Too early for the ferry: The biogeographic history of the Assamiidae of southeast Asia (Chelicerata: Opiliones, Laniatores)

Luciano Palmieri a,*, Gonzalo Giribet b, Prashant P. Sharma a,*

a Department of Integrative Biology, University of Wisconsin-Madison, Madison, WI 53711, USA
b Museum of Comparative Zoology, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT
Opiliones (harvestmen) have come to be regarded as an abundant source of model groups for study of historical biogeography, due to their ancient age, poor dispersal capability, and high fidelity to biogeographic terranes. One of the least understood harvestmen groups is the Paleotropical Assamiidae, one of the more diverse families of Opiliones. Due to a labyrinthine taxonomy, poorly established generic and subfamilial boundaries, and the lack of taxonomic keys for the group, few efforts have been undertaken to decipher relationships within this arachnid lineage. Neither the monophyly of the family, nor its exact placement in the harvestman phylogeny, have been established. Here, we assessed the internal phylogeny of Assamiidae using a ten-locus Sanger dataset, sampling key lineages putatively ascribed to this family for five of the ten markers. Our analyses recovered Assamiidae as a monophyletic group, in a clade with the primarily Afrotropical Pyramidopidae and the southeast Asian Beloniscidae. Internal relationships of assamiids disfavored the systematic validity of subfamilies, with biogeography reflecting much better phylogenetic structure than the existing higher-level taxonomy. To assess whether the Asian assamiids came to occupy Indo-Pacific terranes via rafting on the Indian subcontinent, we performed divergence dating to infer the age of the family. Our results show that Indo-Pacific clades are ancient, originating well before the Cretaceous and therefore predate a vicariant mechanism commonly encountered for Paleotropical taxa.

1. Introduction

Among the arachnid orders, Opiliones (harvestmen) have come to be regarded as the foremost model system for study of historical biogeography. Two decades of molecular phylogenetic study have unveiled high fidelity between harvestman distributions, phylogenetic relationships, and timing of cladogenesis, for various taxonomic levels (Boyer et al., 2007; Derkarabedian et al., 2021; Giribet et al., 2010), and with few known cases of transoceanic dispersal (Baker et al., 2020; Pérez-González et al., 2022; Sharma and Giribet, 2012). As examples, mite harvestmen (Cyphophthalmi) have been intensely studied as models of vicariance biogeography, particularly of temperate Gondwanan landmasses (Baker et al., 2020; Boyer and Giribet, 2007; Giribet et al., 2012, 2016; Oberski et al., 2018). In the largest harvestman suborder, Laniatores, various subfamilies of Gonyleptoidea have featured prominently in efforts to circumscribe areas of endemism in the Neotropics and examine latitudinal diversity gradients (Benavides et al., 2021; Bragagnolo et al., 2015; Castro-Pereira et al., 2021; DaSilva et al., 2016). The geographic provincialism exhibited by various harvestman taxa (i.e., families and superfamilies) has been linked to poor dispersal ability and small species ranges, possibly as a consequence of narrow niche specificity (Giribet et al., 2012). As a result, numerous neglected or obscure groups within Opiliones have prospered from renewed interest and revitalized sampling efforts for biogeographic study.

One exception to this trend is Assamiidae, a group that is broadly distributed in the Afrotropics, the Indian subcontinent, and Australasia (Fig. 1). With over 450 described and accepted species (Kury et al., 2022), assamiids exhibit marked morphological diversity, spanning minute forms that are completely blind (e.g., Irumuinae), to large-bodied species that exhibit a grossly hypertrophied fourth leg coxa and scutum in males (e.g., the genera Paktongius and Mysorea) (Fig. 2). The latter condition is often associated with males of Gonyleptoidea and the phylogenetic placement of Assamiidae implies convergent evolution of this mode of sexual dimorphism. A separate type of sexual dimorphism that is much less common in Opiliones in seen in females of some sub-Saharan African genera; whereas females of most harvestmen...
species are unremarkable, those of genera like Binderella exhibit marked distention of the antero-posterior axis, via elongation of the opisthosomal segments, as well as hypertrophied armature in some cases (Fig. 2A-C). In less exceptional cases, assamiids are generally recognizable for their relatively short pedipalps, which are held over the chelicerae (comparably to Cosmetidae, but without the spoon-shape). However, despite the promise of biogeographic insights and intriguing patterns of body plan evolution within the group, there is to date no internal phylogeny of Assamiidae.

The cause of this neglect is largely driven by its convoluted systematics; there are more than 250 described genera of Assamiidae, which were divided into 17 subfamilies by Roewer (1912, 1935), with these higher taxa based on a combination of six highly variable characters. The lack of support for these subfamilies was highlighted by Starega (1992), and Kury (2007) suggested the existence of at least five major definable groups of Assamiidae in the Roewerian system: the Australasian Dampetrinae, the African Erecinae, the blind African Iruimuinae, the subcontinental Trionyxellinae, and the Indo-Nepalese Assamiinae. Many of these putative subfamilies occur in both the Afrotopics and Australasia (e.g., Dampetrinae, Erecinae), which is contrary to the pattern exhibited by most Opiliones subtaxa. Kury suggested that no key to the subfamilies could be provided, given how poorly supported they were (Kury, 2007). Today, this leaves Assamiidae with a staggering proportion of monotypic genera and relatively large number of described species for a family of Opiliones (e.g., Assamiidae is the largest family of Opiliones in Africa; (Starega, 1992). Given the absence of a taxonomic key for these taxa, as well the limited quality of drawings in Roewer’s descriptions, even identifying specimens to genus can be an arduous task. As a result, few workers have described new assamiid species in the past two decades (Bauer and Prieto, 2009; Lotz, 2011; Santos and Prieto, 2010; Zhang and Zhang, 2015; Martens 2022) and no efforts to assess its internal phylogenetic structure have been undertaken. Some workers have additionally questioned the monophyly of the group altogether (Starega, 1992).

The placement of the family in the broader harvestman tree of life is little better understood. Assamiidae was previously thought to be the sister group of Gonyleptoidea on the basis of similarities of genitalic architecture between assamiids and Stygnopsidae, as well as...
correspondences of external morphological characters. Molecular sequence data sampling up to nine assamiid exemplars in Sanger-based efforts (based on five to ten genes) suggested instead a closer relationship to the largely Afro tropical family Pyramidopidae, which were thereafter placed in the superfamily Assamioidea (Giribet et al., 2010; Sharma and Giribet, 2011; Sharma et al., 2017). This relationship was not recovered in a reanalysis of the ten-locus dataset that included a new family from southeast Asia (Kury et al., 2019), although alternative placements of Assamiidae were also not supported in that work. A single phylogenomic analysis sampling both Assamiidae and Pyramidopidae recovered only partial support for Assamioidea, as a function of matrix completeness (Aharon et al., 2019), an instability likely driven by minimal taxon sampling (Ontano et al., 2021). The identity of the sister group of Assamiidae has therefore not been ascertained.

To begin addressing the relationships of this diverse family, as well as its placement within Laniatores, we generated a Sanger dataset for a subset of assamiid species, using legacy markers for incorporation with previous sampling efforts. Given the difficulty of identifying assamiids, we focused our efforts on sampling terminals that could be identified at least to genus. Here, we present the first molecular phylogeny of Assamiidae, sampling key taxa from both the Afro tropics and Australasia. Leveraging the recent description of several exceptionally preserved Laniatores fossils from Cretaceous Burmese amber, we infer divergence times within Laniatores and assess a well-established vicariance hypothesis for the Indo-Pacific: an Afro tropical origin, followed by colonization of the Indo-Pacific via continental drift of the Indian subcontinent.

2. Materials and methods

2.1. Taxon sampling and imaging

Specimens were collected from sifted litter in sites across Australasia, as well as sampled from natural history collections, principally from the Museum of Comparative Zoology, Cambridge, United States (MCZ) and the Museum d’histoire naturelle, Geneva, Switzerland (MHNG). We selected 59 sequence-grade specimens representing a subset of the putative subfamilies of Assamiidae, with most of these reliably diagnosable to genus. We used routine DNA extraction, amplification and sequencing protocols for partial fragments of two mitochondrial protein-encoding (16S rRNA and cytochrome c oxidase subunit I), one nuclear protein-encoding gene (histone H3), and two nuclear ribosomal genes (18S rRNA, 28S rRNA). To test the monophyly of Assamiidae, as well as its phylogenetic placement within Laniatores, we included newly sequenced specimens in a legacy ten-locus dataset that had previously sampled nine assamiids (Giribet et al., 2010), with further additions of phylogenetically significant Grassatores lineages (Aharon et al., 2019; Cruz-López et al., 2016; Kury et al., 2019). Locality and accession data for all terminals are provided in Supplementary Table S1.

2.2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from 1 to 4 legs of each specimen using the Qiagen® DNeasy Blood and Tissue kit, following the manufacturer’s protocol. Standard PCR reactions, primer selection, and Sanger-
sequencing protocols used in this study followed Sharma et al. (2011). Sequences were assembled and cleaned using Geneious® 9.1.8. Consensus sequences were submitted to BLAST against the NCBI database (https://blast.ncbi.nlm.nih.gov) to identify possible contaminations. Sequences were deposited on GenBank under the accession numbers provided in Supplementary Table S1.

2.3. Phylogenetic analyses

Sequences were aligned using MAFFT v7.221 (Katoh and Standley, 2013), implementing the L-INS-I option for accuracy. Individual gene alignments were visually inspected for ambiguously aligned bases. Alignments of the protein-coding genes were translated into amino acids on AliView v1.26 (Larsson, 2014) to detect premature stop codons and ensure alignment by codon. Ambiguous sequences presenting stop codons were treated as pseudogenes and discarded. The final matrix, consisting of 188 taxa and 7024 bp was assembled on SequenceMatrix v1.7.8 (Vaidya et al., 2011).

Maximum likelihood phylogenetic analyses were carried out with IQ-TREE v1.6.10 (Nguyen et al., 2015) using an edge-linked-proportional partition model with separate models between partitions. The final alignment was split into 13 partitions, and the best nucleotide substitution model for each partition was determined using ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE. We compared the Bayesian information criterion (BIC) of ten independent IQ-TREE runs following Zhou et al. (2018) to optimize the tree search. The tree with the lowest BIC score was selected. Support of the nodes was assessed with ultrafast bootstrapping (Hoang et al., 2018), also implemented in IQ-TREE.

Bayesian inference analysis was performed using MrBayes v3.2.6 (Ronquist et al., 2012). The partition scheme was determined using PartitionFinder 2.1.1 (Lanfear et al., 2017), applying the greedy algorithm (Lanfear et al., 2012) and limiting the substitution models to those implemented in MrBayes. To improve chain mixing and avoid converging on local optima, we used Metropolis-coupled Markov chain Monte Carlo (MCMC), with two parallel runs, implementing one cold chain and three incrementally heated chains. The heating parameter was set to 0.01 to allow swap frequencies from 20 % to 70 %. Two independent runs were run for 60 million generations (sampling all the values every 6,000 generations), with the first 15 million (25 %) discarded as burn-in. The convergence of the chains was assessed by ensuring ESS values > 200 for all variables reported by MrBayes. Maximum likelihood analysis was performed at the Bioinformatics Resource Center computational cluster at UW-Madison, and Bayesian inference was performed on the CIPRES Science Gateway (Miller et al., 2010).

2.4. Divergence time estimation

Divergence time estimation was performed using the Bayesian relaxed molecular clock method implemented in BEAST 2 v2.6.3 (Bouckaert et al., 2014; Drummond et al., 2006). Log normal age calibrations for the final tree.

2.5. Biogeographic inference and ancestral range reconstruction

The biogeographic history of the group was inferred using the pruned BEAST chronogram as the input tree for RASP v4.3 (Yu et al., 2020), preserving only the core Assamiidae (79 terminals) and two closely related outgroup families (Pyramidopidae and Beloniscidae). We specified eight geographic areas corresponding to biogeographic realms and bioregions (Fig. S1). Maximum range size was restricted to three areas to simplify analysis and interpretation of the results. This restricted range size also reflects the biology of Assamiidae Opiliones, which tend to present short-range endemism. The likelihood of ancestral range estimations were tested under the Dispersal-Vicariance Analysis (Ronquist, 1997) using S-DIVA (Yu et al., 2010) with 1000 reconstructions for the final tree.
3. Results

3.1. Assamiidae placement and relationships

Maximum likelihood inference (Fig. 3) of the 10-locus dataset recovered a tree topology with support for assamiid monophyly (bootstrap resampling frequency [BS] = 100%). Assamiidae was recovered as the sister group of Pyramidopidae (BS = 78%), with this clade in turn sister group to Beloniscidae (BS = 86%). Relationships of other Laniaurotes clades were comparable to other analyses of this Sanger dataset in

Fig. 3. Tree topology of Assamiidae inferred under maximum likelihood using IQTREE. Complete tree topologies are provided in Supplementary Fig. S2.
Within Assamidae, we recovered sub-Saharan species as a clade (BS = 99 %), nested within the Asian assamids; a clade of two minute genera, *Arulla* and *Tarnus*, were recovered as the sister group of the African Assamidae (BS = 89 %). The small, blind Irumuinae were recovered as nested within the African clade, as were Polycoryphinae species with hypertrophied opisthosomas in the female (e.g., *Binderella*). The latter were resolved as distantly related to the Asian Polycoryphinae (e.g., *Paktongius*) and Hypoxestinae (*Bandona* and *Podauchenius*) were not recovered as monophyletic, substantiating the disutility of several subfamily definitions.

Within a large Indo-Pacific clade, a Sri Lankan *Trionyxella* was recovered as the sister group of a largely Thai-Malay clade (BS = 86 %), with the latter comprised of several species with hypertrophied males (e.g., *Paktongius* and *Mysorea*). The last major Asian clade consisted of Sahul shelf Dampetrinae (BS = 81 %) sister group to Bornean and southeast Asian Dampetrinae (BS = 70 %), with these in turn sister group to Thai-Malay Erecinae (the genus *Neopygoplus*; BS = 99 %).

Bayesian inference analyses (Fig. 4) recovered largely congruent relationships, with the exception that the Asian Assamidae were

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**Fig. 4.** Tree topology of Assamidae inferred under Bayesian inference using MrBayes. Complete tree topologies are provided in Supplementary Fig. S2.
recovered as a single clade that was reciprocally monophyletic with the African Assamiidae, although the Asian clade was not supported (posterior probability [PP] = 0.67). Differences in tree topology and support values are as depicted in Figs. 3 and 4.

3.2. Divergence dating

Molecular divergence time estimation in BEAST recovered the split between Assamiidae and Pyramidopidae near the Permo-Triassic boundary (262 Myr; 95% highest posterior density interval [HPD]: 213–312 Myr), with the basal diversification of assamiids in the Triassic (233 Myr; HPD: 189–281 Myr). Diversification of the putative subfamilies and genera of Assamiidae spanned the Jurassic and Cretaceous, as depicted in Fig. 5. Age estimates of Assamiidae and other Grassatores families were largely consistent with previous dating efforts (Sharma and Giribet, 2011; Sharma et al., 2017). Confidence intervals for the origins Asian Assamiidae clades all predated 90 Myr, approximately the age of the separation of the Indian subcontinent from eastern Africa.

3.3. Biogeography

The range of distribution of ancestral Assamioidea was estimated to have been Pangean during the Paleozoic, in an area encompassing the ancient regions that comprise the present-day Afrotopics, Eastern Paleartic, and Thai-Malay Peninsula (B + D + E, Fig. 5) ca. 265 Myr. Assamiidae were reconstructed to have diversified across the supercontinent and their distribution followed the subsequent rifting of the constituent terranes. During the Upper Triassic the Assamioidea extended their range distribution and probably occupied the majority of Eastern Pangea, with the split between Pyramidopidae and Assamiidae occurring during this time (ca. 235 Ma). The diversification of the lineages kept following the split of the land masses and by the beginning of Upper Cretaceous all major Assamiidae lineages were well established. Our results show that these events occurred before the rifting of India from east Africa (ca. 95 Mya).

4. Discussion

Previous efforts to resolve relationships within Laniatores had sampled only a handful of assamid species (Giribet et al., 2010; Sharma and Giribet, 2011), whereas genomic datasets for the family remain limited to a single dampertrine transcriptome (Aharon et al., 2019; Fernandez et al., 2017). As the aim of those works was to establish higher-level relationships between Laniatores families, the limited sampling of Assamiidae has precluded understanding of their internal relationships. A previous work that sampled nine species recovered two African exemplars as nested within the remaining Asian taxa, but this result was not strongly supported (Sharma and Giribet, 2011). Here, we endeavored to increase the sampling of key African and Asian assamid lineages, with the goals of establishing the first look at the internal relationships of the family and assessing whether the distribution of the Australasian fauna was attributable to the rifting of India from east Africa (ca. 95 Mya) and its subsequent collision with Eurasia in the Cretaceous (ca. 45–55 Mya) (Ali and Aitchison, 2008). While sampling of Assamiidae is greatly limited in this study in proportion to the family’s described diversity, we were able to sample a sufficiently large number of taxa and lineages (the putative subfamilies) that made it possible to test this biogeographic vicariance hypothesis using divergence time estimation of the Asian taxa.

The internal relationships we recovered for Assamiidae did not place the Asian and Sahul shelf fauna as a derived clade within the African taxa, a key prediction of the subcontinental rifting hypothesis. The ancient ages of the Thai–Malay and Australasian lineages greatly predated 95 Mya, suggesting that the presence of Assamiidae across the Paleotropics is instead consistent with a broad ancestral distribution. This inference is supported by the recent discovery of several modern Laniatores families in Burmese amber, such as Pyramidopidae and Beloniscidae (Selden et al., 2016; Bartel et al., 2021), which we recovered as the sister lineages of Assamiidae. Pyramidopidae were previously considered a strictly Afrotropical family, with one representative in the nearby Canary Islands (Sharma et al., 2011). Molecular phylogenetic work on small-bodied relictual species recently expanded their range to include Belize and Israel (Aharon et al., 2019; Cruz-Lopez et al., 2016), whereas paleontological data showed that their historical range also extended to Burma, though pyramidopids no longer occur in southeast Asia (Bartel et al., 2021). Broad ancestral distributions spanning multiple biogeographic terranes are also found in armored harvestman families like Biantiidae, Podoctiidae, and Triarachnoidea (Derkarabetian et al., 2021a; Sharma et al., 2017). Thus, the discovery that Assamiidae are among the oldest facets of Paleotropical leaf-litter fauna accords with broader biogeographic patterns across Laniatores. We anticipate that this inference could be validated by the discovery of assamid fossils from Burmese amber (ca. 99 Mya); such a discovery would substantiate that Asian Assamiidae were an established part of Cretaceous ecosystems in southeast Asia, long before the arrival of the Indian subcontinent (ca. 45–55 Mya).

The sampling of Assamiidae in this study was greatly limited by the availability of material that can be reliably identified under the unstable Roewerian system. Further limitations of this work were incurred by the unavailability of sequence-grade north Indian and Nepalese fauna (harboring much of the diversity of the putative subfamily Assamiinae), and eastern and southern Africa. The inclusion of these lineages is critical to testing whether subgroups within Assamiidae could accord with the scenario of rifting on the Indian subcontinent; this mechanism of vicariance could explain patterns in the Roewerian system where certain subfamilies are found in both Africa and the Indo-Pacific. Recent advances in sequence capture strategies offer promising vehicles to circumvent limitations of legacy datasets. The demonstrable performance of ultraconserved elements (UCE) in molecular phylogenetic efforts is especially promising with regard to accessing sequence data in degraded and aging tissues, as well as providing sufficient recovery of loci that higher-level relationships can be reliably resolved—often, an insurmountable roadblock for Sanger datasets of ancient chelicerate groups (Derkarabetian et al., 2021b, 2019; Starrett et al., 2016). One potential strategy to overcoming the impediments wrought by the Roewerian system may be to apply UCE sequencing to all of Roewer’s type specimens, toward disentangling the maze of monotypic genera and unstable subfamilies in Assamiidae. Applied broadly across Laniatores, this strategy may provide better resolution of the relationships and composition of Laniatores subfamilies (Aharon et al., 2019; Fernandez et al., 2017; Sharma and Giribet, 2011).

Future efforts to understand assamid diversity should emphasize the sampling of the remaining Roewerian “subfamilies”. Beyond systematics and biogeographic patterns within this group, a robust understanding of internal phylogeny will accelerate investigation of body plan evolution in this morphologically diverse family of harvestmen. Together with a phylogeny, parametric approaches may also enhance investigations of trait correlations across Laniatores phylogeny, with emphasis on the degree of sexual dimorphism and detection of character dependencies (e.g., Gainett et al., 2014; Kerr et al., 2010; Riesgo et al., 2013; Santibáñez-López et al., 2017).

CRediT authorship contribution statement

Luciano Palmieri: Conceptualization, Formal analysis, Writing – original draft, Visualization. Gonzalo Giribet: Resources, Investigation, Writing – review & editing. Prashant P. Sharma: Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.
Fig. 5. (next page). Chronogram and ancestral range reconstruction analysis for Assamiidae. Colors of biogeographic terranes follow the legend on the left. Below: Paleomap reconstructions corresponding to diversification of Assamioidea and diversification of Assamiidae. The reconstruction on the right corresponds to the rift between east Africa and the Indian subcontinent. Inset: Biogeographic regions designated for analysis. Complete chronograms are provided in Supplementary Fig. S3.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References
