**Reanalysis of a legacy dataset refutes the traditional higher-level systematics of Assamiidae (Arachnida, Opiliones, Laniatores)**

Benjamin Klementz

**Abstract**

The Laniatores are the most speciose suborder of Opiliones with lineages typically exhibiting endemic distributions across the world’s tropics. However, the Laniatores family Assamiidae is notable for its wide distribution across Africa and Asia, making it a prime system for biogeographical studies. Yet, the internal evolutionary relationships of assamiids remains elusive, given myriad monotypic genera, poorly described taxa, and questionable subfamily designations. To begin resolving the internal systematics of the family, Palmieri et al. (2023) produced the first molecular assamiid phylogeny based on Bayesian inference of a five-locus Sanger dataset. Given the importance of the family to a broader understanding of Opiliones dispersal, distribution, and biogeography, the robustness of Palmieri et al.’s topology was assessed by reanalysis of their dataset utilizing three methodologies: sequential concatenation under maximum likelihood, partitioned concatenation under maximum likelihood, and multispecies coalescence. Both maximum likelihood tree topologies show high congruence with Palmieri et al.’s analysis with often high nodal support. Multispecies coalescence, however, produces a highly variable topology with low support values, believed to result from the limited number of gene trees available for analysis. Ultimately, nearly all existing assamiid subfamilies are recovered as paraphyletic or polyphyletic, emphasizing the necessity for systematic revisions in the family.

**Introduction**

In recent years, a concerted effort has been undertaken by the arachnological community to resolve the higher-level internal relationships of the so-called “neglected cousins,” an assemblage of arachnid orders characterized by combinations of highly conserved morphologies, difficulty of collection, cryptic habitats, and poor knowledge of basic biology (Harvey 2002). By leveraging advances in sequencing technologies for molecular phylogenetic analysis, great steps have been taken in resolving the internal relationships of orders such as Solifugae (Kulkarni et al. 2023) and Amblypygi (de Miranda et al. 2023), replete with new familial designations, detailed biogeographic histories, and systematic revisions of problematic taxa. Yet, these advances in arachnid systematics have been achieved primarily via phylogenomic analysis of ultraconserved elements (UCEs). UCE probe sets have been developed and employed to great effect in spiders (Kulkarni et al. 2020) and the broader arachnids (Faircloth 2017), as probes with higher specificity to a narrow range of taxa tend to yield more phylogenetically informative UCE loci. For example, an Acari-specific probe set increased captured loci from 1,120 when using an arachnid probe set to 2,058 loci (van Dam et al. 2019). Construction of these probe sets, however, relies upon high quality genomic or transcriptomic resources for design of oligonucleotide bait sequences. Kulkarni et al. (2020), for example, designed their spider-specific probe set through the alignment of three separate spider genomes.

Thus, while UCEs represent a promising frontier in arachnid systematics, the requirement of high-quality genomic data limits the applicability of UCEs to those groups for which these resources exist. Opiliones (i.e., daddy longlegs, harvestmen), while representing the third most speciose arachnid order, possess genomic resources for only a single species, *Phalangium opilio* (Opiliones, Eupnoi; Gainett et al. 2021). With genomic resources restricted only to the suborder Eupnoi, only the more traditional analytical techniques are available for internal phylogenetic resolution of the remaining suborders. Laniatores represent the most speciose suborder of Opiliones, and a subset of Laniatores families (superfamily Gonyleptoidea) exhibit poor dispersal, believed to result from extremely narrow niche specificity (Giribet et al. 2012). As a result, Gonyleptoidea exhibits a high degree of micro-endemism, making the group a useful system for the study of historical and vicariance biogeography. Interestingly, the family Assamiidae contradicts the conserved, endemic distributions seen across the remaining Gonyleptoidea, being distributed across the Afrotropics, Indian subcontinent, and Australasia. However, Assamiidae has long been neglected in biogeographic and phylogenetic analyses due to highly questionable systematics. The family lacks a taxonomic key to even the subfamilial level and is plagued by numerous monotypic genera and limited or poor-quality illustrations of described species. Much of the questionable systematics stems from the work of a single taxonomist, Carl Friedrich Roewer, responsible for descriptions of 250 genera and 17 subfamilies of Assamiidae (Roewer 1912; 1935). The use of abundant homoplastic morphological characters to delimit these groupings have led some taxonomists to question whether Assamiidae is truly monophyletic altogether (Starega 1992).

Aside from biogeography, Assamiidae has also been invoked as a potential system for understanding the evolution of sexual dimorphism. Assamiidae exhibits a wide range of morphological diversity, including several lineages of large-bodied animals (e.g., *Mysorea, Paktongius*)characterized by hypertrophic coxae (the proximal-most podomere of arthropod appendages). This hypertrophy, greatly exaggerated in males, yields a charismatic lateral expansion of the posterior carapace. However, this condition has also been observed in other lineages of Gonyleptoidea, including members of Gonyleptidae, Cranaidae, Ampycidae, and Metasarcidae (Benavides et al. 2021). Yet, multiple evaluations of the higher-level relationships amongst Laniatores have recovered Assamiidae as distantly related to Gonyleptoidea (Aharon et al. 2019; Palmieri et al. 2023). Thus, interpretation of the shared hypertrophic male condition requires multiple, independent origins of this habitus. Cases of convergence with Gonyleptoidea also extend to particular male behaviors, given similarity in male-male combat (Zatz et al. 2011; García-Hernández and Machado 2017), as well as documented instances of paternal care (Martens 1993). Yet, an understanding of the number of origins and the extent of convergent evolution between Assamiidae and Gonyleptoidea requires resolution of Assamiidae’s placement within the suborder and its own species and genera relationships.

As an initial effort to address both the internal and sister group relationship of Assamiidae, Palmieri et al. (2023) published the first molecular phylogeny of Assamiidae based on a Sanger dataset of five legacy markers to better incorporate previously sampled specimens. Of the analyses performed, the authors produced a tree topology based on Bayesian analysis conducted via MrBayes v3.2 (Ronquist et al. 2012). The authors optimized this approach by designing a partition scheme using PartitionFinder v2.1.1 (Lanfear et al. 2017) and improved chain mixing using Metropolis-coupled Markov chain Monte Carlo (MCMCMC) in two parallel runs. There are, however, several reasons to be skeptical of the topology obtained by Palmieri et al. (2023). Suzuki et al. (2002), using a series of computer simulations, demonstrated that the generated posterior probabilities in Bayesian analyses are often too liberal when using concatenated gene sequences, while bootstrap probabilities in maximum likelihood analyses tend to be more conservative. The authors suggest that bootstrap probabilities are more fit to judge the reliability of phylogenetic trees than are posterior probabilities. Posterior tree probabilities in Bayesian analysis are also incredibly sensitive to model violations, and the use of simplistic models often leads to exaggerated probabilities (Yang and Rannala 2012). Given Palmieri et al. (2023) did not report the models determined by PartitionFinder, there is thus no way of determining whether overly simplistic models were assigned to the highly conserved legacy markers used in their analysis. Bayesian analyses can also be hindered by the use of default priors, which can have unexpected influences on the posterior distribution (Nascimento 2017). Yang and Rannala (2012) report that the independent exponential prior on branch lengths in MrBayes can enforce an unreasonable prior on tree length and produce overly long trees in some datasets.

Given the tremendous potential of Assamiidae to biogeographical studies and understanding of the evolution of extreme arachnid sexual dimorphism, a well-resolved internal phylogeny and knowledge of their placement within the Laniatores is of the utmost importance. To better assess the robustness of the topology obtained by Palmieri et al. (2023), the same dataset has been analyzed using three different analytical methods. The first and second analyses utilized a concatenation of the five legacy loci both with and without partitioning under maximum likelihood inference in IQ-TREE (Nguyen et al. 2015), while the third relied upon a multispecies coalescent approach based on gene trees produced in IQ-TREE and implemented in ASTRAL (Zhang et al. 2018). Utilization of maximum likelihood may provide insight into the discrepancies between posterior probabilities and ultrafast bootstrapping in relation to nodal support calculation. The multispecies coalescent approach, on the other hand, could offer a higher degree of accuracy given the increased statistical consistency compared to concatenated maximum likelihood and Bayesian approaches (Mirarab 2019).

**Methods**

*Description of the Dataset*

Specimens used in analyses represent those field-collected across Australasia, as well as 59 specimens procured from several natural history collections (e.g., MCZ, MHNG) representing the putative subfamilies of Assamiidae. Specimens utilized were those confidently identified to genus. Sequencing efforts by Palmieri et al. (2023) procured sequences of the mitochondrial protein-encoding genes 16S rRNA and cytochrome *c* oxidase subunit I (COI), the nuclear protein-encoding gene histone H3, and the two nuclear ribosomal genes 18S rRNA and 28S rRNA. For a full description of extraction, sequencing, and PCR protocols, see section 2.2 in Palmieri et al. (2023). In addition, nine specimens utilized sequences in Giribet et al. (2010). Sequence data for numerous Grassatores lineages were also obtained from Aharon et al. (2019), Cruz-López et al. (2016), and Kury et al. (2019). In total, sequences represent 199 taxa, 95 of which were putatively identified to Assamiidae, with sampling of the three other Opiliones suborders as outgroups, as well as numerous families of both infraorders of Laniatores (Insidiatores and Grassatores) that Palmieri et al. (2023) used to test the monophyly of Assamiidae. For a full list of included taxa, including accession and locality data, see the authors’ supplementary table S1.

*Multiple Sequence Alignment*

Sequences obtained from Palmieri et al. (2023) had already been assembled and cleaned using Geneious 9.1.8, as well as aligned. In order to replicate the steps of multiple sequence alignment, provided FASTA files had gaps manually deleted. The resulting sequences for each locus were aligned using MAFFT v7.490 (Katoh and Standley 2013). MAFFT has several limitations, namely, that library extensions are not performed (*contra* T-COFFEE) and can suffer from poor scalability in instances when long DNA sequences are given. This is particularly problematic under the FFT-NS-2 model as a large CPU time is required for full-length alignment. MAFFT also operates under the assumption that the order of alignable blocks is conserved for all input sequences. On the other hand, MAFFT offers various multiple alignment strategies for the user to determine. Progressive methods (i.e., FFT-NS-1 and FFT-NS-2) first construct a rough distance matrix by counting the number of shared sextuples between every sequence pair. The algorithm then constructs a guide tree from these distances and aligns them according to the tree’s branching order. MAFFT also offers several iterative refinement methods (e.g., FFT-NS-i, NW-NS-i, L-INS-i, E-INS-i, G-INS-i). These methods utilize an objective function to evaluate the consistency between multiple and pairwise alignments. The initial alignment produced by FFT-NS-2 is subjected to iterative refinement, repeated until the score of the objective function is maximized, or the algorithm reaches 1,000 iterations. Alignment in MAFFT utilized the L-INS-i option for its designation as the most accurate of the available models. This method is particularly useful when aligning sequences possessing long flanking regions around a single alignable domain. However, it does assume that the sequences have only a single alignable domain.

*Phylogenetic Analysis: Concatenation*

Individual multiple sequence alignments for all five loci were concatenated via Phyutility v2.6 (Smith and Dunn 2008). The output nexus file was then converted to FASTA format. To assess the topology produced by an unpartitioned dataset, maximum likelihood analysis of the output FASTA file was conducted in IQ-TREE v1.6.12 (Nguyen et al. 2015). The best nucleotide substitution model for the dataset was determined by implementation of ModelFinder (Kalyaanamoorthy et al. 2017) within IQ-TREE. Nodal support was estimated based on 1,000 replicates of ultrafast bootstrap resampling. Additionally, branch support was inferred based on implementation of the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010) again with 1,000 replicates. The resulting tree was rerooted to *Troglosiro* (Opiliones, Cyphophthalmi), the most early diverging suborder of Opiliones (Fernández et al. 2017).

To assess the influence of partitioning on the dataset, the concatenated multiple sequence alignment was assigned seven partitions, one each for histone H3, 16S, 18S, and 28S. COI was assigned data blocks corresponding to its three codon positions, allowing for variation in evolutionary rates across codon positions. Maximum likelihood-based phylogenetic analysis was again implemented in IQ-Tree, using ModelFinder for optimization of nucleotide substitution models within each partition. Nodal support and branch support were inferred by 1,000 ultrafast bootstrap resampling replicates and SH-aLRT with 1,000 replicates, respectively. The resulting tree was rerooted to *Troglosiro.*

IQ-TREE is built upon a hill-climbing nearest-neighbor interchange (NNI) algorithm to determine locally optimal phylogenetic trees. The algorithm initially generates 100 starting tree topologies based on maximum parsimony. The 20 initial trees that produce the highest maximum likelihood scores are retained in a candidate tree list. These locally optimal trees are then randomly perturbed. Hill-climbing NNI is performed on these perturbed trees until they reach a new local optimum. If the new optimum has a higher maximum likelihood score than the initial candidate tree, the candidate is replaced by the perturbed tree and the process is repeated. Ultimately, traversal of the tree space ends when the optimal tree has not been substituted after 100 successive random perturbations. IQ-TREE is limited by its sampling of only local optima and the assumption that the top optima found truly represents the global optimum or ideal tree topology. However, the generation of the initial candidates and random perturbations produced during hill-climbing drastically reduce the probability of the algorithm getting stuck on a local optimum. IQ-TREE also tends to outperform RAxML (Stamatakis 2014), computing trees with higher likelihoods when given the same computing time (Nguyen et al. 2015). However, an additional caveat to the use of IQ-TREE is the use of fast NNI perturbations, thought to produce less accurate trees than subtree pruning and regrafting (Zhou et al. 2018). Finally, IQ-TREE, over successive runs, can produce variable log-likelihood calculations resulting in variable output tree topologies.

*Phylogenetic Analysis: Multispecies Coalescence*

Individual gene trees for each locus were first constructed via maximum-likelihood inference in IQ-TREE. The resulting tree files were concatenated in the terminal. Multispecies coalescent tree construction was implemented in ASTRAL v5.7.8 (Zhang et al. 2018). The resulting output tree was rerooted to *Troglosiro*. ASTRAL functions as a method for constructing a species tree from a set of gene trees that incorporates incomplete lineage sorting and gene tree discordance under the multispecies coalescent model of gene evolution. ASTRAL is also a heuristic method in that from a set of defined bipartitions, it will generate a series of tripartitions. For each of these tripartitions, ASTRAL will calculate the number of quartet trees induced by the input gene trees that associate to that tripartition. Individual tripartitions are then assigned a score based on a recursive formula to produce a species tree optimizing agreement with the largest number of quartet trees from the set of gene trees. ASTRAL is particularly robust given that it tends to be more accurate than concatenated maximum likelihood topologies given higher statistical consistency (Mirarab 2019). However, its statistical consistency is hindered when gene alignments are of limited length and gene tree topologies are generated via maximum likelihood. Even despite this limitation, ASTRAL was deemed appropriate for this analysis given high robustness to missing data as sequence data for all five loci was not obtained for all 199 terminals in this analysis. However, this robustness requires the assumption that the presence or absence of a gene for a species is independent of the gene tree topology and the presence or absence of other genes for that species.

**Results**

*Concatenation without Partition*

The maximum likelihood tree obtained via concatenation without a partition scheme had a best-fit model of nucleotide substitutions of SYM+I+G4 as determined by Bayesian Information Criterion (BIC) scoring in ModelFinder. The symmetrical model (SYM) assumes equal base frequencies and a symmetrical substitution matrix (e.g., A to T substitutions occur at the same rate as T to A substitutions). The SYM model was augmented by the specification of a proportion of invariable sites (I) and gamma distributed rate variation among four discrete rate categories (G4). The proportion of invariable sites was calculated as 0.5888. The final tree had a log-likelihood score of -100837.968 and an unconstrained log-likelihood of -37877.6615. Finally, the tree had a total length of 12.2225, the estimated average number of nucleotide substitutions per site.

In relation to topological similarity to Palmieri et al. (2023), the concatenated, non-partitioned analysis also recovers the monophyly of Assamiidae (Fig. 1), albeit with a bootstrap resampling frequency (BS) of 82.3% and SH-approximate likelihood ratio test value (SH-aLRT) of 82%, as opposed to the strongly supported posterior probability (PP) of 1. While the previous analysis recovers the monophyletic Assamiidae as sister to Pyramidopidae (PP = 0.72), this analysis supports a sister relationship with Epedanidae (BS = 78%, SH-aLRT = 62%). Pyramidopidae is instead sister to Beloniscidae (BS = 59.8%, SH-aLRT = 65%), and this clade now sits as sister to the Epedanidae and Assamiidae clade (BS = 69.8%, SH-aLRT = 52%) with low support (Fig. 1). Outside of Assamiidae, this analysis recovers, when rooted by *Troglosiro* (Opiliones, Cyphophthalmi), the monophyly of the remaining suborders Dyspnoi (BS = 100%, SH-aLRT = 100%) and Eupnoi (BS = 94.4%, SH-aLRT = 94%). The Laniatores are also recovered as monophyletic (BS = 100%, SH-aLRT = 100%). The infraorder Insidiatores appears, however, paraphyletic given the clade of *Fumontana, Larifuga, Rostromontia, Equitius,* and members of Triaenonychidae sit as sister to the remaining Laniatores (infraorder Grassatores). Yet, this placement seems dubious considering the extremely low BS of 26.5% and SH-aLRT of 60%. At the familial level, this analysis largely supports existing Laniatores systematics with nearly all families recovered as monophyletic. The families Biantidae, Samoidae, and Stygnommatidae are polyphyletic across two of three clades in a highly supported polytomy (BS = 100%, SH-aLRT = 100%). Members of the genus *Metabiantes* (Biantidae) are recovered as sister to an unidentified member of Samoidae (BS = 100%, SH-aLRT = 100%). The remaining biantids (genus *Stenostygnus*) appear in a distinct clade (BS = 99.5%, SH-aLRT = 99%) alongside a nested member of Stygnommatidae. Separately, *Stygnomma* (Stygnommatidae) sits sister to the samoid genera *Fijicolana* and *Pellobunus* with low support (BS = 38.6%, SH-aLRT = 59%). Gonyleptidae is the only other family not recovered as monophyletic, appearing instead paraphyletic to the exclusion of a nested Stygnidae and Cranaidae clade (BS = 45%, SH-aLRT = 77%).

Within Assamiidae, Palmieri et al. (2023) recover a monophyletic clade of all Afrotropical species (PP = 1), sister to a weakly supported monophyletic clade of the Asian Assamiidae (PP = 0.67). This analysis, however, recovers a member of the Asian Assamiidae (As135\_Viet) as sister to the remaining Assamiidae (BS = 82.3%). The Afrotropical Assamiidae are, however, still recovered as monophyletic (BS = 96.1%) within the larger Asian clade, sister to the Thai-Malay Peninsula endemic genus *Arulla.*

In relation to assessing the validity of current subfamilial classifications in Assamiidae, this analysis casts similar doubt on the previously morphology-based system. Palmieri et al. (2023) recovers no subfamilies as monophyletic with sampling of greater than a single taxon. Similarly, this analysis recovers largely paraphyletic and polyphyletic subfamilies (Fig. 2). The sole exception is the miniaturized, blind subfamily Irumuinae recovered as monophyletic and nested within the African clade (BS = 62%, SH-aLRT = 79%). The Asian Dampetrinae were recovered as paraphyletic given the nested position of the Erecinae genus *Neopygoplus* (BS = 74.1%, SH-aLRT = 61%), with an identical topology obtained by Palmieri et al. (PP = 0.94). Polycoryphinae appears as a polyphyletic subfamily. The African Polycoryphinae are recovered as highly derived lineages of the clade, but as paraphyletic due to the nesting of another genus of Erecinae (genus *Chilon*; BS = 98.9%, SH-aLRT = 99%). The Asian Polycoryphinae genus *Paktongius* is paraphyletic, given a single taxon, As108\_Laos, sits as sister to remaining Asian lineages in the second major subclade (BS = 76.5%, SH-aLRT = 44%). The singular terminals of Trionyxellinae (BS = 97%, SH-aLRT = 95%) and Mysoreinae (BS = 97.9%, SH-aLRT =96%) are also nesting within a smaller clade of the remaining *Paktongius,* as well as a single terminal of Hypoxestinae, representing the genus *Bandona* (BS = 83.9%, SH-aLRT = 85%)*.* Likewise, Palmieri et al. (2023) also recover a polyphyletic Polycoryphinae, with the African lineages paraphyletic due to the inclusion of *Chilon* (PP = 1). Similarly, they also recover Mysoreinae (PP = 1) and *Bandona* (PP = 0.99) as nested within the Asian Polycoryphinae. Other lineages of Hypoxestinae in this analysis appear polyphyletic within the African assamiid clade. Finally, the remaining lineages of Erecinae sit in a grade sister to the remaining Assamiidae or as sister to the African clade.

The final tree topology had four internal branches with near-zero length estimates (<0.0002). The first near-zero branch subtends the clade of *Neopygoplus,* As116\_Thai, and As117\_Thai, which yields a polytomy relationship with Assamiidae\_DNA104858 when visualized in FigTree v1.4.4. However, comparison to taxon labels in supplementary table S1 of Palmieri et al. (2023) reveals that the terminals correspond to four terminals of *Neopygoplus* (Assamiidae, Erecinae), perhaps suggesting three samples corresponding to the same species. The second near-zero branch subtends two terminals, As085\_WDus and As085\_WAus, samples of the same species of *Chilon* (Assamiidae, Erecinae). Similarly, the third near-zero branch subtended the clade of As092\_EDus and As092\_EAus, both the same species *Dampetrus australis* (Assamiidae, Dampetrinae). The final near-zero internal branch length yielded a polytomy relationship of clades composed of Samoidae, Biantidae, Stygnommatidae, Escadabiidae, Guasiniidae, Kimulidae, Icaleptidae, Zalmoxidae, and Fissiphalidae.

*Concatenation with Partition*

Of the seven partitions imposed on the dataset, four partitions (16S, 28S, COI’s first codon position, and histone H3) had best-fitting general time reversible models with unequal rates and unequal base frequencies (GTR) augmented by empirical base frequencies (F), defined proportions of invariable sites (I), and gamma distributed rate variation among four discrete rate categories (G4; Table 1). The 18S locus, on the other hand, had a best-fitting transition model with equal base frequencies (TIMe), but supplemented again by a proportion of invariable sites and gamma distributed rate variation among four discrete rate categories (G4; Table 2). The second codon position of the COI locus had a best-fitting TIM2 transition model (i.e., AC=AT, CG=GT) with unequal base frequencies, with arguments specifying empirical base frequencies, a proportion of invariable sites, and gamma distributed rate variation among four discrete rate categories (G4; Table 1). Finally, the third codon position of COI had a best-fitting GTR model with empirical base frequencies, gamma distributed rate variation among four discrete rate categories (G4), and an ascertainment bias correction (ASC) that corrects the likelihood conditioned on variable sites, ensuring branch lengths are not overestimated when the alignment does not contain constant sites (Lewis 2001; Table 2). The final tree produced by IQ-TREE had a log-likelihood of -98831.2274 (±2397.5725) and an unconstrained log-likelihood of -29843.0896. Finally, the total tree length was calculated as 23.2846 average nucleotide substitutions per site.

The final tree had three near-zero internal branches (designated as branch lengths <0.0002). The first subtends the clade containing a subset of *Neopygoplus* assamiids (As116\_Thai, Neopygoplus, and As117\_Thai), producing a polytomy relationship with Assamiidae\_DNA104858 (Fig. 3). The second near-zero branch subtends the sister groups As085\_WDus and As085\_WAus (Assamiidae, Dampetrinae), creating a polytomy alongside As087\_WAus. The final near-zero branch length subtends the sister groups As092\_EDus and As104\_EAus (Assamiidae, Dampetrinae), again yielding a polytomy with As092\_EAus.

**Table 1.** The seven partitioned loci included in analysis and corresponding best-fit nucleotide substitution models as determined by ModelFinder under Bayesian Information Criterion (BIC) scoring.

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| --- | --- |
| **Locus** | **Model** |
| **16S** | GTR+F+I+G4 |
| **18S** | TIMe+I+G4 |
| **28S** | GTR+F+I+G4 |
| **COI, codon position 1** | GTR+F+I+G4 |
| **COI, codon position 2** | TIM2+F+I+G4 |
| **COI, codon position 3** | GTR+F+ASC+G4 |
| **Histone H3** | GTR+F+I+G4 |

The partitioned dataset produced a largely congruent topology to Palmieri et al. (2023)’s Bayesian phylogeny and the previous concatenated, unpartitioned dataset (Fig. 3). Assamiidae are once again recovered as monophyletic (BS = 82.9%, SH-alrt = 81%). A sister group relationship with Epedanidae is also recovered in line with the unpartitioned analysis (BS = 71.8, SH-aLRT = 57%). The Pyramidopidae, identified as sister to the Assamiidae in Palmieri et al. (2023), are instead recovered as sister to the Beloniscidae (BS = 62.7, SH-aLRT = 52%), with this clade in turn sister to the Tithaeidae with very low support (BS = 37.8%, SH-aLRT = 38%). When rooted by the Cyphophthalmi genus *Troglosiro*, this analysis also recovers the monophyly of the suborders Dyspnoi (BS = 100%, SH-aLRT = 100%), Eupnoi (BS = 91%, SH-aLRT = 91%), and Laniatores (BS = 100%, SH-aLRT = 100%). However, unlike the unpartitioned dataset, this analysis recovers the Laniatores infraorder Insidiatores as monophyletic, albeit with low support (BS = 45.9%, SH-aLRT = 56%). An identical topology to the unpartitioned dataset is also obtained in regard to the polyphyletic families of Stygnommatidae, Samoidae, and Biantidae. Gonyleptidae is also again recovered as paraphyletic due to the nested Stygnidae and Cranaidae with higher support than the previous analysis (BS = 69.3%, SH-aLRT = 71%).

Within Assamiidae, the African Assamiidae appear monophyletic (BS = 98.5%, SH-aLRT = 98%) and nesting within a subset of Asian Polycoryphinae and Erecinae (BS = 99.5%, SH-aLRT = 97%). Thus, the Asian Assamiidae are again paraphyletic due to the Asian Erecinae As135\_Viet sitting sister to the remaining Assamiidae (BS = 96.4%, SH-aLRT = 93%).

In regard to the evolutionary relationships amongst the currently morphology-based subfamilial classification system, a nearly identical topology is recovered when compared to the unpartitioned dataset (Fig. 4). The small-bodied Irumuinae subfamily is again the sole monophyletic subfamily (BS = 57.1%, SH-aLRT = 66%), nesting within the African clade (BS = 87.1%, SH-aLRT = 77%). Dampetrinae were likewise paraphyletic within the Asian Assamiidae to the exclusion of the nested Erecinae genus *Neopygoplus* (BS = 58.4%, SH-aLRT = 68%). Polycoryphinae is recovered as polyphyletic, with African Polycoryphinae paraphyletic to the exclusion of nested lineages of the Erecinae genus *Chilon* (BS = 98.1%, SH-aLRT = 99%; BS = 98.5%, SH-aLRT = 82%). A sole Polycoryphinae terminal, As108\_Laos, also nests as sister to the clade of *Arulla* (Erecinae) and the African Assamiidae (BS = 85.4%, SH-aLRT = 67%). The individual terminals of Mysoreinae (BS = 98%, SH-aLRT = 94%) and Trionyxellinae (BS = 97.4%, SH-aLRT = 96%) either sit as sister or nested within the remaining Polycoryphinae genus *Paktongius,* respectively. The Hypoxestinae genus *Bandona,* also nests within this clade (BS = 88.9%, SH-aLRT = 82%). The remaining Hypoxestinae lineages nest as a monophyletic clade within the larger African Assamiidae clade (BS = 99.9%, SH-aLRT = 100%).

*Multispecies Coalescence*

The final tree produced in ASTRAL utilizing the five maximum likelihood gene trees had a normalized quartet score (e.g., the portion of input quartet trees satisfied before correcting for multiple individuals) of approximately 0.82 and quartet score of 97065491. Additionally, given the use of only 5 input gene tree topologies, posterior probabilities and other statistics relating to branch length and nodal support are demarcated as unreliable. ASTRAL output warnings for each clade as the effective number of genes impacting the inference ranged from as low as a single gene, to the maximum of five genes.

As opposed to all previous analyses, the topology of the tree inferred via multispecies coalescence did not recover Assamiidae as monophyletic (Fig. 5). The Erecinae taxon As135\_Viet is recovered in a polytomy (PP = 0.83) including the Gonyleptidae, Cosmetidae, Nomoclastidae, Cranaidae, and remaining Laniatores. Additionally, the Erecinae taxon As105\_Phil also sits in a polytomy with the derived members of Grassatores (PP = 0.31), while As098\_Phil sits in a polytomy with lineages of Epedanidae (PP = 0.42). The larger clade of Assamiidae also has little resolution, given it sits in a polytomy with Podoctidae, Agoristenidae, Petrobunidae, and Epedanidae (PP = 0.66).

Within the Laniatores, rather than a monophyletic or paraphyletic Insidiatores sitting sister to the Grassatores, the Grassatores family Stygnidae now sits as sister to the remaining Laniatores (PP = 0.69). Additionally, the infraorder Insidiatores are now polyphyletic, with the genus *Synthetonychia* sister to the remaining Laniatores (PP = 0.61). The other lineages of Insidiatores are monophyletic but occupy a highly derived position sister to the Phalangodidae (PP = 0.70). Many families of Grassatores recovered as monophyletic in previous analyses are now either paraphyletic or polyphyletic. In a single clade, the families Gonyleptidae and Cosmetidae are now polyphyletic, with nested positions of Nomoclastidae (PP = 0.67) and Cranaidae (PP = 0.84). Similarly, the family Kimulidae is now polyphyletic, with the genus *Minuella* sitting in a higher level polytomy, while the genus *Kimula* nests within the clade of Guasniidae, Escadabiidae, Icaleptidae, Zalmoxidae, and Fissiphallidae (PP = 0.83). However, the families Samoidae, Biantidae, and Stygnommatidae are recovered in an identical topology to previous analyses.

Within Assamiidae, the African taxa no longer appear monophyletic, and the previously monophyletic Irumuinae are now polyphyletic. The Trionyxellinae, previously recovered as nested within the Asian Polycoryphinae, are now sister to a single lineage of Irumuinae (PP = 0.87). Mysoreinae (PP = 0.44) and the Hypoxestinae genus *Bandona* (PP = 0.95) are, however, still recovered as nested within the Asian Polycoryphinae genus *Paktongius*.

Dampetrinae were likewise paraphyletic within the Asian Assamiidae to the exclusion of the nested Erecinae genus *Neopygoplus* (BS = 58.4%, SH-aLRT = 68%). Polycoryphinae is recovered as polyphyletic, with African Polycoryphinae paraphyletic to the exclusion of nested lineages of the Erecinae genus *Chilon* (BS = 98.1%, SH-aLRT = 99%; BS = 98.5%, SH-aLRT = 82%). A sole Polycoryphinae terminal, As108\_Laos, also nests as sister to the clade of *Arulla* (Erecinae) and the African Assamiidae (BS = 85.4%, SH-aLRT = 67%). The remaining Hypoxestinae lineages nest as a monophyletic clade within the larger African Assamiidae clade (BS = 99.9%, SH-aLRT = 100%). The Hypoxestinae, Selencinae, Dampetrinae, and Erecinae are also widespread throughout the Assamiidae, although the Erecinae genera *Arulla* (PP = 0.87) and *Neopygoplus* (PP = 0.67) are individually monophyletic.

**Discussion**

For decades, taxonomists have questioned the validity of the internal evolutionary relationships among the assamiid Opiliones (Starega 1992; Kury 2007). The challenge of resolving these relationships is personified by the more than 250 genera and 17 subfamilies questionably “described” by Roewer (1912; 1935), making confident identification of specimens to the subfamily or genus level difficult. Yet, recently, Palmieri et al. (2023) published the first molecular phylogenetic analysis of the group, sampling representatives of eight of the extant Roewerian subfamilies from across their Afrotropical and Asian distributions, in addition to numerous lineages of both Laniatores infraorders (Insidiatores, Grassatores) to evaluate the monophyly and placement of the family within the larger Opiliones tree of life. To evaluate the robustnesses of the obtained topology, the same dataset has here been inferred under three different analytic methodologies. Large-scale congruence in topology, however, lends increased support to the proposed taxonomic changes of Palmieri et al. (2023).

Palmieri et al. (2023) recover the monophyly of Assamiidae under Bayesian inference with maximal support (PP = 1). Likewise, maximum likelihood analyses utilizing both sequential and partitioned concatenated datasets recover Assamiidae monophyly, albeit with lower support (BS = 82.3%, BS = 82.9%). However, a multispecies coalescence approach implemented in ASTRAL failed to recover Assamiidae monophyly, with three Erecinae lineages nesting in distinct Laniatores polytomies. Yet, support for these placements is minimal given As105\_Phil is placed with a posterior probability of 0.31 and As098\_Phil with posterior probability of 0.42. Assamiidae monophyly was also recovered via Bayesian and maximum likelihood inferences of a 10-locus Sanger dataset in Aharon et al. (2019), although the authors’ sampling of assamiid taxa was significantly reduced (PP = 0.61, BS = 66%). Given the dubious nature of the multispecies coalescent topology, and congruence in topology between maximum likelihood and Bayesian phylogenies, Starega’s (1992) morphology-based rejection of Assamiidae monophyly can be confidently refuted.

The placement of Assamiidae within the larger Laniatores infraorder, however, is questionable based on multiple sister group relationships between the concatenated and multispecies coalescent trees. Both concatenated maximum likelihood analyses recover Assamiidae as sister to the family Epedanidae (Opiliones, Laniatores, Grassatores). Analysis in ASTRAL, however, recovers the largest clade of the polyphyletic Assamiidae in a polytomy with unresolved relationships with the families Epedanidae, Agoristenidae, Petrobunidae, and Podoctidae. Yet, Palmieri et al. (2023) recover a sister relationship with Pyramidopidae (PP = 0.72), and several other studies also recover this relationship with reasonably high support (Sharma and Giribet 2011, BS = 79%; Aharon et al. 2019, PP = 1). Of note, however, is that these studies made use of ten, rather than five, molecular markers and fewer sampled lineages. This is significant in that Rokas and Carroll (2005) demonstrate that increases in taxon number correlates with decreases in phylogenetic accuracy, while increasing the number of analyzed loci has a highly significant positive correlation with accuracy.

The phylogenetic placement of the remaining Laniatores families were largely congruent between the novel analyses and previous studies, with the exception of the multispecies coalescent topology. For the sake of brevity, only those families appearing as non-monophyletic will be discussed. Both concatenated datasets recover polyphyletic Samoidae, Biantidae, and Stygnommatidae with identical topologies. Likewise, Sharma and Giribet (2011) and Aharon et al. (2019) recover the same topologies, albeit lacking identical taxon sampling. With repeated recapitulation of this topology, Biantidae, Samoidae, and Stygnommatidae should be primary targets of systematic revision. The concatenated datasets also recover a paraphyletic Gonyleptidae given the exclusion of the nested Stygnidae and Cranaidae, in line with previous studies (Sharma and Giribet 2011; Aharon et al. 2019), suggesting the Gonyleptoidea as another target for revision.

Within Assamiidae, novel analyses lend additional support to the spurious Roewerian subfamily classification system. The subfamily Mysoreinae, containing only two species (*Mysorea thaiensis, Mysorea brevipes*), was recovered in all analyses as nested within a clade of the Asian Polycoryphinae genus *Paktongius*, just as Palmieri et al. (2023) recovered with near maximal support (PP = 0.99), suggesting that at least the sampled *Mysorea thaiensis* may actually be a derived member of *Paktongius*. *M. thaiensis* does exhibit apparent morphological similarity to *Paktongius*, with a charismatic lateral expansion of the posterior scutal territory driven by a hypertrophied fourth walking leg (Zhang and Zhang 2015). Similarly, the genus *Bandona,* putatively a member of the subfamily Hypoxestinae, is also nesting within *Paktongius* in all three analyses and in Palmieri et al. (2023). This case, however, suggests misidentification given the specimen demonstrates the same lateral expansion of the scutum as *Paktongius* and *Mysorea*, rather than the cylindrical body shape seen in other extant species of *Bandona*. Dissimilarity in armature and pigmentation patterns, suggests it may be a novel species of *Paktongius.*

The only subfamily recovered as monophyletic in the concatenated datasets and Palmieri et al. (2023) was the small-bodied, blind Irumuinae, always recovered as nested within a clade of solely African Assamiidae. Dampetrinae is recovered as paraphyletic to the exclusion of the Erecinae genus *Neopygoplus*. Polycoryphinae is polyphyletic due to African lineages nesting within the African clade, rather than appearing as derived members of the Asian Polycoryphinae. Selencinae is also polyphyletic within the African clade. Finally, Erecinae lineages show different affinities, with *Chilon* paraphyletic to the exclusion of a subset of African Polycoryphinae, while *Arulla* sits as sister to the African clade. In total, consistent evolutionary relationships across disparate analyses and under different inference strategies highlights the necessity for the redefinition of existing assamiid subfamilies to increase their suitability for biogeographical studies and comparative genetic and developmental studies.

While the analyses presented here, tend to support the relationships observed in previous molecular phylogenies of both Laniatores and Assamiidae, differences in topology and nodal support values are readily apparent between the three novel analyses. Of particular note, is the failure of the multispecies coalescent approach to recapitulate many of the relationships observed in the concatenated maximum likelihood analyses and Bayesian analysis performed by Palmieri et al. (2023). The tree topology produced by ASTRAL is characterized by numerous polytomies, and a significant proportion of low posterior probabilities for individual nodes. For example, the ASTRAL tree suggested a nested position of the suborder Dyspnoi within Eupnoi with minimal support (PP = 0.44), while all other analyses recover a sister group relationship of the two suborders, in turn sister to the Laniatores. The likely reason for ASTRAL’s poor performance is the estimation of the species tree based on only five gene tree topologies. Rabiee et al. (2019) clearly demonstrate that the accuracy of ASTRAL species tree construction is improved by the additional of more loci. This improvement drastically outweighs any potential benefit of greater taxon sampling, even when branch lengths are short and incomplete lineage sorting is high. Outputs from ASTRAL support the inaccuracy of the analysis relying on only 5 loci, given that each node was label with a warning that posterior probabilities should be ignored given the low number of effective genes impacting the topology. Many nodes were also impacted by effectively only one gene due to the high sequence conservation of the chosen molecular markers. However, simulation studies have shown that concatenated maximum likelihood analyses can be more accurate than ASTRAL when true gene tree discordance is low (Mirarab and Warnow 2015). There is evidence that highly conserved, slowly evolving genes, like the legacy markers used in these analyses, tend to have low gene tree discordance, which could explain the better performance of concatenation as opposed to ASTRAL (Betancur-R. et al. 2014). Additionally, Mirarab (2019) acknowledges that the statistical consistency of ASTRAL can be compromised when gene trees are constructed via maximum likelihood. However, due to time and CPU constraints, Bayesian gene tree topologies could not be constructed in this analysis. In comparing the performance of the two concatenated tree topologies, the partitioned analysis produced a tree with a slightly better log-likelihood at -98831.2274 while the sequential dataset produced a log-likelihood of -100937.968, despite a higher number of free parameters. There is evidence to suggest that under-parameterized models can lead to long-branch attraction and phylogenetic error, perhaps providing an explanation for the slight improvement in likelihood obtained via partitioning (Sullivan and Swofford 1997).

While the results of these analyses in comparison to previously published Laniatores and assamiid phylogenies highlight the necessity for systematic revision of higher-level relationships amongst the assamiids, a fully resolved phylogeny of the group remains elusive. In utilizing the dataset of Palmieri et al. (2023), we are limited to only those taxa that could reliably be identified to genus under the Roewerian system. As such, only eight of the 17 Roewerian subfamilies were surveyed. For example, sequences could not be obtained for Assamiidae from large swaths of India and Nepal, representing the primary range of the subfamily Assamiinae. Likewise, assamiids native to eastern and southern Africa were not surveyed. It is also apparent from previous studies of early-diverging arachnid groups that Sanger datasets simply fail to provide sufficient loci for resolution of higher-level relationships (Starett et al. 2016). To improve resolution of the internal relationships in Assamiidae, future studies should leverage the sequencing of UCEs, given their usefulness in extracting usable sequences from degraded museum specimens and propensity to yield hundreds, if not thousands, of loci when probe sets are tailored to a narrow range of taxa (van Dam et al. 2019; Kulkarni et al. 2020). However, implementation of an Opiliones-specific probe set will first require the generation of additional genomic resources in the order to complement the existing genome for *Phalangium opilio*.

**Figure Legends**

The large size of generated phylogenies prevented insertion in the manuscript in an interpretable format. Figures are instead available in the ~/figures/manuscript\_figures/ subdirectory of this project’s GitHub repository (<https://github.com/bklementz/563-Final-Project>).

**Fig. 1 |** Maximum likelihood phylogenetic tree topology of the Laniatores based on sequential concatenation of the five loci multisequence alignments implemented in IQ-TREE. The numbers on nodes represent both ultrafast bootstrap resampling frequencies and SH-aLRT scores, respectively. Branch lengths measured in average number of nucleotide substitutions per site with scale bar equivalent to 0.07 substitutions per site. Constituent members of Laniatores families appearing non-monophyletic have been highlighted with red (Samoidae), green (Biantidae), blue (Polycoryphinae), and purple (Gonyleptidae) labels. The focal family Assamiidae has been assigned an orange label. Identification of taxon labels to family obtained from Supplementary Table S1 in Palmieri et al. (2023).

**Fig. 2 |** Maximum likelihood phylogenetic tree topology obtained via sequential concatenation, as in Fig. 1, displaying only the monophyletic Assamiidae clade. Numbers on nodes represent both ultrafast bootstrap resampling frequencies and SH-aLRT scores, respectively. Branch lengths measured in average number of nucleotide substitutions per site and scale bar equivalent to 0.2 substitutions per site. The traditional morphology-based subfamilies have been highlighted with green (Erecinae), red (Hypoxestinae), purple (Selencinae), pink (Irumuinae), and yellow (Dampetrinae) labels, aside from the single terminals of Mysoreinae and Trionyxellinae.

**Fig. 3 |** Maximum likelihood phylogenetic tree topology of the Laniatores based on concatenation with partitioning by locus and codon position of the cytochrome oxidase *c* subunit I locus implemented in IQ-TREE. Number on nodes represent both ultrafast bootstrap resampling frequencies and SH-aLRT scores. Branch lengths are measured in average number of nucleotide substitutions per site. Scale bar length is equivalent to 0.2 substitutions per site. Laniatores families have been highlighted with the same color scheme as in Fig. 1.

**Fig. 4 |** Maximum likelihood tree topology obtained via concatenation with partitioning and displaying only the monophyletic Assamiidae. Numbers on nodes represent ultrafast bootstrap resampling frequencies and SH-aLRT scores. Branch lengths are measured in average number of nucleotide substitutions per site. Scale bar length is equivalent to 0.2 substitutions per site. Morphology-based subfamilies have been color-coded in line with Fig. 2.

**Fig. 5 |** Multispecies coalescence tree topology obtained from ASTRAL analysis of maximum likelihood gene tree topologies for each locus implemented in IQ-TREE. Numbers on nodes represent posterior probability estimates. Branch lengths correspond to average number of nucleotide substitutions per site. Scale bar is equivalent to two substitutions per site. Given non-monophyly of Assamiidae, only assamiid subfamilies have been color coded following the schemes defined in Figs. 2 and 4. Non-monophyletic Laniatores families have been appended with dual asterisks (\*\*).

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