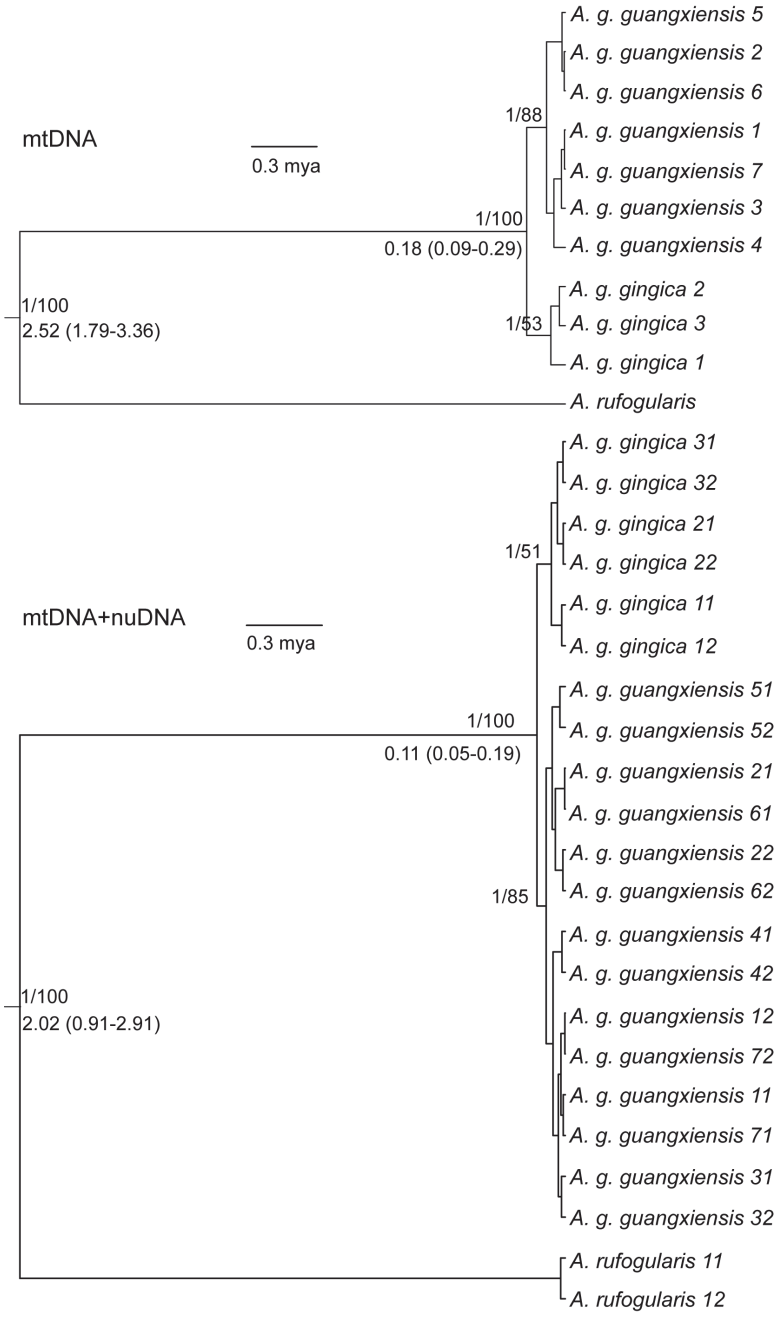

Figure 2. Phylogenetic consensus trees from the mtDNA matrix and complete data matrix. Node values above the branches represented the BI posterior probability and ML bootstrap support. Values below the branches represent the divergence times (median) and 95% highest posterior density (HPD) between lineage groups, note that the divergence times in the multi-locus tree were estimated by species tree analysis. The last number in tip labels in the multi-locus tree represent the two haplotypes phased from diploid nuclear sequences.

Table 1. Mean genetic distances (K2P) between and within subspecies.

Mean distance	mtDNA	ALDOB	FGB	G3PDH	OVOG	nuDNA
Within *guangxiensis*	0.0013 ±0.0005	0.0017 ±0.0009	0.0032 ±0.0012	0.0037 ±0.0017	0.0032 ±0.0015	0.0029 ±0.0007
Within *gingica*	0.0012 ±0.0005	0.0013 ±0.0012	0.0021 ±0.0012	0.0009 ±0.0009	0.0019 ±0.0013	0.0016 ±0.0007
Between subspecies	0.0038 ±0.0009	0.0017 ±0.0011	0.0033 ±0.0012	0.0024 ±0.0011	0.0038 ±0.0018	0.0028 ±0.0007

Standard errors are shown after the symbol "±"

groups (Suppl. material 2). This difference might be explained by the longer coalescence time of nuDNA due to its larger effective population size than mtDNA (Moore 1995), so that in recently diverged taxa lineage sorting would be complete for mtDNA but not yet for nuDNA (Zink and Barrowclough 2008). Between *A. g. guangxiensis* and *A. g. gingica*, mtDNA haplotypes were fully sorted whereas both taxa had a few shared nuDNA haplotypes. Furthermore, the combined mtDNA and nuDNA tree showed that *A. g. guangxiensis* and *A. g. gingica* formed reciprocally monophyletic groups (Fig. 2). Although the monophyly was mainly resolved by mtDNA (Zink and Barrowclough 2008), our results indicate that the two subspecies already exhibit recognizable divergences in nuDNA haplotype frequency, although the divergence was not complete.

In general, molecular phylogenetic study often reveals non-monophyly of avian subspecies (Zink 2004, Phillimore and Owens 2006), which may be due to incorrect taxonomy or rapid divergence. Among Chinese birds, non-monophyly has been documented in some of the subspecies of *Lophura nycthemera* (Dong et al. 2013), *Charadrius alexandrinus* (Rheindt et al. 2011) and *Garrulax chinensis* (Wu et al. 2012), and all subspecies of *Motacilla alba* (Li et al. 2015) and *Leucosticte brandti* (Sangster et al. 2015). Thus, the congruent divergence of morphological and molecular markers in *A. gingica* contrasts with the divergence patterns observed in several other avian species. However, many tropical and subtropical subspecies have been shown to be monophyletic, and sometimes highly divergent, underscoring the necessity of phylogeographic study for taxonomy within species (e.g. Song et al. 2009; Irestedt et al. 2013).

The divergence between *A. gingica* and *A. rufogularis* in southwest China (Chen et al. 2015) occurred approximately 2.02 mya when there was a major uplift of the Yunnan-Guizhou Plateau during the Plio-Pleistocene boundary (1.8–2.6 mya) (Zhang and Fang 2012). The uplift may have promoted geographical isolation in many species during this period (Qu et al. 2015), including *Stachyridopsis ruficeps* (Liu et al. 2012) and *Aegithalos concinnus* (Dai et al. 2011). The dramatic climatic cooling during the Plio-Pleistocene boundary may have resulted in altitudinal shifts in montane species (Hewitt 2000). These two events may have resulted in the divergence of *A. gingica* from *A. rufogularis*, and that between several other species (Liu et al. 2012).

Our results suggest that the divergence between *A. g. guangxiensis* and *A. g. gingica* occurred 0.11 (0.05–0.19) mya, during or after the penultimate glaciation (0.13–0.42 mya). We speculate that *A. g. guangxiensis* and *A. g. gingica* might have had separate

refugia during the glaciation, inducing population differentiation. This Pleistocene refugia scenario has been proposed for several bird species in southeast China, including *Tragopan caboti* (Dong et al. 2010) and *Alcippe morrisonia* (Song et al. 2009).

In any case, geographical isolation has likely played a role in population differentiation. *A. g. guangxiensis* and *A. g. gingica* are currently separated by the karst basin in central Guangxi. This area also represents unfavorable habitat for some montane species, including *Gorsachius magnificus* (Hu and Liu 2014) and *S. ruficeps* (Liu et al. 2012), perhaps due to the large portion of limestone in the karst basin. Early modern human activities dating back to 0.14 mya have been discovered in this area (Shen et al. 2002), and these activities might have interrupted gene flow between bird populations (Zhou and Jiang 2008). These isolation hypotheses may also have affected differentiation between *A. g. guangxiensis* and *A. g. gingica*.

The estimated temporal diversification and historical biogeography of *A. gingica* proposed here is based on a small dataset and thus should ideally be substantiated with additional data. To better explore the underlying diversification process (e.g. speciation-with-gene-flow, Nosil 2008), more sampling using additional nuclear loci is needed (Edwards and Bensch 2009). In addition, ecological niche modelling may help to identify the potential distribution of both subspecies and the main environmental variables which determine the range of each subspecies (Hu and Liu 2014).

Conclusion

Our study demonstrates that the newly found subspecies *A. g. guangxiensis* and nominate *A. g. gingica* formed reciprocal monophyletic groups in a multi-locus molecular phylogenetic analyses. The allopatric distribution of *A. g. guangxiensis* and *A. g. gingica* and a single diagnostic morphological difference underscore the distinctiveness of these two taxa (Zhou and Jiang 2008). The total body of evidence thus meets the traditional requirement that subspecies are geographically non-overlapping and phenotypically divergent (Wilson and Brown 1953; Mayr and Ashlock 1991) and meets the modern requirements that subspecies are either genetically distinct (Zink 2004), diagnosable (Remsen 2010) or both (Braby et al. 2012). Therefore, our results further support the taxonomic validity of *A. g. guangxiensis* and we suggest that this subspecies should be considered as a conservation unit.

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Supplementary material 1

Table S1

Authors: De Chen, Qiong Liu, Jiang Chang, Aiwu Jiang, Fang Zhou, Yanyun Zhang, Zhengwang Zhang

Data type: molecular data

Explanation note: Primers used for PCR amplification and sequencing in this study.

Supplementary material 2

Figure S1

Authors: De Chen, Qiong Liu, Jiang Chang, Aiwu Jiang, Fang Zhou, Yanyun Zhang, Zhengwang Zhang

Data type: molecular data

Explanation note: Phylogenetic trees from the nuDNA matrix and each of the nuclear introns.

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COMMENTARY

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Horst Aspöck, encyclopedist and entomologist extraordinaire – a personal appreciation

Michael Ohl[1]

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Abstract

The paper provides an overview of the life and work of Prof. Dr. Horst Aspöck, the doyen of neuropterology, on the occasion of his 75th birthday. It particularly emphasizes his outstanding contributions to the development of neuropterology since the 1960s.

Keywords

Biography, Neuropterida, Raphidioptera, Neuroptera, Megaloptera, history of science

Introduction

This is a note on one of the most outstanding and productive entomologists of our time. It is not meant to be an exhaustive account of Horst Aspöck's career and achievements over the 75 years of his life so far. My knowledge of Horst is somewhat limited to his entomological, historical and linguistic activities and stems mostly from my professional and personal relationship with him over the last ten years. Numerous biographical essays on Horst have been published on various occasions and from different perspectives, and it is certainly advisable to read them for a more complete picture of Horst than I can provide here (e.g., Auer 1999, Knapp 2004, Gusenleitner 2004,

Thaler 2004, Christian 2009). These authors have covered some aspects of his life, which I will not repeat here, including Horst's extensive fieldwork (e.g. Rausch and Rausch 2004), his role as an academic teacher, his varied roles in scientific societies, and finally the numerous awards and prizes he received. Here I will briefly summarize his life and career, with information derived from these published biographies, and give my personal impressions of his influence on neuropterology and entomology in general, and finally on my personal relationship to him.

Writing such a paper about Horst's personal life and scientific work with a significant biographical and entomological focus is a challenge not only because of the sheer amount of potentially relevant material, but also because Horst's academic work in entomology is inextricably intermingled with the life, scientific work and career of his wife Ulrike. In 1964, they began to work and publish together on Neuropterida, and most of their publications on Neuropterida published since then are coauthored by the Aspöcks together, often with additional coauthors. Trying to carve out Horst's many achievements is often hardly possible, since most of them were actually Horst's and Ulrike's combined achievements. However, since this paper is written in honor of Horst's 75th birthday, I try to follow his personal tracks over time, but I am aware that Ulrike's and his life and work are too closely connected to do that consistently. Horst himself has worked out their close and intimate life-long collaboration in a paper published on the occasion of her 70th birthday (H. Aspöck 2012).

Horst's life and career (Figs 1–7)

Horst was born on 21 July 1939 in Budweis (České Budějovice) in former Czechoslovakian and now Czech Bohemia. At the time of his birth, Czechoslovakia has already been annexed by the German Reich, and as late as early 1945, the World War also arrived in Budweis. Until May 1945, Bohemia and Moravia were the very last war zones of World War II in Europe, until the German Armed Forces capitulated on 8 May 1945. Local inhabitants of German origin had to suffer severe retaliations, and according to the Potsdam Agreement from 1945, all Germans were expelled from the Sudetenland region. Horst's mother, Maria ("Manka") Knapp, was of Austrian nationality, because she was married to the Austrian-born Fritz Aspöck, from whom she had already divorced in 1941. In the fall of 1945, the Aspöck family moved to Linz in the Austrian state of Upper Austria.

Horst lived in Linz with his family until 1957, when he enrolled at the University of Innsbruck to study Biology with a Zoology major. On the 12th July 1962, he received his Ph.D. from the University of Innsbruck with a thesis on the toxicological characteristics of carbamates. Shortly after, Horst became a student assistant at the Institute of Hygiene of the University of Vienna, and he continued working at this very same university until his retirement in 2004. In January 1963, he became research assistant, and in 1966, he was commissioned to establish and lead a new department of medical parasitology at the Institute of Hygiene of the University of Vienna. In 1970,

Figures 1–7. Horst Aspöck, developmental stages and as a gentleman and professor. **1** around 1950 in Linz, Upper Austria **2** In 1961, passport photo **3** In 1963, Prague, Czechoslovakia, during a symposium on "Theoretical questions of Natural Focis of Diseases" **4** In 1970, Igls near Innsbruck, Austria, during a conference of the German-speaking society for tropical medicine **5** In 1973, in Vienna, Austria, as enthusiastic dancers on a ball at the Wiener Konzerthaus **6** 2004, in the Festsaal of the University of Vienna. With vice rector Hans Geord Eichler (left), during the first PhD defense of the Medical University Vienna (previously Medical Faculty of the University Vienna), held in Latin language, as still mandatory in Austria **7** In 2014, in Linz, Austria, with Ulrike, during the 81st International Entomologist's Conference. All photos from the Aspöck photo archive.

he received his habilitation and was promoted to "Universitätsdozent" for medical parasitology. In 1977, Horst was promoted to extraordinary Professor and in 2000 to full university professor for medical parasitology. He worked as the head of the department of medical parasitology at the same institute, until he retired in September 2004. Since retirement he has continued duties in academic teaching and research and in the supervision of students. Over the years, he has received numerous awards and became honorary or corresponding member in several national and international societies. He is a member of the Nationale Akademie der Wissenschaften Leopoldina since 2000 and a member of the Human-Rights-Committee of the Leopoldina since 2003.

In 1963, Horst married Ulrike Pirklbauer, who is better known as Ulrike Aspöck in entomology. They have one son, Christoph, born in 1965.

Horst's way to Entomology and to Neuroptera

As early as 1952, at the age of 13, Horst became a member of the "Entomologische Arbeitsgemeinschaft" at the "Oberösterreichisches Landesmuseum". Getting in close contact with experienced and enthusiastic entomologists was quite influential, and only four years later, in 1956, he joined an entomological field trip to Istria. He regularly gave private lessons in order to cover the costs of his entomological activities. From the very early beginning, he also showed a disposition to classical languages and to linguistic subtleties, which largely influenced his writing and his taxonomic work until today. During his university studies in Innsbruck, Horst continued his entomological activities, amongst others by temporary work at the "Commonwealth Institute of Biological Control" in Delémont, Switzerland. Although he had already developed some interest in lacewings during the field trip to Istria, he started to work systematically on Neuroptera in 1960 (H. Aspöck 2003a). In 1962, Horst began to study snakeflies from Greece and Anatolia from the personal collections of Josef Klimesch and Franz Ressl, two Austrian entomologists, and he soon realized that the diversity of Raphidioptera in the eastern Mediterranean, particularly in Greece, was much larger than expected from the literature and in contrast to the overall uniform external morphology of snakeflies. Right from the beginning of his developing fascination on Neuroptera, he started to publish his observations and results. In 1962 alone, Horst published five papers (see Gusenleitner 2004, 2009, 2014, for bibliographies of Horst's work), two about the biological effect of carbamates as a result of his Ph.D. thesis, but three papers already on Neuroptera. Two of them dealt with the taxonomy of Hemerobiidae, but one was entitled "Gedanken zur Erforschung der Neuropterenfauna Österreichs" (Thoughts on the exploration of the neuropteran fauna of Austria) (H. Aspöck 1962). It is remarkable and quite symptomatic, that one of his very first publications, which Horst has published on Neuropterida, was an overview on the state-of-the-art of the knowledge of Austrian Neuropterida. Retrospectively, this publication can be seen as the very early announcement of his personal research program on Neuroptera for the following decades. Even more, it is a very early example of Horst's tendency to keep the general picture in mind, even when working on small taxonomic projects. In this small paper from 1962, he concluded that the knowledge of the Austrian fauna of Neuropterida is incomplete, and he spent most of the text to present the reader an overview on the diversity of Austrian lacewings, how the various families can be recognized and where they can be found. Consequently, a brief note was added, where Horst asked members of the "Arbeitsgemeinschaft Österreichischer Entomologen" to send him any neuropteran they would collect in exchange for other insects from Central and Southern Europe. In 1963, Horst and Ulrike not only married, but they also began a life-long and fruitful collaboration on Neuropterida, particularly on Raphidioptera. The very first results were *Raphidia ulrikae*, a new Central European snakefly species, which Horst dedicated to his wife (H. Aspöck 1964), and the first publication coauthored by the Aspöcks on new species of *Raphidia* (H. Aspöck and U. Aspöck 1964). In the following decades, they would not only publish, but also travel together to numerous countries on all continents to collect Neuropterida (Figs 8–14, see also Rausch and Rausch 2004).

Figures 8–14. Horst Aspöck in the field. **8** In 1975, Anatolia, Turkey. During a collecting trip in the Pontus-mountains to collect Raphidioptera (photo by Hubert Rausch) **9** In 1975, Doğubayazit, Anatolia, Turkey. Horst, flanked by Ulrike Aspöck (left) and Renate Rausch, dancing with locals **10** In 1971, Catalonia, Spain. From left: Ernst Hüttinger, Horst, Ulrike **11** In May 2014, Anti-Atlas, Morocco, Horst night-collecting **12** In 1995, Talasskaya Oblast, Talasskiy Alatau, Kyrgyztan. Horst collecting larvae of Raphidioptera **13** In 2014, Anti-Atlas, between Tafraoute and the Igmir Oasis, with Ulrike **14** In 2000, Mae Hong Son Pai Province, Thailand, night-collecting with the Austrian entomologist Hans Malicky. All photos except for photo 8 from the Aspöck photo archive.

Entomology

I have only limited knowledge about Horst's research activities in medical parasitology, but these have been presented and cherished in several papers by more competent authors, including Auer (1999), Flamm (2007, 2012) and publications in the Festschrift

"Entomologie und Parasitologie", edited by Ulrike Aspöck (2004), on the occasion of Horst's 65th birthday. The "duality" (Thaler 2004) of contributing significantly to two different scientific fields in two different scientific disciplines is more than unusual and exemplifies probably more than anything else the broad and general perspective of Horst's character. I will here concentrate on his entomological work, but also on an additional "side-interest" in the history of science.

Horst is unusually productive, and the "Aspöck-and-Aspöck" publication list comprises 739 publications until 2014, including more than ten books and numerous book chapters (Gusenleitner 2004, 2009, 2014). In the last half a century, almost exactly 50 years after his first publication on Neuropterida, the Aspöcks are more than anybody else at the forefront of neuropterid research, and it is, thus, not surprising that they have authored a large number of chapters on Neuropterida in many contemporary textbooks on zoology, entomology and in several catalogs. Prominent examples are the chapter in "The Insects of Australia" (H. Aspöck and U. Aspöck 1991), the four chapters on neuropterida, Neuroptera, Raphidioptera and Megaloptera in the second edition of the German written "Lehrbuch der Speziellen Zoologie" (U. Aspöck and H. Aspöck 2003a-d), and their catalog of the Neuropterida of the Western Palearctic (H. Aspöck et al. 2001).

Some of the publications, particularly some books, are clearly milestones in their field and proved to be influential for generations of neuropterologists. Two particularly outstanding examples are the "big green books", as a reference to the color of their book cover, "Die Neuropteren Europas" (The Neuropterans of Europe, H. Aspöck et al. 1980) and "Die Raphidopteren der Erde" (The Raphidiopterans of the World, H. Aspöck et al. 1991). Both of them consist of two volumes and they have scholarly summarized the available knowledge on these two orders as available at that time. They are accompanied by 900 (Neuroptera) and 1300 (Raphidioptera) line drawings and several distribution maps, tables and other kinds of images. Although written in German, both monographs received a worldwide distribution and appreciation, and they are still among the most significant book length monographs on Neuroptera and Raphidioptera ever written. They can clearly be called modern classics in neuropterology.

Although the Aspöcks are especially influential in Raphidioptera research, they have published intensively on virtually all families in Neuroptera, with a specific interest in Berothidae (e.g., U. Aspöck et al. 2013). The majority of publications are on the taxonomy and phylogeny of Neuropterida, although, when feasible, information on the behavior has also been published, including review papers like H. Aspöck (2002a). With the increasing worldwide overview on the diversity and distribution of Neuropterida, particularly Raphidioptera, specific distribution patterns in space and time became obvious, and Horst has published on this historical biogeography intensively. One of the obvious peculiarities of the Raphidioptera is their strictly northern hemisphere distribution, which deserves explanation. One of the more recent publications on global distribution patterns in Raphidioptera is H. Aspöck and U. Aspöck (2004b).

Horst has published intensively in collaboration with his wife Ulrike, and the enormous number of publications with both Aspöcks as authors is remarkable. Only between 2009 and 2014, 71 of a total of 88 publications have been co-authored by

both of them (Gusenleitner 2014). In the same period of time, Horst has published with an incredible number of more than 370 different co-authors. Although this high number is biased due to a single publication on a phylogenomic analysis of insect evolution with almost 100 co-authors (Misof et al. 2014), only a relatively small portion of the 88 publications have been published with Horst as sole author. This large number of co-authored publications clearly emphasizes Horst's understanding of science as a culture of networking and exchanging information, data and material.

The multi-authored publication by Misof et al. (2014) is an indicator of another remarkable character of Horst. In his parasitological research, molecular methods have been standard techniques for quite a long time, particularly when studying viruses and the wide variety of pathogenic organisms. For quite a few decades, Horst has intensively used molecular methods in medical research, usually in close collaboration with competent colleagues and students. In contrast, by far the majority of his entomological activities and publications are based on a careful and exhaustive study of morphological characters in the frame of a comparative approach. However, with the increasing importance of molecular methods in systematic entomologies, he (and his wife Ulrike Aspöck) immediately realized the benefits of the molecular approach. Together they published a first outline of the importance of molecular studies in Neuropterida (U. Aspöck et al. 2003), which was followed by publications on the molecular phylogeny of the Neuroptera (Haring and U. Aspöck 2004, without Horst) and the Raphidioptera (Haring et al. 2011), and a few smaller contributions largely based on the dataset by Haring et al. (2011) with discussions of the implication of the molecular analysis on the biogeography of snakeflies. More recently, Horst and Ulrike contributed to a large genomic project lead by Bernard Misof (Museum Alexander Koenig), which resulted in a series of conference lectures and by two prominent publications on the phylogeny of insects (Peters et al. 2014, Misof et al. 2014). There is more to come soon.

History, language, and catalogs

Horst has always grounded his empirical work on a fundamental understanding of the historical development of the discipline. He has published repeatedly on the history of neuropterology, including detailed and often personal appreciations of deceased colleagues, like the almost 90-pages-obituary for Herbert Hölzel (H. Aspöck 2009) with the telling title "Ein sehr persönlicher Nachruf und ein Stück Geschichte der Neuropterologie" (A very personal obituary and a piece of history of neuropterology). Over the years, Horst has published more than 60 biographical papers, most of which on colleagues in medical parasitology and in neuropterology (Gusenleitner 2004, 2009, 2014). Some of these publications are of broader significance, like a monographic treatment on all authors who have published taxonomic names in Raphidioptera (H. Aspöck and U. Aspöck 2014a).

Besides the many obituaries and personal biographies, Horst has intensively published on the history of neuropterology in Austria (H. Aspöck 1984) and in the German

Figures 15–20. Horst Aspöck among colleagues and friends. **15** In 2013, group photo of the 13th workshop of the German-speaking neuropterologists on Schwanberg Castle, Bavaria, Germany. From left: Johannes Gepp, Melitta Fuchs, Lukas Kirschey, Karl Meissner, Horst and Ulrike, Wilfried Wichard and his wife, Steffen Potel, and Axel Gruppe **16** In 2014, same meeting, with Hubert Rausch and Michael Ohl (from left) **17** In 2011, Berlin, Germany. Annul meeting of the "Deutsche Gesellschaft für allgemeine und angewandte Entomologie (DGaaE), with Ernst Joachim Tröger and Rainer Willmann (from left) **18** In 2011, same conference, with John Oswald (right) **19** In 2014, Vienna, Austria, with Fritz Gusenleitner (right) **20** In 2011, Ponta Delgada, Azores, Portugal. XI International Symposium on Neuropterology, with a group of Chinese neuropterologists. From left: Yongjie Wang, Horst, Xingyue Liu, and Dong Ren.

speaking countries (H. Aspöck and U. Aspöck 2010), on the history of the "International Association of Neuropterologists" (H. Aspöck 2010) (Fig. 20), and on the history of the "Österreichische Entomologische Gesellschaft" (H. Aspöck 2003b). His broad knowledge of the historical literature is best reflected by a series of beautifully illustrated, detailed publications of early descriptions and illustrations of Raphidioptera (H. Aspöck 1998), Mantispidae (H. Aspöck 1999) and Osmylidae (H. Aspöck 2002b).

Horst had a profound education in classical languages, particularly in Latin, and he has developed a good sense for the subtleties of his own mother tongue. In his German publications, Horst develops a complex language, which is not only scientifically accurate and detailed, but also a delight to read. It seems to be logical, at least from my perspective, that Horst has thought about the linguistic background in general and for his science since very early on. This can best seen in the wide variety of taxon names he proposed in Neuropterida, which clearly exhibit a deep understanding of classic languages, combined with careful observation and a sense of humor and fantasy. The etymological origin of all names in Raphidioptera published by him and Ulrike has been presented in a long paper on the etymology of all names in Raphidioptera (H. Aspöck and U. Aspöck 2013).

My personal appreciation

In the title of this contribution, Horst has been called an "encyclopedist", and I am well aware that this term has a somewhat old-fashioned connotation. However, his approach in a sense of broadly compiling data and information is encyclopedic both in a historical and a completely modern sense. "Encyclopedist" is truly an historical word, and it derives from a time at least back to the 18th century, when formal biological nomenclature originated from the work of a true encyclopedist, Carl Linnaeus. Since 1758, the year of the publication of the 10th edition of Linnaeus' "Systema Naturae", which has been assigned the birth of zoological nomenclature, taxonomists have struggled hard to catalog and register all life on Earth. In a sense, the backbone of Horst's work lies in the direct tradition of the "Linnaean enterprise" of a global register of all living forms (Wilson 2005). In the 18th century, Linnaeus was inspired by the "dream of completeness" (Frangsmyr et al. 1990), but even at his time, he and his disciples and contemporaries suffered from what the historian Staffan Müller-Wille paraphrased as "information overload" (Müller-Wille and Charmantier 2012). Taxonomic systems and methods were developed to organize and control the ever increasing amount of available information. Linnaeus' "dream of completeness" was based on a dramatic underestimation of the true amount of the global diversity, and the "Linnaean enterprise" as a full mapping of the Earth's biodiversity is still far from being completed. It has been extensively discussed, which strategies and technologies are needed "for a comprehensive mission to explore and document Earth's species" (Wheeler et al. 2012), and Horst and Ulrike have decided to concentrate on a relatively small group of the hyper-diverse insects, which guarantee to approach completeness as close as

possible. Neuroptera and Raphidioptera seem to be the perfect target. The diversity is sufficiently large for a life-long and challenging research agenda, but small enough to cover a large, if not the largest portion of the global diversity within a career.

This is how I perceived Horst and Ulrike Aspöck, when I first met Ulrike in mid-1994 in the Naturhistorisches Museum in Vienna. Shortly before I had started to work on my dissertation on apoid wasp taxonomy and phylogeny under the supervision of Rainer Willmann at the University of Göttingen, Germany (Fig. 17). Willmann had sporadically published on Neuroptera before, although he was largely concentrating on Mecoptera. In the early 1990s, when I visited the museum in Vienna for the first time, he had published a few papers on the phylogeny of Mantispidae (Willmann 1990, 1994). Based on morphological characters and the fossil record, he argued in favor of a sister-group relationship between the Mantispidae and Rhachiberothidae, with raptorial forelegs as one of their synapomorphies. This was in contrast to the Aspöcks' assumption of a monophyletic Berothidae + Rhachiberothidae, with the Mantispidae being their sister-group. This hypothesis has been confirmed in numerous analyses since then (e.g., U. Aspöck and Mansell 1994). With the scientific conflict between Rainer Willmann and the Aspöcks in mind, being a Ph. D. student of Willmann, and well aware of the extraordinary standing of the Aspöcks in entomology in general and particularly in Neuropterida, I was quite nervously looking forward to meeting Ulrike for the first time. She proved to be exceptionally hospitable and intrigued to learn about me and my scientific project. I felt warmly welcomed by her even as a young Ph. D. student, and this combination of scholarly curiosity, kindness and openness is certainly a typical character of Horst and Ulrike, which I have had the pleasure to experience whenever I meet them.

In the following years, I met Horst on various occasions on entomological meetings and conferences, and his personal appearance always inspired me respect. He was, and still is, always critically listening to presentations, even from students giving their first talk, and he very often asks the very first question during the discussion. We first got into a closer contact, when I started to work on Mantispidae within Neuroptera in the late 1990. I was appointed as curator for Neuropterida at the Museum für Naturkunde in Berlin in 1997, and soon after I decided to complement my interest in Hymenoptera by setting up a new, initially smaller project line on Neuroptera. Mantispidae was an attractive group, not only because of its intriguing morphology and behavior, but also because the diversity was limited, with about 350 valid species known, in contrast to the more than 10,000 species of apoid wasps I was confronted with for many years. Even more, after reading through the relevant literature and after talking to the Aspöcks and other neuropterologists, it became clear that at that time, there was nobody seriously working on the taxonomy and systematics of the Mantispidae with a global perspective. As a first step, I started to compile a personal catalog of all taxonomic names, which can be assigned to Mantispidae. I talked to Horst and Ulrike Aspöck frequently about the catalog, and Horst was always curious to learn about the progress I was making. My conversation with him about Mantispidae was usually accompanied by some kind of "examination" of my knowledge mostly about historical literature on

Mantispidae, and in the first years, I was always nervous not to reveal too many painful and embarrassing knowledge gaps. However, I not only learned a lot from talking to Horst, but I very much enjoyed discussing with him topics of mutual interests. My "personal mantispid catalog" was finally published a few years later (Ohl 2004).

Over the last decade, I met Horst regularly on the meetings of the "Arbeitskreis Neuropteren" (http://www.dgaae.de/index.php/neuroptera.html), formerly the "Arbeitstreffen deutschprachiger Neuropterologen" (Figs 15–16). This has been established as a study group within the "Deutsche Gesellschaft für Allgemeine und Angewandte Entomologie", the largest German entomological society. The meetings of the "Arbeitskreis" take place in the "Kloster Schloss Schwanberg" in the Bavarian area of Lower Franconia, and it is a very stimulating place. The "Arbeitskreis" is a rather small group, organized by Axel Gruppe (Fig. 15), Freising, Munich, and Horst and Ulrike clearly play a central role in this small scientific community. For me, this is an especially pleasant opportunity to talk extensively to Horst about plans, ideas and particularly Neuroptera. Since families are also welcomed on these meetings, I frequently take my own family with me. Horst, now himself being a grandfather, was always very interested in my wife Daniela and my children Mattes, Merle and Mina, particularly when he realized that my son Mattes is already in the process of becoming an enthusiastic naturalist, with serious interests in spider taxonomy and the Latin language.

Besides Neuroptera, Horst and I have a great deal in common, and two particular interests we share are the linguistics and etymology of taxonomic names and the history of entomology, not to speak of neuropterology. Ulrike's and his monographic treatment of the etymology of names in Raphidioptera (H. Aspöck and U. Aspöck 2013) and my own popular science book on the culture of naming in natural history (Ohl 2015) developed simultaneously, though coincidentally. Horst's interests in the many aspects of the history of entomology, neuropterology in general, neuropterology in Austria and many other aspects are best demonstrated by the extraordinary private Aspöck library. Horst is an enthusiastic collector of historical books in natural history, and their personal book collection is far beyond any other personal library I know. Neuropterological literature is predominant, but there are many more historical books, which make their personal book and reprint collection an impressive library. Horst was lucky to start collecting rare books at a time, when the international book market was not dominated by the internet, so that he could buy many rare books for reasonable prices, which would be hardly affordable today. My own book collection is significantly smaller, but we enjoy sharing our knowledge and interest about historical books whenever possible.

The celebration of Horst's 75th birthday is the perfect opportunity to look back to his many accomplishments. It is obvious from the above that Horst has a fearsome intellect and exhibits boundless dedication. He is working hard in pursuit of his exceedingly broad passion, and he is not slowing down significantly. He is a real gentleman, both personally and as a scientist, and he is very thoughtful of the needs of friends and colleagues. He is much sought as a collaborator, first because of his broad knowledge in so many fields, but also because he has the focus and concentration to bring projects to a success. I admire his continuing energy, his productivity and his drive. He is setting a

high standard for the younger generation, including me, and although I am not always sure that I am able to meet these standards myself, I can call myself very fortunate to benefit from extensive interactions with Horst in so many respects. We are all looking forward to much more to come.

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