





Reconstructing the nonadaptive radiation of an ancient lineage of ground-dwelling stick insects (Phasmatodea: Heteropterygidae)

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Abstract. Stick and leaf insects (Phasmatodea) are large terrestrial herbivorous arthropods known for masquerading as plant parts such as bark, twigs and leaves. Their evolutionary history is largely shaped by convergent evolution associated with adaptive radiations on geographically isolated landmasses that have repeatedly generated ground-dwelling ecomorphs. The members of one lineage, however, the Oriental Heteropterygidae, are morphologically rather uniform, and have a predominantly ground-dwelling lifestyle. The phylogeny of Heteropterygidae that comprises approximately 130 described species is controversial and remains uncertain. In particular, the systematic position of the giant Jungle Nymph *Heteropteryx dilatata*, whose males are capable of flight and exhibit the most plesiomorphic wing morphology among extant phasmatodeans, is of major interest to the scientific community. Here, we analysed a set of seven nuclear and mitochondrial genes to infer the phylogeny of Heteropterygidae covering the group's overall diversity. The divergence time estimation and reconstruction of the historical biogeography resulted in an ancestral distribution across Sundaland with long distance dispersal events to Wallacea, the Philippines and the South Pacific. We were able to resolve the relationships among the three principal subgroups of Heteropterygidae and revealed the Dataminae, which contain entirely wingless small forms, as the sister group of Heteropteryginae + Obriminae. Within Heteropteryginae, *Haaniella* is recovered as paraphyletic in regard to *Heteropteryx*. Consequently, *Heteropteryx* must be considered a subordinate taxon deeply embedded within a flightless clade of stick insects. Within Obriminae, the Bornean *Hoplocлонia* is strongly supported as the earliest diverging lineage. Based on this finding, we recognize only two tribes of

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equal rank among Obriminae, the Hoplocloniini **trib. nov.** and Obrimini **sensu nov.** Within the latter, we demonstrate that previous tribal assignments do not reflect phylogenetic relationships and that a basal splitting event occurred between the wing-bearing clade *Miroceramia* + *Pterobrimus* and the remaining wingless Obrimini. The Philippine genus *Tisamenus* is paraphyletic with regard to *Ilocano hebardi*, thus, we transfer the latter species to *Tisamenus* as *Tisamenus hebardi* **comb. nov.** and synonymize *Ilocano* with *Tisamenus*. We discuss character transformations in the light of the new phylogenetic results and conclude that the current taxonomic diversity appears to be mainly driven by allopatry and not to be the result of niche differentiation. This radiation is thus best described as a nonadaptive radiation.

Introduction

The evolutionary history of stick and leaf insects, commonly referred to as the insect order Phasmatodea, appears to be strongly shaped by convergent evolution as a consequence of repeated adaptive radiations in geographic isolation as has been revealed for the stick insect faunas of Australia, New Caledonia, New Zealand, Madagascar and the Mascarene archipelago (Buckley *et al.*, 2009, 2010; Bradler *et al.*, 2015; Glaw *et al.*, 2019; Simon *et al.*, 2019). Thus, similar morphological forms as well as behavioural traits (Robertson *et al.*, 2018) were often acquired independently due to similar selective pressures associated with adaptations to the same habitat in separate geographic areas. For decades this has deceived taxonomists who tried to recover the phylogeny of Phasmatodea based on morphology alone (Bradler *et al.*, 2014). In contrast to adaptive radiations, where species diversification is driven by the occupation of a variety of ecological roles resulting in considerable phenotypic disparity (Givnish, 1997), nonadaptive radiations might also play a major role in stick insect evolution. Nonadaptive radiation is defined as the diversification from a single ancestor that is not accompanied by relevant niche differentiation (Gittenberger, 1991), resulting in a group of allopatric taxa with little or no ecological and phenotypic variation (Rundell & Price, 2009). Adaptive radiation has been extensively studied in the past, whereas the phenomenon of nonadaptive radiation has been largely neglected and appears to be controversially discussed (Wilke *et al.*, 2010). A clade of Oriental stick insects, the Heteropterygidae may be considered to represent a nonadaptive radiation since its members deploy a number of uniform phenotypic and behavioural characteristics associated with living close to the forest floor. Although often generally referred to as ground-dwellers (Bragg, 1998; Hennemann *et al.*, 2016a; Bradler & Buckley, 2018; Bresseel & Constant, 2018), Heteropterygidae can also be found on bark (e.g. *Mearnsiana* Rehn & Rehn; Hennemann *et al.*, 2016a) and in the vegetation, in particular during nocturnal feeding, whereas during daytime they mostly rest among leaf litter, pieces of bark or between roots of trees (Bragg, 2001). However, the group exhibits a consistent egg-deposition mode by burying eggs in the soil (Robertson *et al.*, 2018).

The majority of stick and leaf insects are highly adapted to masquerade as plant parts in order to avoid detection by predators. An elongated twig-like morphotype is prevalent, with some stick insects counting among the longest insects worldwide with body lengths of over 30 cm (Hennemann & Conle, 2008). The Heteropterygidae, however, is not known for extremely long and slender insects but for rather robust forms including the large Jungle Nymph *Heteropteryx dilatata* Parkinson, one of the heaviest insects worldwide with a body weight of over 50 g (Wood, 1976; Beccaloni, 2010). Instead of resembling slender twigs, these stout ground-dwellers are generally coloured brownish and mimic leaf litter or bark (Figs. 1, 2). Adapted to life near the ground, most species are flightless with no or strongly shortened wings, and eggs are always deposited into the soil (Bradler & Buckley, 2018; Robertson *et al.*, 2018). Although these traits limit their dispersal capacity, heteropterygids have managed to disperse across the Indomalayan and Australasian region since their origin approximately 50 million years ago (Robertson *et al.*, 2018; Simon *et al.*, 2019). The radiation gave rise to ~130 described species, which are currently assigned to 27 genera (Brock *et al.*, 2020) of which one, *Woodlarkia* Günther, is believed not to belong to Heteropterygidae (Hennemann *et al.*, 2016a).

Since the clade was originally introduced as Heteropteryginae by Kirby (1896), various taxa have been added and transferred in a rather disorderly way to and within the four traditional subgroups Anisacanthini, Datamini, Heteropterygini and Obrimini (e.g. Redtenbacher, 1906; Rehn & Rehn, 1938; Günther, 1953; Beier, 1968; Klante, 1976; Bradley & Galil, 1977; Zompro, 2004) and Heteropteryginae was eventually elevated to the rank of a family by Zompro (1996) with its aforementioned tribes considered as subfamilies afterwards (see Hennemann *et al.*, 2016a, for a detailed summary). The Anisacanthinae comprising a group of Malagasy stick insects was later considered to be unrelated to the remaining Heteropterygidae and excluded by Zompro (2004). This view was recently supported by molecular data, which simultaneously provided evidence for a monophyletic group combining all stick insects from Madagascar (Glaw *et al.*, 2019; Simon *et al.*, 2019). Also, the monophyly of Heteropterygidae and its three subordinate clades was demonstrated in various studies using molecular



Fig. 1. Photographs of representatives of Dataminae (A, B) and Heteropteryginae (C–F). (A) mating couple of *Dares philippinensis* (Palawan); (B) mating couple of *Pylaemenes* sp. (Tawau); (C, D) male and female of *Heteropteryx dilatata*; (E) *Haaniella echinata* male from Brunei; (F) *Haaniella scabra* female from Kinabalu. Photos by Albert Kang and Christoph Seiler. [Colour figure can be viewed at wileyonlinelibrary.com].

data (Bradler *et al.*, 2015; Goldberg *et al.*, 2015; Robertson *et al.*, 2018; Büscher *et al.*, 2018a; Glaw *et al.*, 2019; Simon *et al.*, 2019). However, the phylogenetic relationships among the three clades Dataminae, Heteropteryginae and Obriminae remained unclear. According to the phylogenetic studies of Heteropterygidae based on morphological data (Klante, 1976; Bradler, 2009), Dataminae are the sister group to Heteropteryginae + Obriminae. This combination is favoured by only one molecular analysis (Bradler *et al.*, 2015), whereas other studies hypothesize either Heteropteryginae + (Dataminae + Obriminae) (Zompro, 2004; Goldberg *et al.*, 2015; Glaw *et al.*, 2019) or Obriminae + (Dataminae + Heteropteryginae) (Robertson *et al.*, 2018; Büscher *et al.*, 2018a; Simon *et al.*, 2019; Forni *et al.*, 2020). The lack of a robust phylogeny for the Heteropterygidae impedes the reconstruction of its biogeographic history as well as the evolution of certain key traits such as wings or secondary ovipositors within this group.

Molecular phylogenetic studies have been useful for revising the problematic traditional classification of Phasmatodea and have substantiated several taxonomic groups (Buckley *et al.*, 2009; Bradler *et al.*, 2015; Robertson *et al.*, 2018; Glaw *et al.*, 2019). Even the uncertain phylogenetic relationships between the major phasmatodean lineages were recently resolved by a phylogenomic analysis based on transcriptomic data (Simon *et al.*, 2019). Simon *et al.* (2019) were able to provide clarity on the relationships of most major phasmatodean clades and placed Heteropterygidae as a taxon derived from a rather ancient node and sister group to all remaining members of the species-rich Old World clade Oriophasmata. Notably, the only poorly supported node (49% bootstrap support) in that study happened to occur within Heteropterygidae, illustrating the problematic nature of Heteropterygidae phylogeny. The estimated relationship presented Obriminae as sister to the poorly supported group of Dataminae + Heteropteryginae,

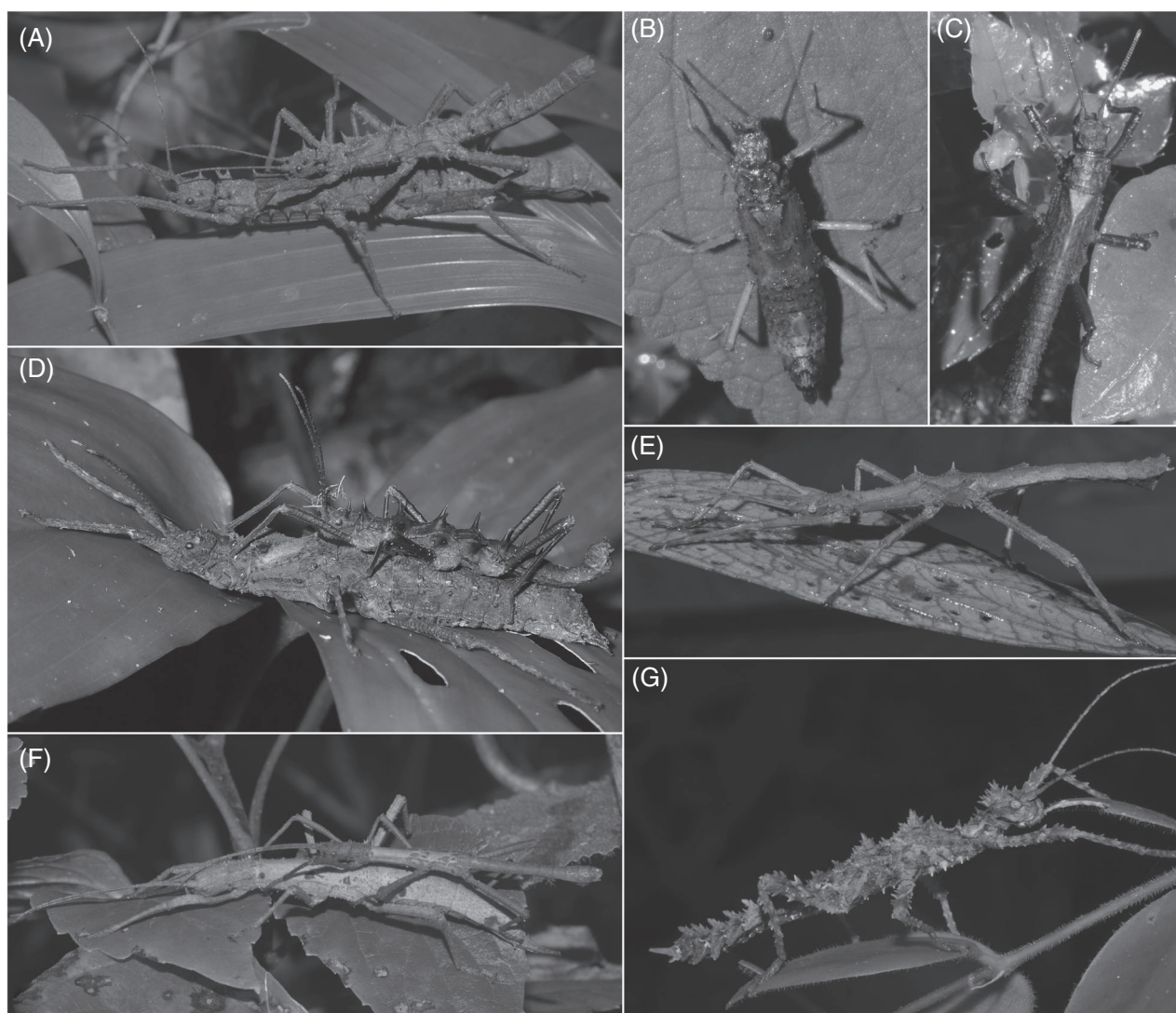


Fig. 2. Photographs of representatives of Obriminae. (A) couple of *Tisamenus* sp. (Sibuyan); (B, C) female and male of *Tisamenus hebardi* **comb. nov.**; (D) couple of *Hoploclonia cuspidata* (Brunei); (E) male of *Brasidas* sp. (Camiguin Island); (F), couple of *Eubulides igorrote* (Mt. Pullol); (G) female of *Aretaon muscosus* (Mulu, Borneo). Photos by Albert Kang. [Colour figure can be viewed at wileyonlinelibrary.com].

which was already shown in the multi-gene study by Robertson *et al.* (2018). Although the latter included less sequence data, their sample size comprised five times as many individuals as the transcriptomic study by Simon *et al.* (2019) contributing to an increase in support to 71% (Bayesian posterior probability) for the same node.

Here, we provide an even more comprehensive data set exhaustively covering the overall diversity of Heteropterygidae by including representatives of most genera and spanning across the group's entire geographic distribution. We used a set of three nuclear and four mitochondrial genes for a phylogenetic analysis to test the monophyly of Heteropterygidae and its subgroups as well as to identify their phylogenetic relationship. One focus was on resolving the systematic position of taxa previously considered as problematic due to the presence of

unusual anatomical traits (Bradler, 2009) such as *Hoploclonia* Stål, which is characterized by a unique secondary ovipositor among Heteropterygidae, and *Miroceramia* Günther and *Pterobrimus* Redtenbacher, the only Obriminae taxa with wings or wing remnants (*Pterobrimus*). In particular, we intended to robustly place *Heteropteryx dilatata* among stick and leaf insects, whose prominent male exhibits the most plesiomorphic fore wing among all extant phasmatodeans (Willmann, 2003; Shang *et al.*, 2011). We combined the phylogenetic data with geographical data to reconstruct the historical biogeography and discuss implications for the classification of Heteropterygidae. Furthermore, our phylogeny provides a comparative framework to facilitate the interpretation of evolutionary processes, for instance, to explore size evolution and survey the highly debated hypothesis on the (re-)evolution of wings in stick

insects (Stone & French, 2003; Whiting *et al.*, 2003; Goldberg & Igić, 2008).

Material and methods

Taxonomic sampling and laboratory protocols

For our phylogenetic analysis, we included 123 representatives of Heteropterygidae covering all of the 26 currently recognized genera (excl. *Woodlarkia*) except for four of which no sample could be obtained (*Hainanphasma* Ho, *Heterocopus* Redtenbacher, *Microrestes* Bresseel & Constant and *Spinodares* Bragg). We added 65 outgroup species from other major phasmatodean lineages resulting in a total of 188 specimens (see Table S1 for details). We predominantly chose new, previously unused outgroup taxa in order to assess and corroborate the gross phylogeny of Phasmatodea based on a novel set of samples. Molecular data of 43 taxa was already published and available on GenBank (Whiting *et al.*, 2003; Buckley *et al.*, 2009; Kômoto *et al.*, 2011; Vera *et al.*, 2012; Schwander *et al.*, 2013; Bradler *et al.*, 2014; Bradler *et al.*, 2015; Goldberg *et al.*, 2015; Robertson *et al.*, 2018; Glaw *et al.*, 2019) and for nine of these we were able to acquire additional material to sequence missing genes.

Samples were either stored in ethanol (70–100%) or dried. A unique sample code was given to every specimen and whenever possible, the voucher was stored at the Biodiversity Museum at the University of Göttingen, at the Royal Belgian Institute of Natural Sciences or in a private collection (see Table S1). Dried tissue was soaked in pure water before dissection. We removed muscle tissue from coxae and/or femora of each specimen. When the amount of gathered tissue was insufficient, one or two whole legs were used. Prior to DNA extraction, complete evaporation of ethanol residues was ensured. DNA was extracted from each sample using the Quick-DNA Miniprep Plus Kit (Zymo Research Europe GmbH, Freiburg, Germany). The manufacturer's protocol was followed for solid tissues and DNA was eluted in 60 µL of the provided elution buffer.

Polymerase chain reaction (PCR) amplifications were performed in 10 µL reactions containing the following reagent volumes: 5.12 µL water, 1 µL dNTP (2 mM; RotiMix PCR-1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 1 µL 10x DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, U.S.A.), 0.08 µL DreamTaq DNA Polymerase (Thermo Fisher Scientific), 0.4 µL forward primer, 0.4 µL reverse primer and 2 µL template. Three nuclear and four mitochondrial target genes were amplified for each sample. Nuclear data included 18S rRNA (18S), 28S rRNA and Histone subunit 3 (H3) and mitochondrial data was sampled from cytochrome oxidase subunit I and II (COI and COII), 12S rRNA (12S) and 16S rRNA (16S) (Buckley *et al.*, 2009; Robertson *et al.*, 2013). The 18S gene was amplified and sequenced using a combination of three overlapping primer sets (Robertson *et al.*, 2013). The PCR thermal cycling program was set to an initial step of 95°C for 1 min, 40 cycles of 95°C for 1 min, respective annealing temperature for 1 min and 72°C for 1.5 min, followed by a final extension step of 72°C for 8 min. See Table S2 for more

information on primers and specific annealing temperatures. Successful amplification of PCR products was verified by gel electrophoresis. We applied an enzymatic cleanup method using 1–2 µL of ExoSAP-IT™ Express (Thermo Fisher Scientific) to purify 2.5–5 µL of PCR product which was subsequently Sanger-sequenced by Microsynth Seqlab (Göttingen, Germany). DNA sequences and corresponding electropherograms were examined and edited in GENEIOUS v. 11.0.5 (Biomatters Ltd., Auckland, New Zealand). We used the implementation of BLASTN (Nucleotide Basic Local Alignment Search Tool; Altschul *et al.*, 1990) in Geneious to compare our data to reference sequences available at NCBI and subsequently removed identified contaminants. Final sequences were deposited in GenBank under accession numbers MN924966–MN925870 (see Table S1).

Phylogenetic analysis

Nucleotide sequences were combined with previously published data and aligned for each gene separately with MAFFT v. 7.450 (Katoh & Standley, 2013) under the G-INS-I algorithm using --globalpair --maxiterate 1000. Subsequently, we used MACSE v. 2.03 (Ranwez *et al.*, 2018) to deal with length variability of alignment extremities by trimming each multiple sequence alignment (MSA) from the beginning and the end until a coverage of 50% was reached (–prog trimAlignment –align alignment.fasta –min_percent_NT_at_ends 0.5). Internal gaps (gappy columns) of ribosomal genes (12S, 16S, 18S and 28S) with less than three nucleotides per column were removed using a custom-made Perl script. All MSAs were visually inspected for ambiguously aligned sequence sections and – if necessary – manually corrected in GENEIOUS v. 11.0.5 (www.geneious.com). Protein coding genes (COI, COII and H3) were translated into the corresponding amino acid sequences to ensure the correct frameshift and internal gaps were manually removed.

Concatenation was carried out with FASCONCAT v. 1.1 (Kück & Meusemann, 2010) and resulted in a 5343 bp supermatrix (File S1). We partitioned the supermatrix into 13 data blocks, namely, the four ribosomal genes and each codon position of each of the three protein coding genes. We used PARTITIONFINDER v. 2.1.1 (Guindon *et al.*, 2010; Lanfear *et al.*, 2012; Lanfear *et al.*, 2016) to identify the optimal partitioning scheme and best-fit model (greedy algorithm and linked branch lengths). All models were considered (models = all) and model selection was performed under the corrected Akaike information criterion (AICc; model_selection = aicc). PartitionFinder merged the third codon positions of the COI and COII partitions and kept all other data blocks separate resulting in 12 subsets (Table S3).

Topology and support of certain phylogenetic relationships may be affected by missing data or unstable 'rogue' taxa (Wilkinson, 1996). We generated single-gene trees for each of the seven loci in IQ-TREE v. 1.6.10 (Nguyen *et al.*, 2015) under default settings and used the partitioned concatenated supermatrix to generate 300 standard nonparametric bootstrap trees (Chernomor *et al.*, 2016). The set of bootstrap trees served as input for ROGUENAROK v. 1.0 (Aberer *et al.*, 2013), which

we used with default settings to identify rogue taxa. Although several taxa were detected, only *Orestes guangxiensis* scored considerably high (score = 7.197). After viewing the affected single-gene trees for rogue behaviour, we removed the 28S sequence of this taxon from the dataset.

A Maximum Likelihood (ML) phylogeny was inferred from the final supermatrix with a partitioned analysis in IQ-TREE v. 1.6.10 (Nguyen *et al.*, 2015; Chernomor *et al.*, 2016) using the subsets and substitution models suggested by PartitionFinder. The analysis was run with random starting trees and 1000 ultrafast bootstrap pseudo-replicates (UFBoot; Hoang *et al.*, 2018). We also conducted a single branch test by performing the Shimodaira-Hasegawa-like approximate likelihood ratio test with random starting trees and 1000 replicates (SH-aLRT; Guindon *et al.*, 2010). Since both ML trees resulted in the same topology, we wanted to determine the actual best-scoring log-likelihood tree by running 50 independent ML analyses based on random starting trees. We also assessed nodal support by estimating 500 standard nonparametric bootstrap (BS) trees. The support values were mapped on the best-scoring ML tree after the convergence of BS replicates was verified using the 'bootstopping' criterion implemented in RAXML v. 8.2.12 (Stamatakis, 2014). All trees were rooted with Aschiphasmataidae (sister taxon to Neophasmatodea; Simon *et al.*, 2019) and visualized in FIGTREE v. 1.4.4 (<https://github.com/rambaut/figtree>).

In addition, we wanted to further test the relationships among the three major heteropterygid groups. In order to assess phylogenetic support and to detect potentially conflicting signal, we performed a four-cluster likelihood mapping analysis (Strimmer & von Haeseler, 1997) on the partitioned supermatrix defining Dataminae, Heteropteryginae, Obriminae and the outgroup as four taxonomic groups (clusters). We conducted this analysis in IQ-TREE with the number of randomly drawn quartets set to 10 000. For revealing potential incongruences among single genes, we repeated the analysis for each gene, separately.

Divergence time estimation

Phylogenetic relationships and divergence times were obtained by Bayesian Inference (BI) in BEAST v. 2.6.1 (Bouckaert *et al.*, 2019; see File S2 for the input file). We employed the partitioning scheme suggested by PartitionFinder using a linked tree, but leaving clock and site models unlinked. Instead of using the substitution models proposed by the likelihood-based method of PartitionFinder, we chose the model averaging method with transition-transversion split option and empirical frequencies as implemented in the BEAST package BMODEL-TEST v. 1.2.1 (Bouckaert & Drummond, 2017). We selected the calibrated Yule model and a relaxed clock with lognormal distribution and a clock rate of $1e^{-7}$ as tree and clock priors, respectively (Drummond *et al.*, 2006; Heled & Drummond, 2012). Calibration was achieved by assigning an age prior on the most recent common ancestor (MRCA) of all included taxa (Euphasmatodea) applying a normal distribution of 81 ± 7 approximating the time estimates of Simon *et al.* (2019). An additional MRCA prior was created to enforce monophyly

of Neophasmatodea, thus yielding Aschiphasmataidae as the outgroup.

The Markov chain Monte Carlo (MCMC) analysis was run for 100 000 000 generations, sampling every 5000 iterations. Convergence and effective sample sizes were assessed in TRACER v. 1.7.1 (Rambaut *et al.*, 2018). Trees sampled before reaching the equilibrium plateau (16%) were discarded as burn-in and remaining trees were summarized in a maximum clade credibility tree using TREEANNOTATOR v. 2.6.0 (BEAST package; Bouckaert *et al.*, 2019; refer to File S3 for the tree file in nexus format). The resulting tree was examined and edited in FIGTREE v. 1.4.4 (<https://github.com/rambaut/figtree>).

Ancestral range estimation

The geological history of Southeast (SE) Asia and the Southwest Pacific is quite complex. For estimating ancestral ranges, we first roughly grouped geographical areas in three sections following the division by Wallace's Line and Huxley's Line resulting in continental SE Asia West of Huxley's line, South Pacific SE Asia East of Wallace's Line and the Philippine Islands between both lines in the North (see Fig. 3). We further subdivided these areas based on prior information on their geological history (Hall, 2002; Lohman *et al.*, 2011) and their present position: Borneo + Palawan Island (B), Northern SE Asia including Cambodia, China, Japan, Taiwan, Northern Thailand and Vietnam (N), Southern SE Asia including Peninsular Malaysia, Sumatra and Southern Thailand (S), Eastern SE Asia including Buru Island, Seram Island and Viti Levu (E), the Northern Philippine Islands (PN) and the Southern Philippine Islands (PS) (Fig. 3).

Historical biogeographic inference was carried out with BIOGEOBEARS v. 1.1.2 (Matzke, 2013, 2018) as implemented in R 3.5.3 (R Core Team, 2019) using the six defined areas and the time-calibrated BEAST tree with outgroups removed. Every specimen was assigned to one area and the number of possible ranges was restricted to 18 by allowing a maximum of two adjacent areas to form a range except for the three continental areas (B + N + S) and the Philippine Islands with Borneo and Palawan (PN + PS + B) (Table S4). The analysis was performed under the Dispersal-Extinction-Cladogenesis model (DEC; Ree & Smith, 2008) as well as under the ML interpretations of the Dispersal-Vicariance (DIVALIKE; Ronquist, 1997) and the BayArea models (BAYAREALIKE; Landis *et al.*, 2013). We refrained from using the +j parameter associated with long distance dispersal and founder-event speciation due to recent criticism (Ree & Sanmartín, 2018). We evaluated the relative probability of each model based on the Akaike information criterion (AIC) model weights.

Species delimitation

As for most phasmatodean lineages, morphological variability and hidden diversity are also causing contentious species boundaries in Heteropterygidae. In an attempt to create an approximate

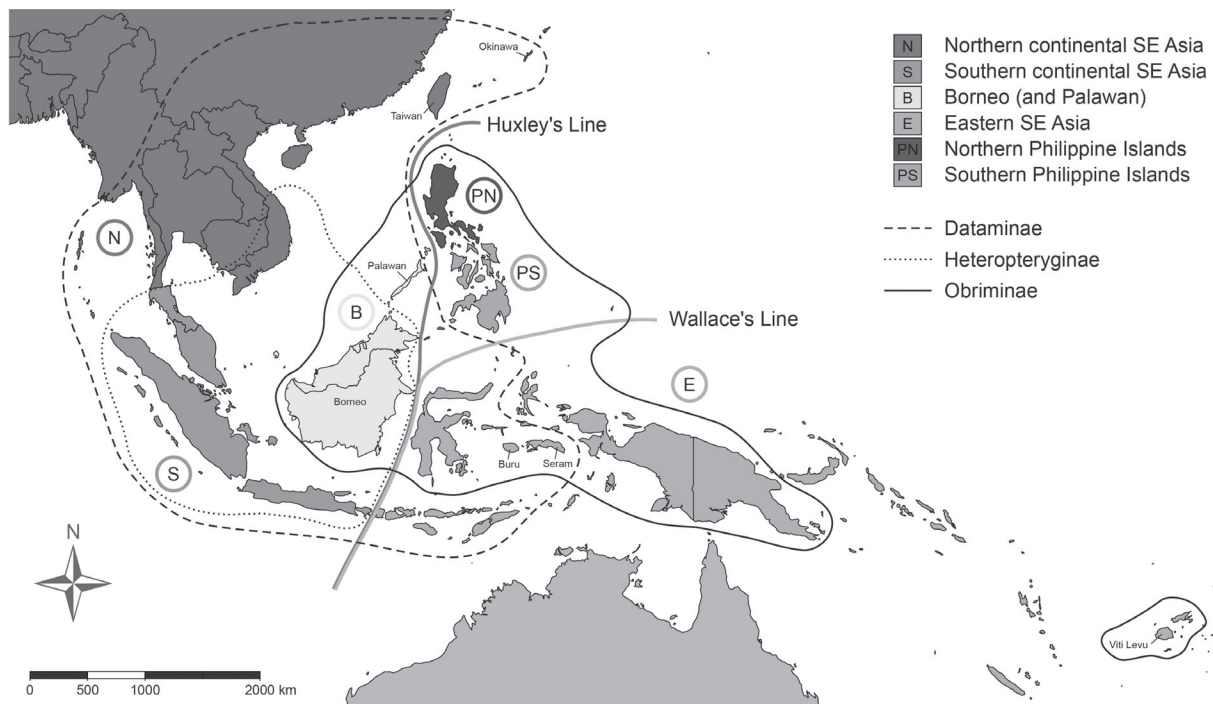


Fig. 3. Map of Southeast (SE) Asia including Huxley's and Wallace's Lines. Distribution of the three heteropterygid subfamilies are shown as solid line (Obriminae), dotted line (Heteropteryginae) and dashed line (Dataminae). The six geographical areas as used for historical biogeographical analysis are colour-coded according to the inserted caption on the right. [Colour figure can be viewed at wileyonlinelibrary.com].

reference point for the delineation of (molecular) species, we considered two independent approaches.

The Poisson Tree Processes (PTP) model determines putative molecular species based on the number of substitutions on a rooted, nonultrametric phylogenetic tree and the implemented bPTP version additionally calculates Bayesian support values (Zhang *et al.*, 2013). We used the ML tree inferred from the concatenated supermatrix, removed the outgroups and ran the bPTP analysis for 200 000 generations with a burn-in of 0.15 on the web server (<http://species.h-its.org/ptp>). Convergence of the MCMC chain was visually checked. In order to compare the suggested number of species, we also performed a multi-locus coalescent-based guide-tree approach using the trinomial distribution model in TR2 (Fujisawa *et al.*, 2016). We generated seven gene trees in IQ-TREE v. 1.6.10 (Nguyen *et al.*, 2015) and rooted them with Aschiphasmataidae. Since TR2 requires an ultrametric tree, we used the BI tree as guide tree. Outgroups were removed from gene trees and guide tree. The resulting tree was visualized in R using APE v.5.0 (Paradis & Schliep, 2018).

Results and discussion

Phylogenetic analyses

Heteropterygidae have been in the focus of attention in previous studies (Klante, 1976; Zompro, 2004; Hennemann *et al.*, 2016a), but the lack of formal cladistic analyses did not

allow for a definitive statement about their phylogenetic relationships. Our dataset with over 100 heteropterygid specimens presents the most comprehensive collection of molecular data used for any subgroup of Phasmatoidea so far.

All phylogenetic trees inferred from the partitioned concatenated supermatrix resulted in largely congruent topologies with varying but mostly moderate to strong node support. The monophyly of Heteropterygidae and its subgroups was confirmed, and all phylogenies consistently represent the heteropterygid relationships to be Dataminae + (Heteropteryginae + Obriminae). The ML trees with support values assessed using UFBoot (Figs. 4–6) and SH-aLRT (Fig. S1) share identical topologies and maximum support for the three heteropterygid subgroups Dataminae, Heteropteryginae and Obriminae. However, although Heteropterygidae were resolved with 99 and 100% using SH-aLRT and UFBoot, respectively, the node shared by Heteropteryginae and Obriminae was better supported with UFBoot (93%) than SH-aLRT (74%). The phylogenetic relationships inferred from the search for the best ML tree resulted in an almost identical topology with the only difference being the placement of *Haaniella scabra* Redtenbacher + *H. grayii* Westwood (Fig. S2). The standard nonparametric bootstrap support (BS), which was mapped on this tree is generally lower than observed with the other methods, but even a consensus tree generated from the 500 bootstrap trees presents itself with mainly the same topology (data not shown). Slight differences from the ML topologies were found in the BI phylogeny (Figs. S3, 7), especially within Dataminae and Heteropteryginae (see below).

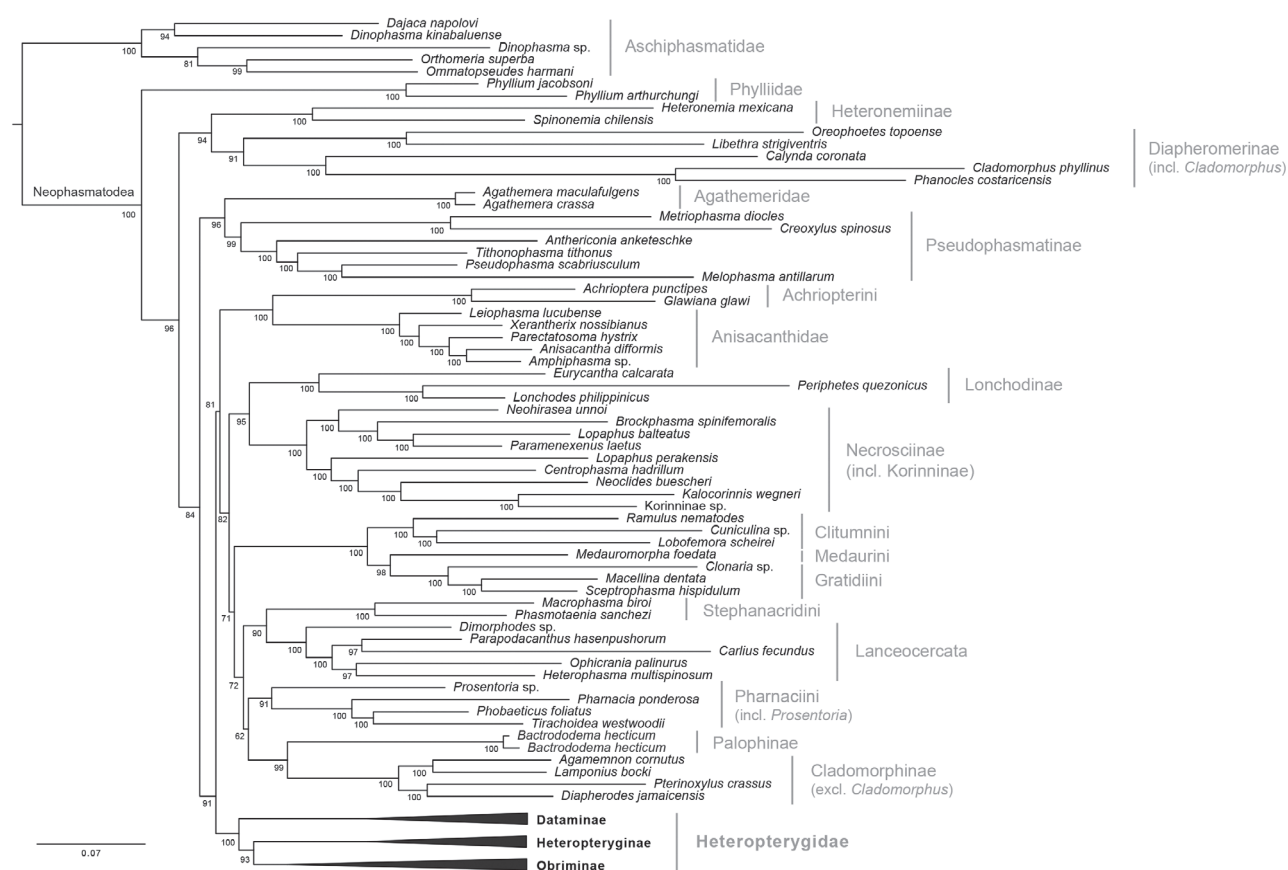


Fig. 4. First part of the ML phylogenetic tree inferred from the concatenated dataset featuring the outgroup taxa. The three subfamilies of Heteropterygidae are included. Support values (UFBoot) are given at each node.

Whereas Heteropterygidae and its subgroups were recovered with maximum support (BPP, Bayesian Posterior Probability = 1), the split of Heteropterygidae and Oribminae was only moderately supported (BPP = 0.85).

The results of the four-cluster likelihood mapping analysis substantiated the hypothesized relationships among Heteropterygidae as observed in the phylogenetic analyses (Fig. S4). The recovered sister group relationship of Heteropterygidae + Oribminae was favoured with 57%, whereas the alternative hypotheses presenting sister relationships of Dataminae + Heteropterygidae and Dataminae + Oribminae were weakly supported with 18.6 and 24.4%, respectively. The likelihood mapping conducted for each gene separately showed that only H3 sequence data was clearly favouring an alternative hypothesis with Dataminae + Oribminae (58.1%). The 16S data showed a higher level of support for the same alternative hypothesis with only 35.9%, whereas the principal hypothesis was supported by 32.4%.

Phylogeny and systematics

Our ML and BI topologies, based on a predominantly novel taxon sampling, largely corroborated clades repeatedly

recovered in past studies (Fig. 4) such as Aschiphasmata, Cladomorphinae, Clitumnini, Diapheromerinae, Gratidiini, Lanceocercata, Lonchodinae (comprising monophyletic Lonchodinae and Necrosiinae), a Malagasy clade (Achriopterini + Anisacanthidae), Medaurini, Pharnaciini, Phylliidae and Pseudophasmatinae (e.g. Buckley *et al.*, 2009; Bradler *et al.*, 2014, 2015; Robertson *et al.*, 2018; Simon *et al.*, 2019). We found no support for the recently established principal lineages Oriophasmata (Old World Phasmatodea) and Occidophasmata (New World Phasmatodea) (Simon *et al.*, 2019) in the ML trees, which is not surprising since standard Sanger sequencing data has been shown to be largely incapable of resolving the deeper nodes among Phasmatodea. Occidophasmata comprising Diapheromerinae, *Agathemera* Stål (= Agathemeridae) and Pseudophasmatinae, however, was recovered with overall good support in the BI phylogeny (BPP > 0.95; Fig. S3) substantiating the uncertain placement of *Agathemera* as sister to Pseudophasmatinae, which had only been moderately supported in the transcriptomic study (BS = 76; Simon *et al.*, 2019). In all phylogenies, we found the leaf insects (Phylliidae) to form the sister group of the remaining Neophasmatodea, a pattern observed already in a few studies before (Kômoto *et al.*, 2011; Bradler *et al.*, 2015; Robertson *et al.*, 2018). Albeit there were some noteworthy and well-supported novel results in our

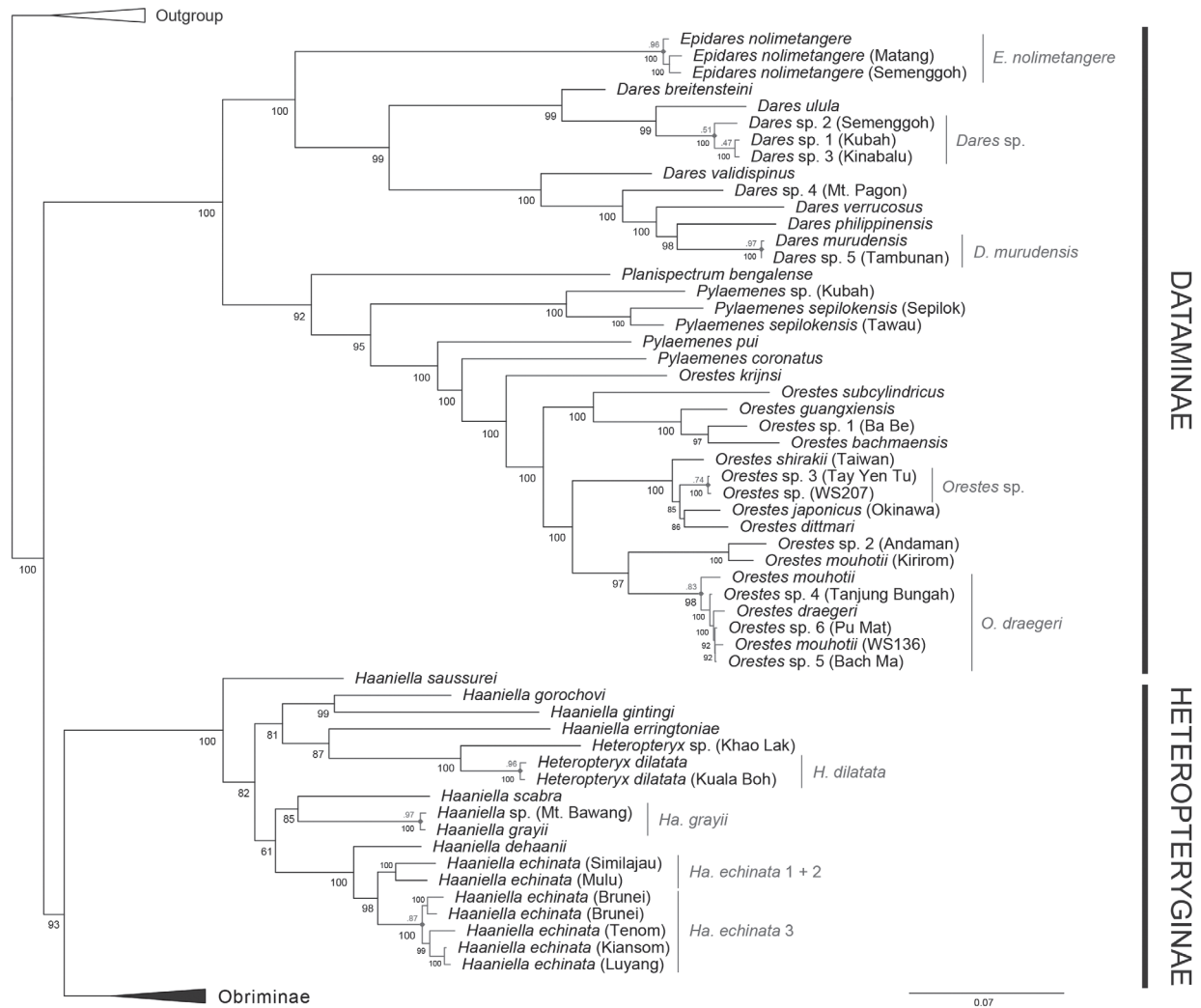


Fig. 5. Second part of the ML phylogenetic tree including the two heteropterygid subfamilies Dataminae and Heteropteryginae. Support values (UFBoot) depicted at each node. Red branches and diamonds at nodes show point of species delimitation according to the bPTP analysis. [Colour figure can be viewed at wileyonlinelibrary.com].

topology. The Brazilian *Cladomorphus phyllinus* Gray, eponym of the Cladomorphinae, clustered among the Diapheromerinae and appears to be unrelated to the remaining members of Cladomorphinae. Robertson *et al.* (2018) already demonstrated that another alleged member of the Cladomorphinae, *Otocrania* Redtenbacher, belongs to Diapheromerinae and assigned the taxon accordingly. These results highlight the need for a comprehensive taxonomic revision of these two New World lineages that are actually not closely related at all (Simon *et al.*, 2019). Within Cladomorphinae, *Pterinoxylus* Serville was recovered as sister taxon of *Diapherodes* Gray, which corroborates the topology of Robertson *et al.* (2018) and refutes the assumption by Hennemann *et al.* (2016b) that *Pterinoxylus* is sister taxon of Hesperophasmatinae (= *Hesperophasma* Rehn + *Agamemnon* Moxey in our analysis). The African Palophinae that were not represented in the sampling of Simon *et al.* (2019) are found to form the sister group of Cladomorphinae, which is another

result congruent with recent findings (Robertson *et al.*, 2018). We found further support for the subordinate placement of Korinninae within Necrosiinae (Goldberg *et al.*, 2015; Robertson *et al.*, 2018; Büscher *et al.*, 2018a) and confirmed that the yet to be described ootheca-producing taxon investigated by Goldberg *et al.* (2015) as a member of the Korinninae, since it is recovered with maximum support as sister taxon to *Kalocorinnis wegneri* Bragg. Within Necrosiinae, *Lopaphus* Westwood appears to be polyphyletic with *Lopaphus balteatus* Chen & He being recovered as sister taxon of *Paramenexenus laetus* Kirby with maximum support and unrelated to *Lopaphus perakensis* Redtenbacher. The males of *L. balteatus* and *P. laetus* furthermore share a strong synapomorphic character, a male vomer with four apical teeth (Bradler *et al.*, 2014). The taxonomic placement of *L. balteatus* needs to be further revised to confirm its potential congeneric status with *Paramenexenus*. We could not corroborate the Clitumninae,

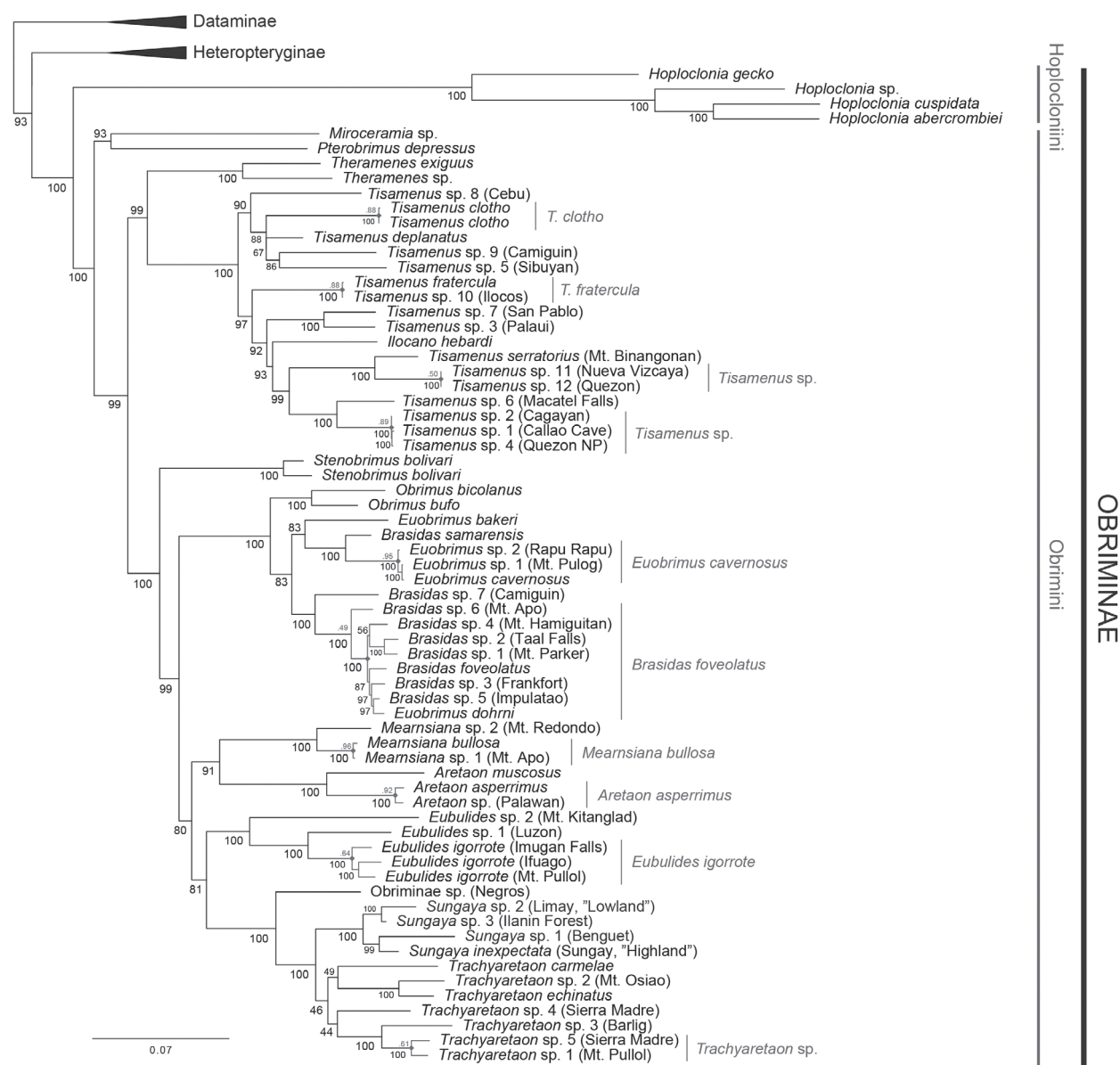


Fig. 6. Third part of the ML phylogenetic tree including the heteropterygid subfamily Obriminae and the newly introduced tribal division into Hoplocioniini trib. nov. and Obrimini sensu nov. Support values (UFBoot) depicted at each node. Red branches and diamonds at nodes show point of species delimitation according to the bPTP analysis. [Colour figure can be viewed at wileyonlinelibrary.com].

which were suggested to comprise Pharnaciini, Clitumnini and Medaurini by Hennemann & Conle (2008) and recently gained support by transcriptomic data (Simon *et al.*, 2019). As in numerous analyses before (Buckley *et al.*, 2009; Bradler *et al.*, 2014; Robertson *et al.*, 2018), Pharnaciini did not cluster with Clitumnini and Medaurini. Instead, Gratiidiini (not included by Simon *et al.*, 2019) appear to be sister group to Clitumnini + Medaurini, and the Clitumnini genus *Prosentoria* Brunner appears to be closer related to Pharnaciini. Bradler *et al.* (2015) recovered a clade comprising all four taxa, Clitumnini, Medaurini, Gratiidiini and Pharnaciini. Considering all these controversial results, the phylogenetic relationships of

these four taxa need further investigation in the future. In contrast, we were able to satisfactorily resolve some long-standing phylogenetic uncertainties in regard to Heteropterygidae.

In accordance with recent molecular analyses, our results reaffirmed that Malagasy Anisacanthinae are not part of Heteropterygidae as initially assumed based on morphology (Redtenbacher, 1906; Günther, 1953; Klante, 1976), but are in fact more closely related to other stick insects from Madagascar (Robertson *et al.*, 2018; Glaw *et al.*, 2019; Simon *et al.*, 2019). As mentioned above, we recovered Heteropterygidae as monophyletic with maximum support in both ML and BI phylogenies (Figs. 4, 7). In earlier molecular studies with fewer

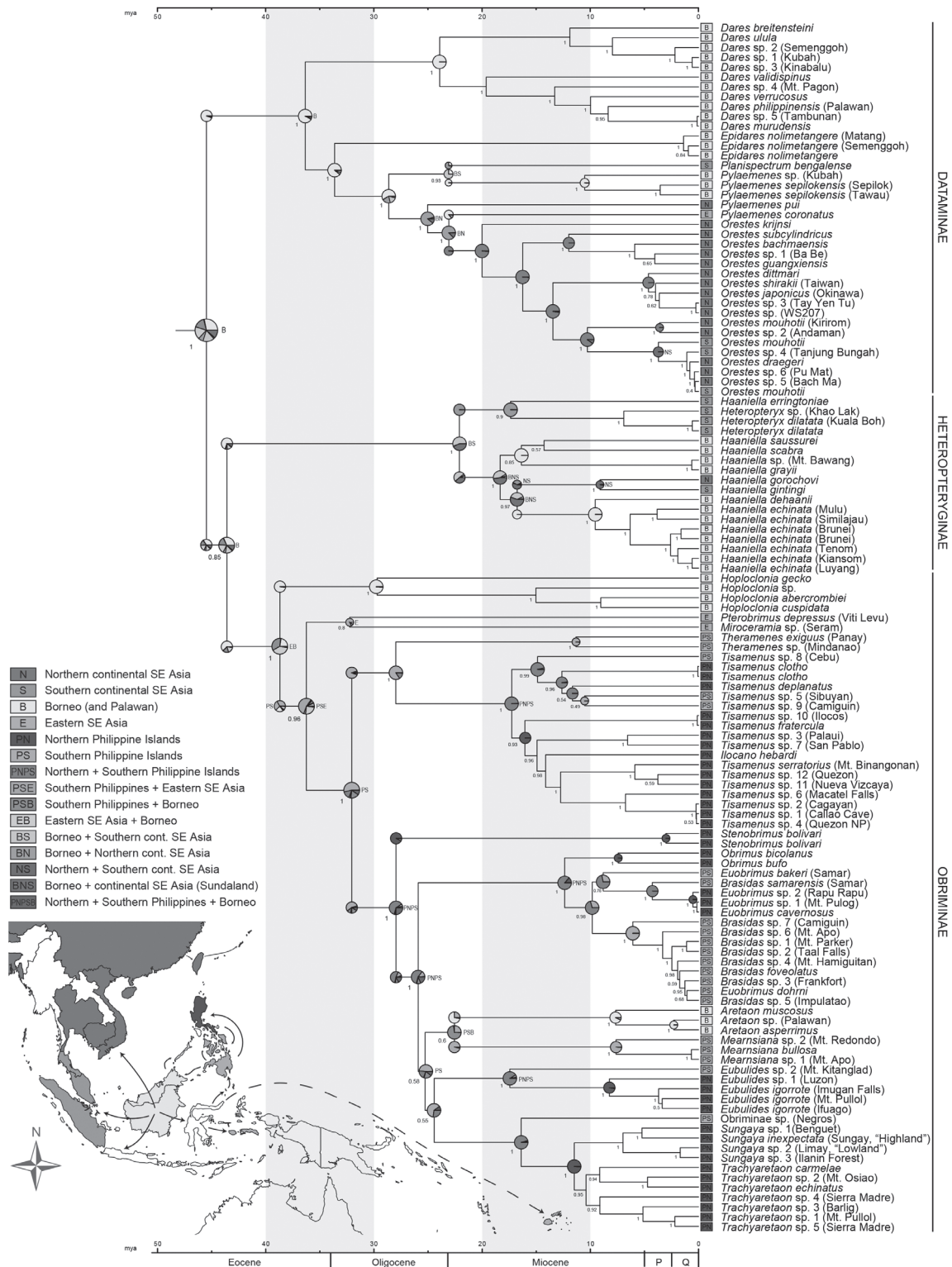


Fig. 7. Divergence times and estimated ancestral ranges for Heteropterygidae. Chronogram derived from BI in BEAST2 with posterior probabilities at nodes (see entire tree in Fig. S3). Inserted map depicts sampled countries in colour and the most likely colonisation routes. Colour code follows the inserted caption on the left. Range distribution of specimens is given at the tips of the tree. Pie charts with the likelihood for ancestral ranges estimated under the DEC model are presented at each node. For nodes with low probability, the most likely range is given in letters corresponding to the inserted caption. P, Pliocene; Q, Quaternary. [Colour figure can be viewed at wileyonlinelibrary.com.]

taxa included, Heteropterygidae were either recovered as not monophyletic at all (Whiting *et al.*, 2003; Buckley *et al.*, 2009; Forni *et al.*, 2021) or as a weakly supported clade (Bradler *et al.*, 2014, 2015; Goldberg *et al.*, 2015; Büscher *et al.*, 2018a). A larger taxon sampling obviously leads to a significantly more robust phylogeny as was also observed by Robertson *et al.* (2018) who recovered Heteropterygidae with 0.96 BPP.

The enlarged number of taxa also greatly increased support for the three heteropterygid subgroups. The placement of taxa with an inconclusive combination of morphological characters and controversial assignation is now well-supported. Our results confirmed the winged species of *Miroceramia* as a member of the Obriminae and not as previously assumed as member of the Heteropteryginae (Günther, 1953; Beier, 1968; Bradler & Galil, 1977; Bragg, 1998). In the otherwise flightless clade Obriminae, *Miroceramia* is the only volant representative and was found to be the sister taxon of *Pterobrimus*, the only obrimine member with wing remnants (Redtenbacher, 1906).

In addition to the well-supported heteropterygid subgroups, our increased taxon sampling further revealed Dataminae as sister group to Heteropteryginae + Obriminae. This topology has only been obtained once based on molecular data (Bradler *et al.*, 2015), whereas transcriptomic data failed to resolve this relationship yielding a weakly supported clade of Dataminae + Heteropteryginae (BS = 49; Simon *et al.*, 2019). Although the main transcriptomic data set was based on translated amino acid sequences, the analysis based on the nucleotide data set in fact recovered Heteropteryginae + Obriminae with slightly higher support (BS = 51). Despite that, our results are in accordance with morphological studies (Klante, 1976; Bradler, 2009) and further corroborate the heteropterygid phylogeny under the inclusion of the aforementioned genera with ambiguous morphological traits.

Evolution of morphological traits

Hennemann *et al.* (2016a) have already discussed morphological traits that support the sister group relationship of Heteropteryginae + Obriminae such as the spinose area apicalis. The number of ventral sensory areas is also mentioned in this context: Although Obriminae have two on the basisternite and Heteropteryginae have a central one on the furcasternite, all three are present in Dataminae (Redtenbacher, 1906; Hennemann *et al.*, 2016a). Our phylogeny substantiates that the presence of three sensory areas is a potential groundplan characteristic of Heteropterygidae, which was retained in Dataminae and independently reduced in Heteropteryginae and Obriminae. In contrast, other trait combinations have not been considered in detail by Hennemann *et al.* (2016a), for instance, the secondary ovipositor. Since this structure is lacking in Dataminae, they concluded that its presence in Heteropteryginae and Obriminae supports their close relatedness. However, Bradler (2009) clarified that the secondary ovipositor of *Hoplocloonia* (Obriminae) is formed dorsally by a different segment than in all remaining Obriminae as well as Heteropteryginae and questioned the taxonomic placement of *Hoplocloonia* and the single origin of

this structure. In fact, our topology recovered *Hoplocloonia* as sister group to all remaining Obriminae with strong support and is in consequence clearly refuting all previously established obrimine tribes (Zompro, 2004; Hennemann *et al.*, 2016a). *Hoplocloonia* has been traditionally regarded as closely related to *Ilocano* Rehn & Rehn and *Tisamenus* Stål (Zompro, 2004; Hennemann *et al.*, 2016a). Rehn & Rehn (1938) had even temporarily synonymized *Hoplocloonia* with the latter and recognized the lineage to be distinct from the remaining Obriminae. The possession of a secondary ovipositor dissimilar to that of all other Heteropterygidae (formed dorsally by the tenth abdominal segment instead of the epiproct, which corresponds to the tergum of the 11th segment) was already outlined by Redtenbacher (1906), but was obviously overlooked or even misinterpreted by subsequent authors (Günther, 1953; Bragg, 1998) until this knowledge was revived by Bradler (2009). Hennemann *et al.* (2016a) acknowledged this morphological difference, yet assumed the secondary ovipositor formed dorsally by the epiproct to be a synapomorphy of Heteropteryginae + Obriminae and the genus *Hoplocloonia* to be a subordinate taxon among Obriminae, which reduced the ancestral secondary ovipositor and evolved an alternatively formed novel one. Since no intermediate state between the two ovipositor types appears conceivable, this transformation is hard to explain. Moreover, the assumption of Hennemann *et al.* (2016a) is not supported by our phylogeny. With *Hoplocloonia* recovered as sister to all remaining Obriminae, we are able to propose a more reasonable and straightforward scenario: The secondary ovipositor that was primarily absent in Heteropterygidae (i.e. as in Dataminae) evolved three times independently within this group, (i) in *Hoplocloonia*, (ii) in the remaining Obriminae and (iii) in the Heteropteryginae, thereby convergently involving the epiproct in the two latter lineages.

Another feature is the peculiar presence of wings in Heteropterygidae. Although all Dataminae are entirely wingless and all Heteropteryginae have wings or wing remnants, there are only two-winged obrimine genera as mentioned above. *Pterobrimus* exhibits only wing remnants in the form of small, lobi-form tegmina (Redtenbacher, 1906), which prompted Rehn & Rehn (1938) to assume that *Pterobrimus* is not even related to the other Obriminae. *Miroceramia*, in contrast, has long tegmina and hind wings similar to those of the male of *Heteropteryx dilatata* (cf. Bradler, 2009), explaining its previously assumed position among the winged Heteropteryginae (Redtenbacher, 1906; Günther, 1953; Bradler & Galil, 1977; Bragg, 1998).

The well-developed wings in *Miroceramia*, males of *Heteropteryx dilatata* and some male *Haaniella* not included in this study (*H. mecheli*, *H. macroptera*, *H. parva*, *H. aculeata*) suggest the capability of active flight, whereas all remaining winged heteropterygids are brachypterous. Considering the canopy-dwelling *H. dilatata* female in an otherwise ground-dwelling clade, Bradler & Buckley (2018) proposed that a secondarily arboreal adaptation might have triggered an atavistic regain of wings. Our results place both volant lineages *Heteropteryx* and *Miroceramia* as subordinate taxa within otherwise flightless groups and as close relatives to partially winged taxa. Whether wings were lost in Dataminae

and Obriminae (excluding *Miroceramia* + *Pterobrimus*) or were primarily lacking in Heteropterygidae and re-evolved in Heteropteryginae and Obriminae is part of an on-going debate on the possibility of character regain (Collin & Miglietta, 2008). Whiting *et al.* (2003) proposed the possibility of wing regain in stick insects, which was controversially discussed and deemed unlikely by some (Stone & French, 2003; Telford & Budd, 2003; Trueman *et al.*, 2004; Goldberg & Igić, 2008). For the ancient lineage of ground-dwelling Heteropterygidae and sister group to all remaining Oriphasmata, we argue that wings might have been primarily absent and re-evolved in the common ancestor of Heteropteryginae and Obriminae, which has been most recently also corroborated by Forni *et al.* (2020), who analysed wing evolution across the whole phasmatodean diversity. The regain of wing structures in a ground-dwelling lineage was advantageous not necessarily for the purpose of flight but for the possession of a stridulatory or timbal organ, which is present in all winged females and partially winged males (Carlberg, 1989; Bradler, 2009; Hennemann *et al.*, 2016a). Although most obrimane lineages secondarily lost or reduced wings, further development of the wings involving the capability of flight has independently evolved in *Miroceramia* and male *Heteropteryx*. The aforementioned fully winged male *Haaniella* species (e.g. *H. macroptera*) are in fact morphologically similar to *H. erringtoniae* and might therefore be closely related to its sister taxon *Heteropteryx*. However, Forni *et al.* (2020) recovered *H. macroptera* as sister to brachypterous *H. erringtoniae* (referred to as *H. muelleri* therein). It is furthermore noteworthy that the male of *Heteropteryx* displays a set of tegmina that is rather unique among extant stick insects (Bradler, 2009) substantiating the hypothesis that an atavistic regain of an ancestral trait is possible in Phasmatodea (Whiting *et al.*, 2003).

Heteropterygidae systematics and taxonomy

As stated before, our results do not corroborate previous assumptions concerning the internal relationships of Obriminae that were divided into several tribes by past authors. Zompro (2004) introduced three tribes, the Miroceramiini (comprising the winged *Miroceramia* and the wingless *Mearnsiana*), Eubulidini (*Eubulides* Stål, *Heterocopus*, *Ilocano*, *Pterobrimus*, *Stenobrimus* Redtenbacher, *Theramenes* Stål, *Tisamenus*) and Obrimini (containing all remaining Obriminae genera). This arrangement was criticized by Hennemann *et al.* (2016a) who considered Zompro's tribes as being poorly supported. In light of our recovered phylogeny, we can confirm that none of the tribes Zompro (2004) established are monophyletic. For instance, *Mearnsiana* is not at all related to *Miroceramia*, and instead of being a high-ranking taxon among Obriminae the genus is rather subordinate as sister taxon to *Areataon* (Fig. 6). However, the new tribal arrangement proposed by Hennemann *et al.* (2016a) has not been corroborated by our data either. According to Hennemann *et al.* (2016a), Miroceramiini consists solely of *Miroceramia* and their newly proposed tribe Tisamenini comprises *Ilocano*, *Hoploclonia*, *Pterobrimus* and the eponymous *Tisamenus*. The latter are described by

the authors as the 'basalmost forms amongst the subfamily Obriminae with the two very closely related and exclusively Philippine *Tisamenus* Stål, 1875 and *Ilocano* Rehn & Rehn, 1939 being more basal than the Bornean *Hoploclonia* Stål, 1875' (Hennemann *et al.*, 2016a: 20). Despite 'basalmost' and 'basal' being inappropriate terms for describing taxa in a phylogenetic context in general (Krell & Cranston, 2004), Tisamenini must be refuted as being polyphyletic, as must their newly arranged Obrimini. Based on the compelling evidence provided here and before (Bradler, 2009; Robertson *et al.*, 2018), we can only recognize two tribes within Obriminae that reflect its two principal lineages of equal rank (as sister groups) as recovered before (Robertson *et al.*, 2018), consisting of *Hoploclonia*, corresponding to Hoplocloniini **trib. nov.** and all remaining Obriminae, forming the Obrimini **sensu nov.**

Hoplocloniini **trib. nov.**

<http://zoobank.org/D95474E2-4D6D-492B-B0B6-A4796FB0D53B>

Type genus. *Hoploclonia* Stål, 1875: 7397.

Diagnosis. The tribe Hoplocloniini **trib. nov.** is well-supported by molecular data (see above) and characterized also by a unique autapomorphic trait among Heteropterygidae found in the female sex: the possession of a secondary ovipositor (oviscapt) ventrally formed by the operculum (abdominal sternum 8) and dorsally by the longitudinally deeply incised abdominal tergum 10 (Bradler, 2009: Fig. 15c therein).

Included taxa. Hoplocloniini **trib. nov.** only contains the genus *Hoploclonia* with currently three described and one undescribed species from Borneo.

Obrimini, Brunner v. Wattenwyl, 1893 **sensu nov.**

Type genus. *Obrimus* Stål, 1875: 7431.

Diagnosis. The tribe is well-supported as monophyletic within Obriminae with females whose secondary ovipositor is dorsally formed by the enlarged epiproct (tergum of abdominal segment 11).

Included taxa. The Obrimini **sensu nov.** comprise the bulk of species of Obriminae including *Areataon*, *Brasidas*, *Eubulides*, *Euobrimus*, *Mearnsiana*, *Miroceramia*, *Obrimus*, *Pterobrimus*, *Stenobrimus*, *Sungaya*, *Theramenes*, *Tisamenus*, *Trachyareataon* and probably also *Heterocopus* that could not be included in this study.

Also in accordance with the study of Robertson *et al.* (2018), *Miroceramia* + *Pterobrimus* are recovered as sister group to all other, predominantly Philippine Obrimini. The latter clade can be further divided into the sister lineages *Theramenes* + *Tisamenus* (including *Ilocano*) and *Stenobrimus* + all remaining Obrimini genera. The latter comprises two major groups of taxa: (i) *Brasidas*, *Euobrimus* and *Obrimus*, a clade already suggested before based on the presence of metasternal pseudoforamina (Zompro, 2004) and (ii) *Eubulides* + (*Sungaya* + *Trachyareataon*) and *Areataon* + *Mearnsiana*. We corroborated most currently recognized genera of Obriminae

as being monophyletic, albeit with uncertainty regarding the taxonomic boundaries of *Euobrimus* and *Brasidas* that need further investigation. Moreover, we clearly demonstrate that the monotypic *Ilocano* is deeply nested within the species-rich *Tisamenus*. Since our phylogeny renders *Tisamenus* paraphyletic, we hereby transfer *Ilocano hebaridi* to *Tisamenus*, establishing *Tisamenus hebaridi* **comb. nov.** and *Ilocano* becoming a junior synonym of *Tisamenus*.

The Dataminae and Heteropteryginae have never been divided into tribes, yet there are some well-defined internal clades present. Within Dataminae, it remains unclear whether the predominantly Bornean *Dares* (BI tree) or a clade formed by *Dares* + *Epidares* (ML tree) are the sister group to all remaining genera, with the latter also recovered by Robertson *et al.* (2018). *Pylaemenes* appears to be paraphyletic according to our analyses with the species from mainland Asia and Wallacea forming two distinct lineages unrelated to the Bornean species. This issue might be elucidated by the inclusion of further datamine species from other parts of the Greater Sunda Islands (e.g. Sumatra, Java). The monophyly of *Orestes* could be confirmed and the position of *Orestes krijnsi* as sister taxon to all remaining species is in fact coinciding with morphological analysis of the egg, which is distinct to other *Orestes* (Bresseel & Constant, 2018). Heteropteryginae consists of two currently recognized genera *Haaniella* and *Heteropteryx* of which the former appears to be paraphyletic with some *Haaniella* spp. closer related to *Heteropteryx* as demonstrated before. This clearly contradicts current views on the monophyly of *Haaniella* (Zompro, 2004; Hennemann *et al.*, 2016a) and has to be further investigated under inclusion of the type species *Haaniella muelleri* Haan.

Species delimitation

The two approaches we applied yielded different species delineations (Figs. 5, 6, S5). Although each method was based on either the BI or the ML phylogeny, we deemed the analyses to be comparable, since most topological differences affected deeper nodes and not the specimens in question. The trinomial distribution model used by tr2 delineated significantly fewer species than PTP/bPTP. Evidently, accurate calculations could not be performed in many cases, thus many nodes remained unresolved, especially those involving short terminal branches (hash signs; see Fig. S5). The poor performance of the software, which is built to accept hundreds of loci, is probably due to the low number of gene trees and their varying amounts of species coverage. Additionally, the lack of information and the accumulation of unresolved splits caused the software to excessively merge otherwise separate species.

We assumed the analysis performed under the bPTP model to be more reliable, since it is based on the differences in the number of substitutions and therefore rather independent of missing data. We conclude that this method is a better fit for our data, since the results show only few ambiguously resolved species boundaries. In total, a sensible number of 88 molecular species was estimated (Figs. 5, 6) with four instances where the support to merge or split the species was only slightly higher

than the alternative (*Brasidas* sp. 6, *Dares* sp. 2, *Sungaya* sp. 2 + 3 and *Tisamenus* sp. 11 + 12; see Table S5).

Within Dataminae, the *Epidares* individuals from different localities with varying colouration and armature represent one single species, highlighting once more the enormous intraspecific disparity a species can exhibit. We reveal two undescribed *Dares* species, one from Mt. Pagon (Brunei) and one with specimens from several localities, which is recovered as a distinct species from its sister taxon *Dares ulula* Westwood. Furthermore, the *Dares* sp. individual from Tambunan is conspecific with *Dares murudensis* Bragg. The two specimens identified as *Pylaemenes sepilokensis* Bragg from Sepilok and Tawau are revealed not to be conspecific. The *Orestes* sp. from the Tay Yen Tu Nature Reserve (Vietnam) appears to be identical to the *Orestes* sp. listed as being from India by Robertson *et al.* (2018), which raises doubts on the locality given by these authors. Several specimens allegedly representing *Orestes mouhotii* Bates do not cluster together, most probably because of erroneous identifications in previous studies (Kômoto *et al.*, 2011; Goldberg *et al.*, 2015; Robertson *et al.*, 2018). The true *O. mouhotii* is represented by an individual from the Kirirom National Park (Cambodia; Bresseel & Constant, 2018), which is the sister species of a yet undescribed *Orestes* species from the Andaman Islands. The erroneous remaining *O. mouhotii* individuals are in fact members of *O. draegeri* Bresseel & Constant, as are the *Orestes* spp. members from Tanjung Bungah, and Pu Mat and Bach Ma National Park. A potentially new species is represented by the *Orestes* sp. from Ba Be National Park in Northern Vietnam.

Within Heteropteryginae, our most spectacular discovery is that of a second Jungle Nymph species of the hitherto monotypic *Heteropteryx* from Khao Lak (Thailand) that is genetically clearly separated from *H. dilatata* occurring in Peninsular Malaysia. In *Haaniella*, we reveal a high degree of cryptic diversity among the Bornean individuals that were identified as *H. echinata* Redtenbacher, suggesting the existence of three separate species in this lineage (one from Brunei and Sabah (Malaysia), and two from Sarawak (Malaysia): Mulu National Park and Similajau National Park). The *Haaniella* sp. from Mt. Bawang (West Kalimantan) was recovered to be identical to *H. grayii*.

Within Oribriminae, we corroborate the view of Robertson *et al.* (2018) that *Hoploclonia abercrombiei* Bragg and *H. cuspidata* Redtenbacher are separate species (in contrast to Seow-Choen, 2016) and reveal a further presumably undescribed *Hoploclonia* sp. from Mt. Pagon (Brunei). Among the numerous species and forms of *Tisamenus* (including the former *Ilocano* **syn. nov.**), we analysed 18 individuals that represent 13 different species. The specimens from isolated Philippine Islands such as Camiguin, Sibuyan, Cebu, Palau were all confirmed as separate species. Since no exhaustive taxonomic comparison with type specimens was conducted, the species revealed here do not necessarily represent undescribed taxa. The two specimens we included as *Stenobrimus bolivari* in our analysis are probably comprising two distinct species. *Euobrimus* sp. 1 (Mt. Pulog, Bicol) and sp. 2 (Rapu-Rapu Island) appear to be conspecific with *E. cavernosus* Stål from

Mt. Pulog (Bicol, Luzon). Among *Brasidas*, we found *Brasidas* spp. 1–5 (and potentially sp. 6) and *Euobrimus dohrni* Rehn & Rehn to represent *Brasidas foveolatus* Redtenbacher, whereas *Brasidas* sp. 7 from Camiguin Island was found to be likely a new species. *Mearnsiana* is represented by two species, with *Mearnsiana* sp. 1 from Mt. Apo (Mindanao) confirmed to be *M. bullosa* Rehn & Rehn and *Mearnsiana* sp. 2 from Mt. Redondo (Dinagat Island) representing a new, undescribed species for this so far monotypic genus. In contrast, the *Aretaon* sp. from Palawan appears to be the same as *Aretaon asperrimus* Redtenbacher, thus revealing a range extension for this species. The three forms of *Eubulides igorrote* Rehn & Rehn have been confirmed as one species, however with low support (64%) when compared to other splits in our analysis. The presently monotypic *Sungaya* is revealed to comprise three, maybe even four different species. Although the originally described asexual population of *S. inexpectata* Zompro from Sungay and the sexual population from Benguet represent most probably different species, there is only little support for the individuals from Limay and Ilanin Forest to be conspecific (51%, see Table S5). In any case, the male from Limay described as the alleged male of *S. inexpectata* by Lit & Eusebio (2008) is based on a specimen pertaining to a yet undescribed *Sungaya* species. All included *Trachyaretaon* spp. appear to represent distinct species, with *Trachyaretaon* sp. 1 (Mt. Pullol, Luzon) and sp. 5 (Sierra Madre, Luzon) probably being conspecific (61%). The results from the presented species delimitation analysis will hopefully serve as a helpful and reliable foundation to facilitate numerous future taxonomic descriptions.

Historical biogeography

The DEC biogeographical model, featuring parameters for narrow and subset sympatry as well as narrow vicariance, was inferred as best-fitting model for ancestral range estimation based on the time-calibrated BI tree (see Table S6 and File S4 for details). The divergence time analysis estimated the radiation of Heteropterygidae to have started ~45.5 mya (56.1–34.9 mya; Fig. S3), a range of dates similar to previous estimates derived from fossil dating (Bradler *et al.*, 2015; Robertson *et al.*, 2018; Simon *et al.*, 2019). These were however recently challenged by Tihelka *et al.* (2020), who proposed a significantly older origin – an age that cannot to be satisfactorily determined from the data they provided but which we assume to be approximately 90 mya for this clade (misspelled by the authors as ‘Heteropterygida’). This discrepancy can be partly explained by the usage of different sets of fossils as calibration points. Tihelka *et al.* (2020) included fossils that were intentionally excluded in the study by Robertson *et al.* (2018), such as *Echinosomiscus primitivus* Engel & Wang, a fossil insect preserved in Cretaceous amber (~99 mya) described as an adult male related to a subordinate lineage comprising Lonchodinae and Clitumninae (Engel *et al.*, 2016). However, this extremely small fossil most probably does not belong to Phasmatodea at all (Bradler & Buckley, 2018) and was used as calibration point for Phasmatodea or Euphasmatodea (Simon *et al.*, 2019; Forni *et al.*, 2020;

Forni *et al.*, 2021), whereas Tihelka *et al.* (2020) included it as calibration point within the much more subordinate Oriophasmata. Another important fossil specimen included by Tihelka *et al.* (2020) is a Jurassic heelwalker (Mantophasmatodea) described by Huang *et al.* (2008) that needs to be critically reassessed. Bradler & Buckley (2011) emphasized the importance of rigorously interpreted and unambiguously placeable fossils as reliable calibration points on phylogenetic trees. In addition to the usage of different fossils, also the varying definition of upper bounds appears to result in incongruent divergence times estimations. Although the rising discussion on the contradictory phasmatodean divergence times deserves further attention (see also Forni *et al.*, 2021 for another significantly older estimation), a full-fledged analysis and discussion of this topic is beyond the scope of the present study. Since the general divergence times of extant Euphasmatodea estimated by Simon *et al.* (2019) based on phasmatodean fossils largely correspond to those obtained by Misof *et al.* (2014) based on a broad array of nonphasmatodean fossils across all hexapod lineages (but see Tong *et al.*, 2015 and Kjer *et al.*, 2015 for a critical discussion), we consider our time estimation to be a scientifically sound basis for reconstructing the Heteropterygidae radiation.

Ancestral range estimates under the DEC model favoured the common ancestor of Heteropterygidae to have originated in Borneo (Fig. 7). Borneo, however, was part of continental Sundaland, an expansion of the South Asian peninsula joining the Thai-Malay Peninsula, Western Indonesia and Borneo. Despite sea level fluctuations, Sundaland, namely the geographic areas B + N + S (Borneo, Northern and Southern SE Asia; Fig. 3), was considered as one connected terrestrial area from Eocene to Early Miocene (Hall, 2002). Hence, we assume the ancestral range of Heteropterygidae and its early descendants to be Sundaland, neglecting any inconsistencies caused by the ancestral range estimates.

Diversification of Dataminae was estimated to have started in Borneo (=Sundaland) between 45.6 and 28.1 mya (36.4 mya). Our analysis suggests that dispersal may have happened primarily to the North and East of Sundaland. The split between lineages distributed in the North and in Borneo can be explained by the advancing South China Sea, separating continental Asia from present Borneo since the Late Oligocene (Hall, 2002; Hall, 2013). Subsequent dispersal was mainly via temporarily available land bridges between adjacent areas such as Borneo or the Malay Peninsula (*Planispectrum bengalense* Redtenbacher) or continental Asia and Taiwan and Okinawa (*Orestes*). The invasion of Wallacea by *Pylaemenes coronatus* Haan (and other species not included here) may be attributed to long-distance dispersal, but remains unclear due to the lack of data.

The divergence of Heteropteryginae and Obriminae occurred in Sundaland in the Middle Eocene (~43.6 mya; 54.0–33.4 mya) causing the former lineage to occupy the Southwestern region and the latter to colonize the East. Extant species of Heteropteryginae began to radiate in the early Miocene (~22.1 mya; 28.2–16.0 mya) and split into two lineages, of which one dispersed to Southern continental SE Asia and the other diversified in Borneo, including a secondary colonisation of Southern and Northern SE Asia by *Haaniella*

gintingii Hennemann et al. and *H. gorochovi* Hennemann et al. Note that these two species are more closely related to the other non-Bornean species in the ML tree, thus resulting in two geographically separated heteropterygine clades (Fig. 5).

The reconstruction of ancestral ranges for Obriminae is far more challenging, since the geological history of the involved regions of oceanic SE Asia and Southwest Pacific counts among the most complex (Hall, 2002; Schellart *et al.*, 2006). Countless land connections and separations as well as constant shifts of terrestrial areas render numerous alternative dispersal routes (Hall, 1998). The ancestral distribution of Obriminae was estimated to be Borneo or Eastern SE Asia and may be interpreted as Eastern Sundaland. Hoplocloiniini diverged from Obrimini between 48.3 and 29.6 mya (~38.7 mya) and remained in Borneo, whereas Obrimini expanded to the East and may have colonized Western Sulawesi before its separation from Borneo and the formation of the Makassar Straits in the Middle Eocene (Hall, 2002, 2009, 2013). The ancestral range of Obrimini is however estimated to be Southern Philippines and Eastern SE Asia corresponding to the divergence into a Philippine and a Wallacean clade. The lineage splitting off at ~36.3 mya (44.68–27.23 mya) to disperse eastwards and colonize regions from Wallacea to the Southwest Pacific, is comprised of *Miroceramia* (Sulawesi + Seram) and *Pterobrimus* (Fiji), whose capability of flight was likely to have facilitated the dispersal using locally emergent land as stepping stones. Hennemann *et al.* (2016a) questioned the type locality of *Pterobrimus* being Fiji, but here we reaffirm that the *Pterobrimus* specimen used here and before by Buckley *et al.* (2009) was collected by Daniel Otte (Philadelphia) on Viti Levu (Fiji). The dispersal to Fiji may be explained by long-distance dispersal and founder-event speciation, yet, our divergence time estimates coincide with the existence of the Vitiaz arc, a continuous volcanic arc extending from the Philippines to Fiji in the Late Eocene to Early Oligocene (Rodda, 1994; Hall, 2002). The Vitiaz arc has likely contributed to biotic migration processes across the whole Southwest Pacific (Oliver *et al.*, 2018) and has already been considered to explain the colonisation of Fiji (e.g. Duffels & Turner, 2002; Liebherr, 2005). The geological dynamics of the fractured landmasses might have triggered diversification of the obrimine Wallacean lineage and its distribution across the entire arc (Oliver *et al.*, 2018), but subsequently led to the extinction of most species. Consequently, the few known species presently show a disjunct distribution, yet, represent the oldest lineage within our taxon sample (32.2 mya; 40.2–22.0 mya).

For the obrimine Philippine clade, we inferred one invasion of the Philippine Islands, which gave rise to a remarkable radiation resulting in the most species-rich lineage of Heteropterygidae. However, recovering the route and order of colonisation is difficult due to the oceanic and tectonic origin of the islands (Hall, 2002) and, correspondingly, the proposal of numerous alternative scenarios regarding their reconstruction. In this regard, several potential colonization routes have been illustrated (Esselstyn & Oliveros, 2010; Matsui *et al.*, 2010; Lohman *et al.*, 2011; Brown *et al.*, 2013; Kyriazis *et al.*, 2018; Huang *et al.*, 2019), but the most common routes via Palawan or the Sulu archipelago are not compatible with our data, since

they only affect more recent invasions from Borneo during the Late Miocene and the Pliocene. Our data, however, suggests a colonization event from the South, potentially from Sulawesi, which was temporarily close to the Southern Philippine Islands during the Oligocene (Hall, 2002; King & Ebach, 2017). The Sangihe-Talaud archipelago, an island arc similar to the fragmental landmasses of the Vitiaz arc, was already suggested to have served as stepping stones for other terrestrial animals (Evans *et al.*, 2003; Lohman *et al.*, 2011; Setiadi *et al.*, 2011). Alternatively, the ancestral range shared by Obrimini in the Late Eocene was the Vitiaz arc itself, which permitted the Wallacean lineage to disperse eastwards and the Philippine clade to disperse North. The presence of one representative of Obrimini (*Theramenes olivaceus* Westwood, not included in this study) on the Talaud Islands is an indication for the probability of this route and the inclusion of this species within a biogeographic framework might illuminate the ancestral distribution of the clade even further. Within the Philippine clade, the genus *Theramenes* has a Southern distribution and is sister group to members mostly from the Northern island of Luzon. Generally, we observe a distributional pattern from South to North among most lineages as well as at least two secondary invasions to the South. Although the biogeographic analysis estimated a joining of the island groups in the Oligocene, geological reconstructions suggest that the proximity of the current configuration of the Northern and Southern landmasses was not reached until the Late Miocene (Hall, 2002). Hence, diversification of most lineages probably started before the colonization of the North and dispersal among the entire archipelago occurred more recently, most likely during sea-level lowstands in the Pliocene and Pleistocene. We also propose that the invasion of Borneo by *Aretaon* did not occur in the Early Miocene as estimated, but instead via the fragmental Sulu island arc in the Middle/Late Miocene (Hall, 2013). The dispersal to Palawan occurred subsequently from Borneo during low sea levels in the Pliocene or Pleistocene.

Nonadaptive radiation

Stick and leaf insects repeatedly underwent adaptive radiations as demonstrated in the past for various geographic areas, often islands, such as Madagascar, the Mascarene archipelago, New Caledonia and New Guinea (Buckley *et al.*, 2009, 2010; Bradler *et al.*, 2015; Simon *et al.*, 2019). These radiations are characterized by extensive niche differentiation accompanied by morphological and behavioural adaptations that regularly misled previous scientists who underestimated the degree of evolutionary convergence exhibited by phasmatodeans. For instance, the Madagascan radiation includes slender giant-sized, winged canopy-dwellers such as *Achrioptera* and closely related sturdy, flightless ground-dwelling ecomorphs such as *Parectatosoma* (Glaw *et al.*, 2019), the latter originally assumed to be a subgroup of Heteropterygidae (Klante, 1976). In contrast, the Heteropterygidae are generally uniform ground-dwelling ecomorphs exhibiting a stocky body form, predominantly brownish colouration, inability of flight (with exception of male *Heteropteryx*, long-winged *Haaniella*

spp. and possibly *Miroceramia*) and consistent egg deposition into the soil, which is a derived strategy applied by comparatively few taxa (Goldberg *et al.*, 2015; Robertson *et al.*, 2018). This oviposition strategy is mostly achieved by the use of the aforementioned secondary ovipositors in Heteropteryginae and Obriminae (see video, File S5) or by burying the egg with the fore legs after an acrobatic shot between the antennae (see video, File S6) as also described by Abercrombie (1992). Yet, the Heteropterygidae vary significantly in body size, with Dataminae being comparatively small phasmatodeans whereas Heteropteryginae can be impressively large. The tarsal attachment microstructures have been suggested to be partly associated with body size in Heteropterygidae, although the nonuniformity of this trait may be indicative of the occupation of different niches (Büscher *et al.*, 2018a, 2018b, 2019) as they reflect adaptations towards different substrate conditions (Büscher & Gorb, 2019; Büscher *et al.*, 2020). Nevertheless, no notable niche differentiation appears to have occurred in Heteropterygidae, the only exception being *Heteropteryx* whose green, leaf-imitating females and flighted males adapted to a secondary tree-dwelling life style (Bradler & Buckley, 2018). Whether *Miroceramia* retained a ground-dwelling life style despite the well-developed wings is not known, since no ecological information was ever documented. A change in colour as in *Heteropteryx* females has however not occurred (Bates, 1865; Günther, 1934).

Our results emphasize the nonadaptive radiation of Heteropterygidae and that speciation was mainly driven by allopatry (or parapatry) due to jump dispersal events and colonization of new areas. Unlike adaptive radiations, the allopatric populations continued to occupy the same niches and retained the ancestral ecological traits (Gittenberger, 1991; Rundell & Price, 2009). Poor dispersers have generally a higher potential for allopatric and nonecological speciation, especially when occupying disruptive landmasses (Czekanski-Moir & Rundell, 2019). This is particularly true for Obriminae, which dispersed across highly fragmented and isolated areas (Philippine Islands) resulting in the highest diversity among heteropterygids. In contrast to nonadaptive radiations associated with cryptic diversity (e.g. Kozak *et al.*, 2006; Slavenko *et al.*, 2020), Heteropterygidae have undergone slight trait diversification. However, these morphological differences have possibly accumulated after speciation and derived from minor ecological differences in the respective microhabitats in vicariant isolation (Rundell & Price, 2009). Additionally, phenotypic divergence might have occurred via character displacement as ranges of allopatric species overlapped (Brown & Wilson, 1956). Shifts in body size in Dataminae and Heteropteryginae may have resulted from (secondary) co-occurrence, a pattern observed in allopatric as well as sympatric animal species (Moritz *et al.*, 2018). Although the morphospace among heteropterygids is rather uniform, the potential for disparification appears to be generally common. In fact, some conspecific populations have been suggested to be distinct species but were here recovered as phenotypic variations with minimal genetic difference (e.g. *Epidares nolimetangere* Haan, *Eubulides igorrote*, *Haaniella echinata*). The phenotypic disparity among extant heteropterygid species may even be explained by ancestral plasticity (Levis & Pfennig, 2016;

Czekanski-Moir & Rundell, 2019) that was not caused by adaptations to new environments, a process that is difficult to assess and to discern from alternative or concurrent evolutionary mechanisms. Future extensive morphological examinations are crucial to fully understand the evolutionary patterns that shaped the nonadaptive radiation of Heteropterygidae.

Conclusions

Our study demonstrates that a comparatively small genetic data set of a limited number of loci may still resolve phylogenetic questions regarding the relationships of evolutionary younger lineages even in the era of phylogenomics. Considering that the recovered phylogenetic relationships of Heteropterygidae are in conflict with previous molecular phylogenies based on fewer taxa, but agree with hypotheses and analyses based on morphological characters, the usage of an increased and comprehensive taxon sampling proved essential to obtain optimal support. The historical biogeography of Heteropterygidae and its ancestral range in Sundaland (mainland Asia) coincides with the origin of Oriphasmata in the Oriental region. Despite their low vagility, heteropterygids have expanded their distribution across the Oriental and towards the Australian region during a relatively short time period and provide an example of potential dispersal routes applicable to other Oriphasmata lineages such as Lanceocercata, Lonchodinae or Phylliidae. In contrast to other phasmatodean lineages, diversification in Heteropterygidae resulted from allopatric speciation without subsequent ecological divergence (nonadaptive radiation). Therefore, morphological and behavioural traits associated with adaptations to the forest floor were essentially retained. The occurrence of disparate traits among conspecific individuals in Heteropterygidae is not uncommon and may provide an insight into preceding nonadaptive processes leading to diversification via character displacement. Having provided a basis for species delineation, future studies should focus on morphological examination to assess whether a specific morphological trait lies within the scope of phenotypic disparity or delimits species' boundaries. Ultimately, the combination of morphological and molecular analyses will further improve our understanding of trait evolution and diversification in nonadaptive radiations and how these evolutionary processes shaped the diversity of these lineages.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Phylogenetic relationships inferred from the partitioned concatenated supermatrix. Support values were assessed with a single branch test (SH-aLRT) using 1000 replicates rounded to whole numbers.

Figure S2. Phylogenetic relationships resulting from the tree search based on the partitioned concatenated supermatrix.

Node support was independently assessed using 500 standard nonparametric bootstraps summarized and mapped on the best-scoring ML tree.

Figure S3. Time-calibrated BI phylogeny inferred from the partitioned concatenated supermatrix. Support values corresponding to Bayesian posterior probabilities are indicated at each node.

Figure S4. Likelihood mapping plots resulting from the four-cluster likelihood mapping analysis for the concatenated dataset and for each single gene. The three areas of a triangle show the support for the possible groupings. A sister group relationship of Heteropteryginae and Obriminae is supported in most cases.

Figure S5. Species delimitation analyses. The results of the trinomial distribution model using tr2 are mapped on the BI tree and putative species are depicted in grey boxes. Positive values imply interspecific relationships, whereas negative values suggest the clade to be one species. Hash sign (#) illustrates nodes with insufficient data. Asterisk (*) shows the best position of delimitation suggested by tr2. On the right, species as delineated by PTP are depicted (more detailed in Figs. 5, 6).

Table S1. Taxon and geographical information of each specimen and GenBank accession numbers for each gene used in this study. Accession numbers MN924966–MN925870 were newly generated in this study. Empty cells imply absence of available DNA sequence. Specimens in alphabetical order.

Table S2. Primers and corresponding information used in this study

Table S3. Best partitioning scheme (subsets) and best-fit substitution models determined by PartitionFinder v.2.1.1.

Table S4. Input for the BioGeoBEARS analysis. List of all heteropterygid specimens and their distribution coded in six geographical areas. Allowed ranges are as follows: PN, PS, E, B, N, S, PN + PS, PN + B, PS + E, PS + B, E + B, E + S, B + N, B + S, N + S, PN + PS + B, B + N + S and null-range.

Table S5. Results from species delimitation analysis using PTP and bPTP.

Table S6. Results of biogeographic model comparison in BioGeoBEARS.

File S1. Supermatrix of the final concatenated alignment (seven genes, 5343 bp) in Fasta format.

File S2. Input (xml) file for BEAST2 created in BEAUti.

File S3. Phylogenetic tree and PP values as depicted in Figs. 7, S3 with divergence times and confidence intervals in Nexus format.

File S4. Results of ancestral range estimation (BioGeoBEARS) including most likely ancestral ranges and probability pie charts for each of the three tested biogeographic models DEC, DIVALIKE and BayAreaLIKE.

File S5. Video of oviposition in Obriminae (*Tisamenus* sp. 'Cagayan'). The beak-like secondary ovipositor is used to insert the egg(s) into the soil.

File S6. Video of oviposition in Dataminae (*Epidares nolimetangere*). The female is digging in the soil with the forelegs. The abdomen is pointed upward. An egg is shot from the ovipositor right between the antennae from which it is then gently removed and deposited in the soil with the forelegs.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article

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