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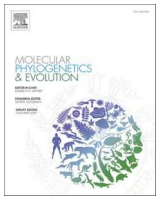

Phylogenomic analyses of Blattodea combining traditional methods, incremental tree-building, and quality-aware support

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ABSTRACT

Despite the many advances of the genomic era, there is a persistent problem in assessing the uncertainty of phylogenomic hypotheses. We see this in the recent history of phylogenetics for cockroaches and termites (Blattodea), where huge advances have been made, but there are still major inconsistencies between studies. To address this, we present a phylogenetic analysis of Blattodea that emphasizes identification and quantification of uncertainty. We analyze 1183 gene domains using three methods (multi-species coalescent inference, concatenation, and a supermatrix-supertree hybrid approach) and assess support for controversial relationships while considering data quality. The hybrid approach—here dubbed "tiered phylogenetic inference"—incorporates information about data quality into an incremental tree building framework. Leveraging this method, we are able to identify cases of low or misleading support that would not be possible otherwise, and explore them more thoroughly with follow-up tests. In particular, quality annotations pointed towards nodes with high bootstrap support that later turned out to have large ambiguities, sometimes resulting from low-quality data. We also clarify issues related to some recalcitrant nodes: Anaplectidae's placement lacks unbiased signal, Ectobiidae s.s. and Anaplectoideini need greater taxon sampling, the deepest relationships among most Blaberidae lack signal. As a result, several previous phylogenetic uncertainties are now closer to being resolved (e.g., African and Malagasy "*Rhabdoblatta*" spp. are the sister to all other Blaberidae, and Oxyhaloinae is sister to the remaining Blaberidae). Overall, we argue for more approaches to quantifying support that take data quality into account to uncover the nature of recalcitrant nodes.

1. Introduction

The current golden age of phylogenomics is characterized by huge datasets (Pyron, 2015), highly efficient analytical algorithms, and improved implementation of evolutionary models (e.g., Zhang et al., 2018; Minh et al., 2020b). It is easier than ever to reconstruct an impressive-looking phylogeny. Yet, none of these advances diminish the importance of assessing phylogenetic support (Simon, 2022) and exposing the defects of a phylogenetic hypothesis. For instance, although calculation of node support values is nearly ubiquitous, their usefulness in phylogenomics is limited (Simon 2022). Topologies and support values derived from huge datasets can still be confounded by outlier genes (Walker, et al., 2018), problematic missing data patterns (Dell'Ampio, et al. 2014), and estimation error (Bossert et al., 2021).

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Phylogenetic uncertainty is so pernicious that multiple tests of support are needed to fully describe it, even with small datasets (Evangelista et al., 2018). Phylogenetic uncertainty falls into several categories. Lack of support can occur when ancient populations were short-lived and little evidence of them remains (Townsend 2007). Conflicting support can also occur (Pease et al., 2018). One example is when true gene trees are discordant with the true species tree (Degnan and Rosenberg, 2009). Topological uncertainty of all types can derive from biases in nature (Strimmer and von Haeseler, 1997; Shen et al., 2017), such as nucleotide compositional bias (Foster and Hickey, 1999), or from experimental error—e.g., stochastic error (Shimodaira, 2002), missing data patterns (Dell'Ampio et al., 2014), or assembly errors creating outliers (Shen et al., 2017). These effects often occur simultaneously. For instance, long branch attraction (LBA) can result as a combination of real-world bias and the lack of correctly handling that bias with appropriate modelling of rate variance (Wagele and Mayer, 2007).

These mosaic sources of uncertainty can be identified and overcome by contrasting genetic loci of differing quality (Townsend, 2007; Wagele and Mayer, 2007; Evangelista et al., 2020; Mongiardino, 2021; Duchene et al., 2022). For instance, a phylogenetic analysis of loci with many quickly substituting sites will reveal relationships deriving from LBA (Wagele and Mayer, 2007) but perhaps has higher support on short internodes (Källersjö et al., 1999). The opposite might reveal a topology that is lacking LBA but also lacking support for short internodes (Evangelista et al., 2020). Thus, by leveraging heterogeneity in locus quality, we can infer phylogenies with better character support and better describe uncertain nodes.

We apply this approach using a phylogenomic dataset of cockroaches and termites (Blattodea). Recent studies have investigated the phylogenetic relationships of these insects with transcriptomes (Evangelista et al., 2019b; Liu et al., 2023), anchored hybrid enrichment (Evangelista et al., 2020; Evangelista et al., 2023), and mitochondrial genomes (Bourguignon et al., 2018; Li, 2022; Wang et al., 2023; Han et al., 2024). While most recent systematic work has focused on relationships within superfamilies (Djernæs et al., 2020; Djernæs and Murienne, 2022; Deng et al., 2023; Malem et al., 2023; Wang et al., 2023; Han et al., 2024) some work has been done at the level of the whole taxonomic order (Evangelista et al., 2019b; Evangelista et al., 2023; Liu et al., 2023). These studies have made strides in pushing forward our understanding of phenotypic evolution and classification (Djernæs et al., 2020; Deng et al., 2023; Evangelista et al., 2023; Liu et al., 2023; Han et al., 2024).

Less focus has been given towards deeply exploring support, or lack thereof, for the Blattodea phylogeny. Recent studies have used statistical frameworks for ruling out alternative placements (Evangelista et al., 2019b; Djernæs et al., 2020; Evangelista et al., 2020; Evangelista et al., 2023; Liu et al., 2023; Wang et al., 2023), tested signal for controversial relationships with quartet mapping (Evangelista et al., 2019b; Liu et al., 2023), assessed topological support using state-of-the-art approaches (Evangelista et al., 2023; Liu et al., 2023; Wang et al., 2023; Han et al., 2024), incorporated a multi-species coalescent framework (Evangelista et al., 2023; Han et al., 2024), and explored the effect of evolutionary models and gene tree choice (Evangelista et al., in review, 2024). Yet, only one study has attempted to analyze the effect of data quality on tree uncertainty in these insects Evangelista et al. (2020).

Evangelista et al. (2020) examined the effects of locus quality on support for the phylogeny of superfamily Blaberoidea. Their results are intended to justify more efficient analyses of smaller, but highly informative, datasets. This study has, however, three shortcomings. The first is a taxonomic sampling that lacks breadth in key areas of the tree. Second, despite their findings, there are drawbacks to filtering low-quality data (Tan et al., 2015). Third, there is a lack of attention to robust testing of controversial relationships. Here, we address all three issues with a multi-faceted approach to tree inference and support testing. In our taxon- and locus-expanded analyses, we curate a series of support tests to establish support for novel relationships, differentiate lack of support from conflicting support, and identify the sources of uncertainty where possible. We end up with a phylogenomic hypothesis based on hundreds of loci and with a critical assessment of support.

Better understanding the Blattodea phylogeny can resolve some historical questions. First, Anaplectidae is a rogue lineage that has not been consistently placed in molecular (Djernæs and Murienne, 2022; Deng et al., 2023; Evangelista et al., 2023; Liu et al., 2023) or combined studies (Djernaes et al., 2015). Similarly, while Lamproblattidae has recently been established as sister to Xylophagodea (Evangelista et al., 2019b; Evangelista et al., 2020; Liu et al., 2023), other studies disagree (Djernæs et al., 2020). Third, there is particularly troubling incongruence of transcriptomic data (Evangelista et al., 2019b) with morphological and other genomic data (Engel et al., 2009; Bucek et al., 2019; Hellemans et al., 2022) for the position of *Mastotermes* relative to all other termites. One historical problem in Blaberoidea phylogenetics has been a lack of taxon sampling within *Ectobiinae*. Also, *Anaplectoidea* likely comprises a deep lineage in Blaberoidea but its placement has only just begun to be tested (Evangelista et al., 2023; Wang et al., 2023). Sixth, Blattellidae is one of the largest cockroach families but its internal relationships have never been thoroughly assessed. Finally, a large amount of attention has previously been paid to Blaberidae systematics (Legendre et al., 2014; Legendre et al., 2015; Legendre et al., 2017; Evangelista et al., 2018; Djernæs et al., 2020; Evangelista et al., 2020; Evangelista et al., 2023; Wang et al., 2023), but almost no progress has been made in establishing sub-familial interrelationships.

While address these major aims, and some more minor ones, in phylogenomic analyses combining traditional concatenation, a coalescent method, and a novel incremental tree-building approach with follow-up support testing. Contrasting these three approaches provides diverse perspectives on the phylogenetic relationships that consider emergent (Gatesy et al., 2018), incomplete lineage sorting (Degnan and Rosenberg, 2009), and data deficits (e.g., occupancy, noise). Each approach also has its own measures of topological support that provide systematic insight. Finally, by utilizing statistical tests (Table S2.6) we can verify findings of the aforementioned analyses.

2. Methods

2.1. Data collection and processing

We compiled transcriptomes, full genomes, and target enrichment data of Blattodea Brunner, 1882 used in previous studies (Evangelista et al., 2019b; Evangelista et al., 2020; Evangelista et al., 2023). We also include newly sequenced taxa (see the following for detailed methods: Evangelista et al., 2020; Evangelista et al., 2023). Newly included taxa were: (i) rare taxa with weak systematic hypotheses (*Proscratea* sp., *Chrastoblatta dimidiata*, *Onycholobus ectobioides, Parellipsidion* sp., *Phyllodromica maculata*); (ii) phenotypically aberrant taxa (e.g., *Stayella rohdei*); (iii) taxa chosen to increase representation among Afrotropical taxa (*Derocalymma* sp., *Zuluia* sp. cf. *lithostrata*, *Hyposphaeria* sp. cf. *pilosa*, *Pronauphoeta viridula, Rhabdoblatta* sp. Madagascar, *Ectobius* sp. cf. *textilis*, *Nondewittea globulifera*); and (iv) taxa chosen to improve taxon sampling of Oxyhaloinae and Gyninae (*Heminauphoeta* sp., *Brachynauphoeta foulpointensis, Ateloblatta* sp., *Jagrehnia madecassa*, *Leozehntnera maxima*, *Pseudocalolampra* sp. cf. *pardalina*, gen. cf. *Pseudocalolampra*, *Alloblatta nugax*). See Supplementary data for total taxon list, including accession numbers. 203 samples were in the initial dataset. 17 taxa were outgroups, with *Ladona fulva*—a dragonfly—being the most distant outgroup. Taxon and molecular data sampling protocols, and preliminary bioinformatics generally follow protocols in Evangelista et al. (2020). Major departures occur after the stage of 1:1 ortholog assessment. Instead of limiting the data to 265 loci targeted *a priori*, as in Evangelista et al. (2020), we began with all 4033 single-copy orthologous regions. These were first reduced to loci covered in > 25 taxa. Further reductions were accomplished using the combination of manual and automated filtering discussed below.

2.2. Generating the dataset: Homology assessment and locus filtering

Preliminary alignments were done in MAFFT v. 7.487 (–retree 2 –maxiterate 2 –adjustdirection; Katoh and Standley, 2013). Loci were sorted by the percentage of total gap characters (-,?, or N) and minimum number of codons, and subsets were visually examined. Since the combined dataset was designed based on transcriptomes, it is expected that the vast majority of sequences will be protein coding, and thus the number of stop codons is in some way informative for the quality of the alignment. Thus, the minimum number of stop codons per alignment were counted and all alignments with more than 15 stop codons were considered too difficult to align (n = 2152). Those with ≤ 15 % gap characters (n = 605) were determined to be largely free of errors, and rarely had stop codons. Loci with a moderate percentage of gap characters (15–50 %) were examined and determined to often need manual modifications (e.g., trimming messy ends, introns, or other rapidly evolving regions; n = 446). Most loci with > 50 % of gap characters appeared to have too many errors to be easily fixed through manual editing. All of these with more than five stop codons were removed (n = 1915). 558 additional loci were also removed for intractable alignment problems. The remaining "hard to align" loci were manually adjusted and retained. At this point, there were 1560 loci in the dataset.

All alignments were automatically trimmed using a custom script with the following parameters. To remove taxa for which the locus was poorly sequenced, we trimmed the 15 % of taxa with the most missing data (avg. of 10 taxa per alignment). We trimmed low occupancy nucleotides from the ends of the alignment until reaching a position with 80 % occupancy. If these steps reduced the number of taxa below 25, the locus was removed from the dataset (n = 372). All loci were reading-frame adjusted and then realigned more thoroughly in MAFFT (–localpair –maxiterate 1000). We repeated the steps of counting stop codons, manually reexamined alignments with > 5 stop codons present, and discarded any alignments deemed inappropriate for the final analysis (n = 5). The final number of loci was 1183, comprising ~ 1 million nucleotide positions. This dataset was analyzed in the following phylogenetic pipeline (Fig. 1).

2.3. Alignment information content assessment

We assessed the information content of each locus alignment for the purpose of better designing the concatenation analysis to reduce erroneous inferences. The assessment was designed based on information from previous studies (Evangelista et al., 2020; Vasilikopoulos et al., 2021). The quality metrics assessed were: number of outlier tips in gene tree (rogue taxa), substitution rates and heterogeneity (locus rates), and missing data proportions (locus completeness).

We assessed rogue taxon information through analysis of gene trees. 1183 gene tree histories were inferred using IQ-TREE2 (Minh et al., 2020b) (-m MFP -rcluster 10 -mrate G,R -wsr -allnni -T AUTO) and rooted with an outgroup prioritizing the most distant outgroup tip. Three automated gene tree pruning methods and one clade-based pruning method were used to identify and filter rogue taxa (see Aberer et al., 2013). First, we calculated the root-to-tip distances (r2td) of every tip taxon in each gene tree. We then fit a gamma distribution to these values and simulated 9999 random branch lengths on that distribution. We chose a gamma distribution because its shape is adaptable for the very different potential topologies of gene trees, and it could effectively model right-skewed branch length distributions. Any actual tips that were longer than the 95th percentile of randomized branch lengths were pruned from the gene tree. Second, the same process was repeated but with pairwise-tip-distances (ptd) and the 99th percentile was used as a cutoff. Different cutoff points were used because the ptd method was much more sensitive to outliers. In the third method, the r2td and ptd methods were repeated recursively on the same gene tree until the topology was stable and no outlier tips remained. An additional monophyly-based pruning method used the MonoPhy R package (Schwery and O'Meara, 2016) (AssessMonophyly, outlierlevel = 0.75) on all raw gene trees, and gene trees pruned under the prior three methods. However, clade-based filtering was only used as a comparison since there is a limited expectation of clade monophyly in gene trees due to incomplete lineage sorting. We recorded the average percentage of rogue taxa (outlier tips) pruned from an alignment under each method. The avg. percentage of outlier tips was then used to categorize loci into four class tiers (Table S2.3.3, Fig. S2.3.3).

From manual inspection of the locus alignments with the most rogue tips, we identified that outlier tips were related to one or more potential

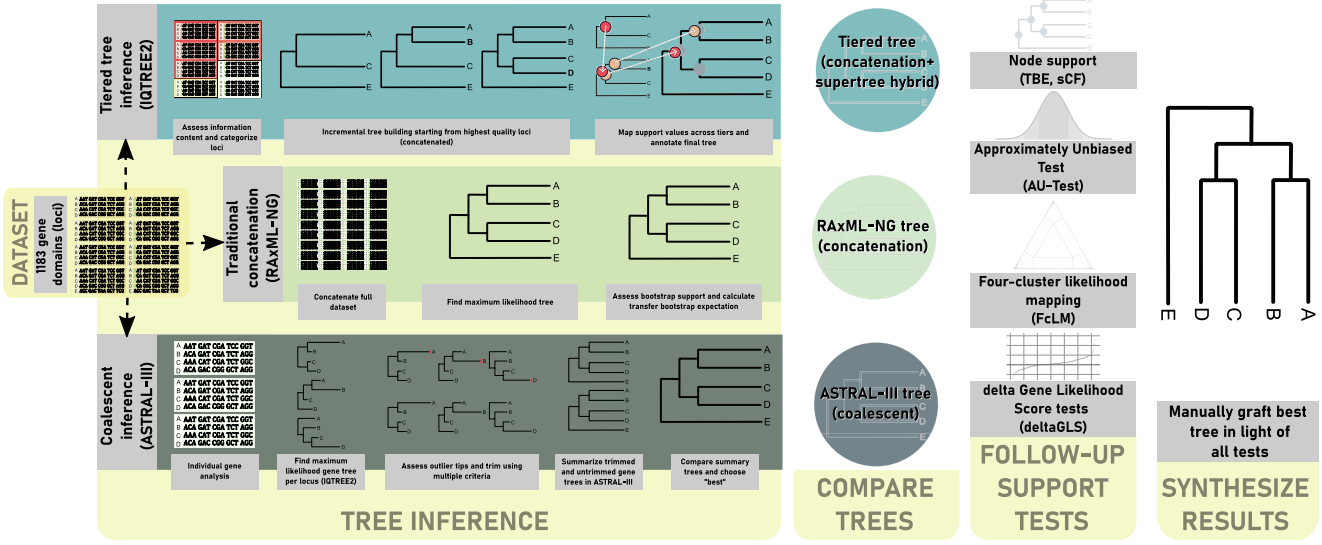


Fig. 1. Overview of phylogenetic pipeline after dataset was composed. Starting from left, data set (comprising 1183 gene domains) was analyzed three ways: (top) a tiered analysis leveraging information about locus information content (IQ-TREE2), (middle) a traditional concatenation analysis in RAxML-NG, and (bottom) a coalescent method in ASTRAL-III. Then, all trees (and individual tiers of tiered analysis) were compared to each other and to the literature. Follow-up support tests were designed to test controversial, low supported, or uncertain relationships. In the final step, all results were synthesized and illustrated in a manually grafted tree. Details for each step are given in methods section and supplementary methods.

issues (e.g., saturated sites, short locus length, alignment errors, sequencing errors, intrusion of non-homologous loci). Therefore, we decided to further categorize loci by: (i) median pairwise sequence distance and (ii) mean rate heterogeneity. (i) Median pairwise sequence distance was calculated by taking the median of the pairwise distance matrix (calculated in IQ-TREE2). (ii) Rate heterogeneity used rate categories calculated in IQ-TREE2. We identified the maximal rate of the slowest rate category and the minimal rate of the fastest rate category and normalized them by the max value for each category. The ratio of the two normalized values, a number between 0 and 1, is a measure of heterogeneity, loci with higher values being more heterogeneous and those with lower values being more homogeneous. Numbers from i and ii were assigned scores (Tables S2.3.1 and S2.3.2). Scores were summed to determine the "rate class" used to further categorize loci (Table 1, Figs. S2.3.1, S2.3.2).

Finally, we calculated the taxon occupancy of each locus. These were used to, again, categorize loci based on their optimality for reliable phylogenetic inference (Table 1; completeness class). Locus occupancy for each taxon was also calculated (Fig. S2.3.4).

2.4. Tiered concatenation inference

Locus quality categories were combined into four tiers, as shown in Table 1. Concatenated data was analyzed in increasing numeric order (i.e., from Tier 1 up to Tier 4) and the maximum likelihood (ML) tree from each tier served as a constraint tree for the subsequent tier. Nodes with < 95 % ultra-fast bootstrap (UF-bootstrap) support were collapsed in the constraint tree.

ML trees and support values were obtained through a partitioned analysis in IQ-TREE2 (Chernomor, et al. 2016; Kalyaanamoorthy et al., 2017; Hoang et al., 2018; Naser-Khdour et al., 2019). Partition files were generated with gene boundaries and codon position (1st, 2nd, 3rd) boundaries. The input code for each tier analysis is given in Supplementary Material S2.4. Preliminary rounds of the Tier 3 and 4 analyses helped to identify extreme outlier branch lengths signaling long branch attraction. After source alignments were reviewed, five problematic tips were removed from the analysis (see Supplementary Material S2.5). Note that at each tier, only loci present for newly added taxa were included. For instance, if a locus met the criteria for Tier 3 except that it did not have data present for a taxa newly added in Tier 3, it was not included in that analysis. This was to minimize the redundancy of each tree search and maximize computational efficiency.

The tiered tree contained 198 tips and support values from ultrafast bootstrap. However, not all support values were meaningful because the Tiers 2–4 analyses were constrained by previous analyses. All constrained nodes would have 100 % support if taxon sampling were the same throughout all tiers. However, newly added taxa intruding on a split in some of the pseudoreplicate trees could reduce that split's support. Regardless, these split support values are not comparable to the remaining and we thus developed the R code "*AwareSupport*" to map annotated support values onto our tree. The functions, including a tutorial script and example data, are available on GitHub. The algorithms are described in detail in Supplementary Material S2.6.1 and summarized in Fig. S2.6.1. We deployed *AwareSupport* with trees from our four-tiered analysis, each of which was rooted in a non-dictyopteran taxon. Each support value on the final tree has both a magnitude (i.e., UF-bootstrap proportions) and an annotation that identified the tier of the analysis where this support value derived from. In combination, we refer to these as quality-aware support (QA-support).

We additionally calculated site concordance factor (sCF) branch support values for each tier. sCF values indicate the proportion of sites supporting a given branch, after removing missing data (Minh et al., 2020a). sCF values range from 0–100 % with any values over 33 % indicating varying degrees of support for the relationship shown. 33 % support indicates completely equal support for all three alternative quartets.

Since QA-support values have not been used before, they should be put in context with more traditional support values. Using the best topology from the tiered analysis we estimated UF-Bootstrap values from the complete concatenated dataset in IQ-TREE v 2.0.7. The input alignment length was 978,759 nucleotides long with 531,489 parsimony informative characters. Modelfinder (options: -mset GTR -rcluster 25 -m TESTNEWMERG -nt 60) took 2584 h of CPU time and 58 h of wall time using 3.2 GB RAM and 60 threads to identify 196 partitions. Tree searches using the calculated partitioning scheme all failed; therefore we performed an intermediate run using option -te to calculate likelihood of the tree under the full dataset. The resulting output files were used in a final IQ-TREE2 run to calculate UF-bootstrap trees (options -bsam GENESITE -bb 1000 -wbt). The CPU time for this final tree search was 517 h (118 h wall-time). Comparisons between the UF-bootstrap values and the QA-support values were done using custom scripts in R.

Tier 4 relationships were compared to two subsequent tree inferences and numerous support tests (Fig. 1; and see below). For ease of interpretation, several *ad hoc* taxonomic terms were used (Table S1.1).

2.5. Stand-alone tree inference methods

We used RAxML-NG to estimate a tree from the full concatenated dataset using the partitioning scheme identified by ModelFinder in IQ-TREE2. With 50 starting trees (25 random, 25 parsimony) we identified the maximum likelihood tree (lnL -15177209.1) in $\sim$ 304 h CPU time. We also pursued bootstrapping using the autoMRE stopping criteria and 1000 maximum BS replicates. After allowing the analysis to run for $\sim$ 1 month wall-time, it had only completed 219 bootstrap replicates, despite being parallelized over 4 threads and 10 workers. We accepted these results as they were, without reaching the autoMRE stopping criterion. We mapped bootstrap support values over the RAxML-NG topology using the transfer bootstrap expectation (TBE) method (Lemoine et al., 2018).

Finally, the 1183 gene tree histories discussed above were used to

Table 1
Description of the four tiers of concatenated data for phylogenetic analysis.

Tier name	Rogue-taxon class	Rate class	Completeness class*	No. loci	Mean locus length	Align. length	No. distinct patterns	Parsimony informative sites	No. Taxa	Description
1	A, B	A, B	A	172	1083	186,414	104,519	75,825	35	Backbone. Mostly transcriptome data.
2	A, B, C	A, B	A, B	261	1064	277,518	189,336	132,637	95	Expanded backbone.
3	A, B, C	A, B, C	A, B, C	390	1108	432,147	333,669	238,746	180	All reliably sequenced taxa. To reduce computational burden, we eliminated loci lacking data for taxa newly added.
4	A, B, C, D	A, B, C	A, B, C, D	70	812	56,835	41,828	29,344	203	Remaining taxa and data of any quality.

Notably, only loci present for newly added taxa were included at each tier. For instance, if a locus met the criteria for Tier 3 except that it did not have data present for a taxa newly added in Tier 3, it was not included for that analysis. Hence, the total amount of data does not increase between every tier.
*Note that completeness is a feature of taxa, whereas the other categories (# of rogue taxa, substitution rates) are features of loci.

infer a species tree in ASTRAL-III (Zhang et al., 2018) with default parameters. Upon examination, it appeared that numerous clades presumed to be monophyletic were recovered with outlier or intruder taxa. Prior study has shown improvements in species tree inference through filtering of erroneous gene trees (Vasilikopoulos et al., 2021). ASTRAL-III species trees were inferred again using all sets of pruned gene trees and compared. However, species trees from all three trimmed sets did not deviate significantly from one another. We used this species tree as input to *Anomaly Finder* (Linkem et al., 2016) to identify nodes where the most probable gene trees do not match the species tree (Supplementary data). Anomalous nodes are expected in rapid radiations (Degnan and Rosenberg, 2009) but it is unclear how common they are in nature.

2.6. Follow-up support tests

After evaluating the tree by comparing support among tiers and between methods, we used additional tests of support and phylogenetic signal: the Approximately Unbiased Test (Shimodaira, 2002), Four-Cluster Likelihood Mapping (Strimmer and von Haeseler, 1997), and deltaGLS (Shen et al., 2017) correlation tests using ANOVA. Each of the tests have different uses and limitations (Table S2.6), but ultimately rule out improbable topologies and identify phylogenetic bias.

AU Test and FcLM were carried out using IQ-TREE2, and deltaGLS tests were carried out using custom scripts included in the *AwareSupport* R package. The alignments models and trees implemented in each test were customized based on the topological test being done, and they are listed in Supplementary data. In particular, most FcLM tests were carried out using one real dataset and three simulated datasets (Misof et al., 2014) to test for the effect of missing data patterns (simulation 1 and 2), nucleotide compositional bias (simulation 1 and 3), and both (simulation 1). Simulated datasets were generated in Phyloinformatica v0.93.

The deltaGLS values were calculated for each locus containing taxa relevant to the topological test. The log-likelihood of the data under each topology was calculated using the pml function (Schliep, 2011) under the GTR substitution model (estimated nucleotide frequencies), and gamma rate modelling. If more than two hypotheses were estimated, they were compared in a pairwise fashion. To eliminate the effect of outlier genes, we omitted deltaGLSs in the outer 5 % of the distribution. We used two methods to determine how deltaGLS values supported the alternative topologies: a likelihood ratio framework using a *t*-test, and a parametric bootstrap test. The *t*-test assessed if the deltaGLS magnitudes were significantly different among the differing hypotheses (alpha = 0.001). Results were assumed valid if one of the three best fitting distributions was normal, or if the sample size of genes was > 30. The bootstrap test was used to compare the number of genes supporting each topology (alpha = 0.01). We generated null deltaGLS values under a normal distribution with mean 0 and standard deviation estimated from the data. Then, the number of actual deltaGLS values favoring each hypothesis were compared to the null data, and if they exceeded those in 1 % of simulations (n = 1,000) they were considered significant. If either test was significant then the worse hypothesis was rejected.

We also investigated whether deltaGLS values were significantly correlated with potentially biasing factors of the loci: among site rate heterogeneity, mean pairwise sequence distance, the number of rogue taxa in the alignment, and two measures of nucleotide compositional bias. For each pairwise comparison of topologies, we used an ANOVA to look for significant correlations. Any strong correlations were then examined considering the overall signal among deltaGLS values to determine if the results could be biased.

2.7. Synthesizing a final tree

We synthesized the results from all stages of the analysis to decide on a final tree topology. We took the preferred tree and manually modified it in Mesquite (Maddison and Maddison, 2017) in order to reflect the findings from follow up support tests and collapsed splits that were largely uncertain. We then mapped support values (QA-support, RAxML-NG TBE) onto the Tiered tree.

3. Results & discussion

We inferred species trees using two well-known approaches (RAxML-NG concatenation, ASTRAL-III coalescent inference) and one novel method (tiered tree inference), each with support values using multiple approaches (Fig. 1). With some notable exceptions, each tree mostly agrees with the topologies obtained from previous phylogenomic studies (Evangelista et al., 2019b; Blaser et al., 2020; Evangelista et al., 2020; Evangelista et al., 2023; Liu et al., 2023) but differs from others, which are mostly Sanger-data based (Legendre et al., 2015; Wang et al., 2017; Evangelista et al., 2018; Djernæs et al., 2020; Li, 2022; Wang et al., 2023).

Below, we first overview some major incongruences among trees. Then, we assess the tendency of the support values on the tiered phylogenetic inference since this is a novel approach. Afterwards, we discuss how we used the information from the tiered phylogenetic analysis as a starting point for assessing some controversial phylogenetic questions and discuss how these results compare with those from other analyses. In each case, we finish by reviewing all the results in light of previous studies and coming to new conclusions about the evolutionary history of Blattodea.

3.1. Overview of differences between tree inferences

The topologies obtained from each analysis differed in important ways, as did their support values. Poorly sequenced taxa were variably placed among different tree inferences. The ASTRAL-III tree, estimated from 1183 genomic loci, placed some poorly sequenced taxa in the wrong superfamily or family (*Ectobius* sp. cf. *textilis*, *Chrastoblatta dimidiata*, *Anaplecta pulchella*), while the tiered inference and RAxML-NG tree placed them correctly with respect to their traditionally hypothesized classifications. On the other hand, the coalescent and RAxML-NG analyses more accurately placed *Cyrtotria* sp. and *Lanxoblatta* sp.

Incongruence in placement of poorly sequenced taxa is likely due to error, but other differences between coalescent and concatenation results could be indicative of places where ILS was predominant. This is likely the case in Blaberidae because the subfamily interrelationships were incongruent among the ASTRAL-III tree and other analyses (Tiered tree and RAxML-NG tree). Indeed, Evangelista, et al. (in review 2024) demonstrated that concatenation would fail to recover a robust Blaberidae backbone. Perhaps most significantly, our ASTRAL-III tree recovered *Diploptera* + Oxyhaloinae as sister to all other Blaberidae (minus AM-"*Rhabdoblatta*" spp.). This relationship was supported, in part, by numerous support tests (discussed below) and may be in agreement with the predominant hypotheses from previous research (discussed in Evangelista et al., in review 2024). Another major difference is that the ASTRAL-III tree placed *Parellipsidion* sp. as sister to *Anaplectoidea klossi* as opposed to falling within Ectobiidae. The ASTRAL-III tree also differed from the others in the placement of Anaplectidae as sister to Blattoidea *s.s.* Finally, the RAxML-NG tree was unique in placing *Buboblatta vlasaki* sister to Anaplectidae, which together were sister to Kittrickea (*s.s.*).

3.2. Comparisons of support values on the tiered tree

We used an algorithm (see Section 2.4 and S2.6.1) to map support values from each tier of the tiered phylogenetic inference onto the final tree. Each value was then annotated to identify the tier, and thus the quality of the dataset, it derived from. We refer to these final annotated support values as quality-aware support (QA-support).

First, we want to know if mapping support values in this way biases support values in some way. There were not strong differences in

summary metrics of tree support between the original Tier 4 support values and QA-support (90 ± 22.0 raw vs. 91 ± 21.9 QA-support). However, when we omit meaningless support values (support values on constrained nodes) we do see differences in bootstrap support (67 ± 30.0 raw vs. 90 ± 22.8 QA-support).

To better understand how QA-bootstrap supports compare to non-tiered bootstrap values that correct for rogue taxon placement (TBE; Lemoine et al., 2018). We calculated the latter in IQ-TREE2 and compared them on the Tier 4 topology. There was similar overall magnitude of support from the full data BS analysis and QA-bootstraps (89.7 ± 21.0 IQ-TREE2 TBE vs. 90.0 ± 22.8 QA-support). We also compared support across 30 arbitrarily chosen deep nodes (Supplementary data) on the Tier 4 topology. Comparing QA-support and IQ-TREE2 TBE, 14 of those nodes had very high (>93 %) support from the full dataset, while QA-support annotations provided insight that suggested some level of weakness due to less-than-ideal quality data (follow up tests corroborate low or conflicting support in 11 of these 14 nodes; Supplement S5, S6 and Supplemental data). Four different splits supported by QA-support were never recovered from the full data analysis due to a single rogue terminal (*Anaplecta pulchella*) in pseudoreplicate trees (this tip was not volatile in the tiered analysis). There were four additional nodes that received modest support in both analyses, yet the additional quality annotation of QA-support provided some hint towards investigating support for that split. Below, we discuss examples of this. Finally, six nodes had low magnitude of QA-support but high support from full data IQ-TREE2 TBE and two nodes had similar interpretations from both analyses. Thus, QA-support provided an advantage for 22 of the 30 nodes examined.

3.3. Assessing support for recalcitrant nodes and revising the evolutionary history of Blattodea

We established two sets of guidelines (Table 2, S5.2) to aid interpretation of the results. The discussion below is limited to issues of support. Implications for cockroach evolutionary history and systematics are discussed in the subsequent section and in S7.

3.3.1. Relationships in Solumblattodea

Three contentious relationships in Solumblattodea involve Anaplectidae, the relationships of *Mastotermes* and *Zootermopsis* to other termites, and *Buboblatta*. Anaplectidae has been a rogue taxon in multiple studies (Djernaes et al., 2015; Legendre et al., 2017; Wang et al., 2017; Bourguignon et al., 2018; Evangelista et al., 2018; Li, 2022). The second relationship was not considered problematic (Engel et al., 2009; Bucek et al., 2019; Hellemans et al., 2022), until a large phylogenomic study showed ambiguous support for which termite lineage is sister to the others (Evangelista et al., 2019b). Finally, the position of *Buboblatta* (Latindiinae) differs from what would be predicted by a recent study (Han et al., 2024). We do not investigate this last problem as rigorously due to taxon sampling limitations.

Ccc Fig. S5.2 shows strong support for *Lamproblatta* + Anaplectidae, but only in the lower-quality Tier 3 analysis. This relationship has been found previously (Bourguignon et al., 2018; Li, 2022; Deng et al., 2023; Evangelista et al., 2023; Liu et al., 2023) but not in all studies (Djernaes et al., 2015; Legendre et al., 2015; Wang et al., 2017; Evangelista et al., 2018; Djernæs and Murienne, 2022). According to our interpretation guide (Table 2), this is condition vii. Monophyletic Anaplectidae is supported maximally, but from the Tier 3 data—condition iii. We further analyzed support for these relationships. Tier 3 and Tier 4 analyses have enough data to be informative on the position of Anaplectidae and the bipartition was not constrained in either analysis. Anaplectidae + *Lamproblatta* was recovered in both tiers with moderate to high support 92 % in Tier 3 and 100 % in Tier 4; Fig. 2A). The ASTRAL-III and RAxML-NG trees show Anaplectidae in different positions, but with low support values (Supplementary data).

The magnitude of QA-support alone erroneously suggests that the position of Anaplectidae is highly supported. UF-bootstrap values are maximal in Tier 4, the lowest quality data. Lacking Tier 1 and Tier 2 quality data, we did further testing. sCF values show 36.8 % (Tier 3) and 24.1 % (Tier 4) of sites support the relationship under parsimony. FcLM tests show nucleotide compositional bias may be driving the *Lamproblatta* + *Anaplecta* sister relationship. The AU Test rejected all inferred placements (Tier 3 placement, RAxML-NG and ASTRAL-III) in favor of *Anaplecta* as sister to Blaberoidea, which is the precladistic hypothesis for *Anaplecta*'s placement (Princis, 1965). However, deltaGLS tests suggested this was likely a result of long branch attraction as it was strongly correlated with higher mean-pairwise sequence distance. deltaGLS tests favored the Tier 3 relationship, but support for that relationship was correlated with compositional bias. Thus, the relationship is still unresolved. More clarity could be gained by sequencing higher quality *Anaplecta* genomic data having more biogeographically and morphologically diverse taxon sampling, choosing loci to avoid potentially biased regions, and repeating these tests.

The second example of misleadingly high support deals with the earliest divergences among Isoptera Brullé, 1832. Evangelista et al. (2019b) left doubt as to the relative positions of *Mastotermes* and *Zootermopsis*. Tiered inference (Fig. S5.2), ASTRAL-III, and RAxML-NG all recovered *Zootermopsis nevadensis* as sister to the remaining termites. This conflicts with the established understanding that Mastotermitidae is sister to all other termites (Engel et al., 2009; Bucek et al., 2019; Hellemans et al., 2022) – condition vii.

Support values for basal termite nodes are high across all analyses (e.g., Fig. S5.2), but examining each tier (Fig. 2A) shows the relationship is volatile. Only Tier 2 and 3 have sampling sufficient to test this issue. Tier 4 should not be examined all of the oldest termite nodes were constrained in that analysis. In Tier 2*Mastotermes* is sister to all other termites and most nodes have maximal bootstrap support. However, the relationship was unconstrained in Tier 3 where *Zootermopsis* was then sister to all other termites. We tested the relationship between data quality and these termites relationships with deltaGLS (Supplementary data). Signal overwhelmingly favored (*Mastotermes* (remaining Isoptera)), while the pattern of signal supporting (*Zootermopsis* (remaining

Table 2
Guide to interpreting quality aware node support values.

Data quality[1]	Support	Congruent with previous studies	Incongruent with previous studies
High	**High**	**i**Relationship strongly supported	**v**Previous hypotheses overturned
	Low	**ii** Relationship weakly supported. *Identify the magnitude of, and possible bias in, signal.*	**vi** Relationship contentious. *Identify the magnitude of, and possible bias in, signal.*
Low	**High**	**iii** Relationship supported but suspect. *Identify changing support over tiers. Identify the magnitude of, and possible bias in, signal.*	**vii** Relationship contentious. *Identify changing support over tiers. Identify the magnitude of, and possible bias in, signal. Collect more data and re-evaluate.*
	Low	**iv** Relationship supported but highly suspect. *Identify changing support over tiers. Compare signal for possible alternative relationships. New data and/or analyses needed.*	**viii** No new systematic information gained. *New data and/or analyses needed.*

[1]In our analyses, we consider Tier 1 and Tier 2 to be "high quality data" and Tiers 3 and 4 to be "low quality data". We considered any support values < 90 % to be "low support" to varying degrees. The eight conditions (i–viii) correspond to the eight possible interpretations written. Italic text describes our proposed next steps to resolve ambiguity.

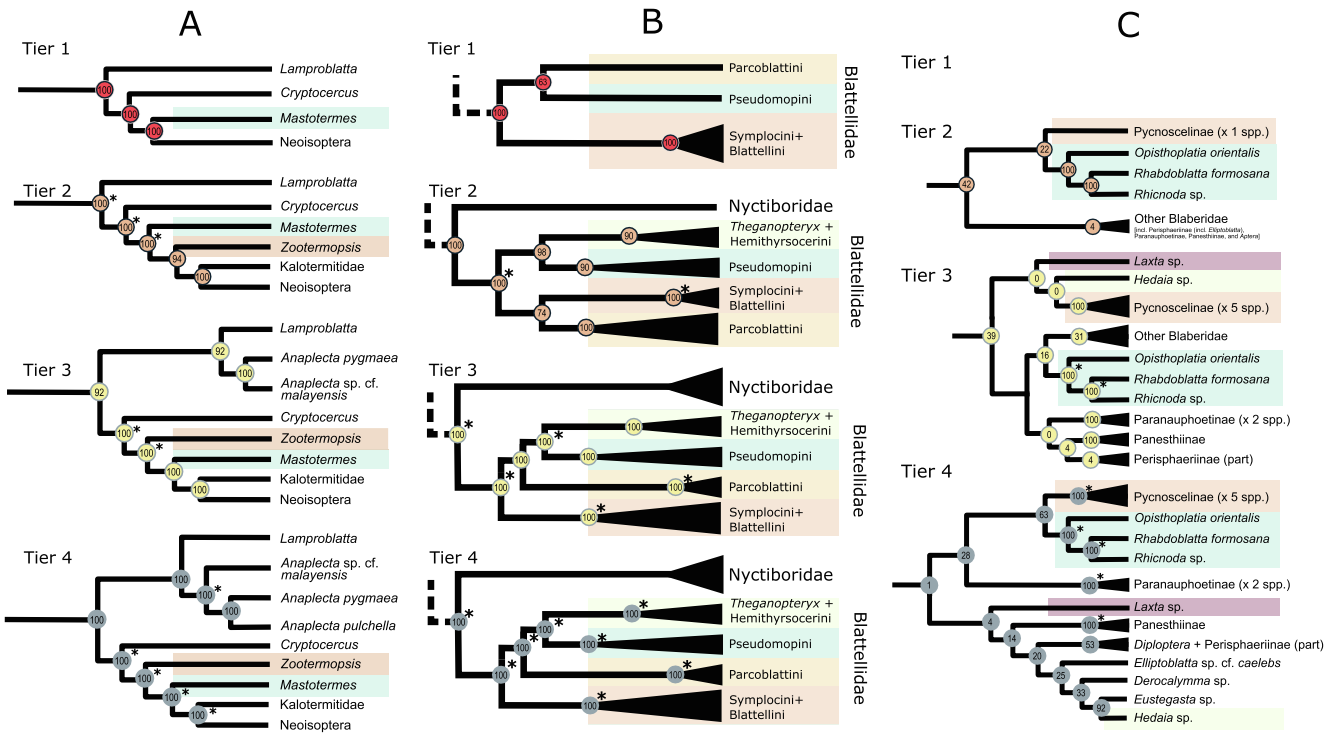


Fig. 2. Comparison of specific relationships among four-tiered analysis. A–Kittrickean relationships. B–Blattellid relationships. C–The position of Pycnoscelinae. Clades in topological flux are colored so multiple tiers can be more easily compared. * Indicates support influenced from constraining that node in the analysis. These node supports are not comparable to the others. In C, Tier 1 is empty because no Pycnoscelinae were sampled in this analysis.

Isoptera)) suggests long branch attraction and among-lineage rate heterogeneity. Resolving these ambiguities about *Mastotermes darwiniensis* as sister to the remaining termites confirms the parsimonious evolution of numerous traits, including the single loss of plicatum, gain of ocelli, loss of ootheca, and loss of *Blattabacterium* spp. that is apparent among non-mastotermitid termites.

For the third example, we recovered *Buboblatta* as sister to Blattoidea (tiered tree, ASTRAL tree) or within Blattoidea (RAxML-NG tree). *Buboblatta* is currently classified as Latindiinae, which, together with Nocticolinae, likely form a monophyletic group with Corydiidae (Han et al., 2024). Grandcolas (1996) placed *Buboblatta* in Corydiidae based on the male genitalia and their hindwings lack a folding anal-fan (Evangelista et al., 2019a), a diagnostic feature of Corydiidae. The current diagnostic characters for Latindiinae (Han et al., 2024) and the poor knowledge of the type genus *Latindia* precludes an in-depth morphological assessment. Paraphyly of Latindiinae might suggest unusual plesiomorphic phenotypes, and ecology (Han et al., 2024) for Solumblattodea. Our support tests did not rule out placing*Buboblatta vlasaki* in Corydioidea (Supplementary data). Our failure to include wide geographic sampling of Latindiinae and Nocticolidae, and non-overlapping sampling with Han et al. (2024), prevents any strong conclusions. A more thorough analysis could resolve the question: was the ancestor of Solumblattodea Latindiinae-like (e.g., minute body-size, Corydioid-like hindwings) or not (perhaps having a mix of blattoid and corydioid traits)?

3.3.2. *Anaplectoidea and Ectobiidae*

Familial relationships within Blaberoidea Saussure, 1864 are congruent with past studies (Evangelista et al., 2019b; Evangelista et al., 2020; Liu et al., 2023; Wang et al., 2023), but few are supported from Tier 1 (Fig. S5.1). For one, Ectobiidae *s.s.*'s monophyly seems to have low support in the Tier 3 and RAxML-NG trees. When examining each tiered tree, placement of Ectobiidae *s.s.* was established in Tier 1. *Ectobius* forms a sister group with *Ectoneura* in Tier 2(100 % UF-Bootstrap). Placement of *Mediastinia* sp. and *Parellipsidion* sp. in Tier 3 received low support. Ectobiidae *s.s.* was monophyletic in Tier 4 with low support (55 % UF-bootstrap, 30.8 sCF), and polyphyletic relative to *Parellipsidion* sp. in Tier 3 (trees in Supplementary data). *Parellipsidion* sp. is sister to *Anaplectoidea klossi* in the ASTRAL-III tree.

Anaplectoidea's placement mirrors an earlier analysis (Evangelista et al., 2023) but contrasts its precladistic placement in Blattellinae (Roth 1996). We tested the placement *Anaplectoidea* and Ectobiidae *s.s.* concurrently. FcLM tests showed that the position of *Parellipsidion* was ambiguous, with equivalent signal for being sister to *Anaplectoidea* or Ectobiinae. Moderate signal supported *Anaplectoidea* as sister to all other Blaberoidea. When constraining *Anaplectoidea* as sister to all other Blaberoidea, *Parellipsidion* sister to other Ectobiinae was more strongly supported. AU Tests and deltaGLS tests were not definitive. See Supplementary data for complete results of all tests. We consider both *Anaplectoidea* and *Parellipsidion* with ambiguous placement in Ectobiinae, Anaplectoideini, or a combined clade.

Support for the monophyly of Ectobiinae (=Ectobiidae *s.s.*) with respect to *Anaplectoidea klossi, Parellipsidion* sp., and *Mediastinia* sp. is low (Supplementary data), and this should be resolved with new sampling [but see Evangelista et al. (2020) and Roth (1998) for evidence of *Mediastinia* being placed in Ectobiinae]. Even with the present ambiguity, the topology suggests expanded hind-wing apical field (present in Ectobiinae, *Anaplectoidea*, and many Pseudophyllodromiinae) could have been present in the ancestor of Blaberoidea. Recent analyses suggest independent gains may be more likely though (Evangelista et al., 2023). Further systematic exploration of *Anaplectoidea* is important for piecing together the evolution of ootheca laying strategies (see Section 3.4).

3.3.3. *Blattellidae s.s. relationships*

Tiers 1 to three show shifting support for relationships inBlattellidae *s.s.* (Fig. 2B). The tiered tree is concordant with the ASTRAL-III tree and previous study (Evangelista et al., 2019b; Evangelista et al., 2020; Liu et al., 2023) – condition iii. Yet, Tier 2 recovers different groupings, but with lower frequency (74 %)—condition vi. The Tier 2 relationships

were recovered in the RAxML-NG tree and were partially recovered in some other recent works (Jin et al., 2022; Li 2022; Wang et al., 2023), while yet other studies (Bourguignon et al., 2018; Djernæs et al., 2020; Wang et al., 2023) suggest support for different sets of relationships (conditions iii and iv; Table 2). While the final tired tree and ASTRAL-III trees seem the most reliable, signal in the Tier 2 dataset and RAxML-NG tree suggests data artefacts. We found low sCF values for relationships at all tiers (32–36 %). Fortunately, further testing clarifies the issue. FcLM and deltaGLS show that the relationship in tiers 1 and 3 are supported without any discernible bias (Supplementary data)and several outlier genes favor the Tier 2 topology. The relationship was also present in seven out of 11 additional tree inferences with the Tier 2 dataset. We infer that the Tier 2 inference, 74 % of Tier 2 bootstrap pseudoreplicates, and the RAxML-NG tree were influenced by outlier loci. Despite strong overall support for the Tier 1 and Tier 3 relationship (Fig. 2B), the Tier 2 relationship could easily have been recovered with different genetic sampling. Thus, by comparing the tiers, we were able to identify a vulnerability in the analysis that would otherwise have gone unnoticed.

3.3.4. Four questions about Blaberidae

Blaberidae Saussure 1864 has low node support and most QA support values derive from tiers 3 and 4 (Fig. S5.3). Uncertainty in Blaberidae intrarelationships has been prominent in past phylogenies. It is suspected that rapid radiations (Evangelista et al., 2020; Legendre et al. unpublished data) and at least eight anomalous nodes (Supplementary data; Evangelista et al., in review 2024) contribute to this challenging reconstruction. There is little, if any, consensus about Blaberidae subfamilial relationships despite thorough study (Legendre et al., 2017; Bourguignon et al., 2018; Evangelista et al., 2018; Djernæs et al., 2020; Evangelista et al., 2020; Liu et al., 2023; Wang et al., 2023). Thus, we will mainly compare our topology to the study with the most comparable taxon sampling—Evangelista et al. (2020)—and other recent studies when appropriate. Evangelista et al. (2020) proposed four major clades for Blaberidae: African Epilamprinae (AM-"Rhabdoblatta" spp.), Neotropical Epilamprinae (Epilamprinae *s.s.*), "Peri-Atlantic Blaberidae", and "Peri-Indian Blaberidae". The present analysis contradicts these latter two clades, and conflicts elsewhere along the backbone.

What is the first split in Blaberidae? AM-"Rhabdoblatta" spp. as sister to all other Blaberidae was proposed recently (Evangelista et al., 2020) and mirrors a recent study that included East Asian *Rhabdoblattella* (Wang et al., 2023). The monophyly of all Blaberidae except AM-"Rhabdoblatta" spp. was established in Tier 4 due to rogue placement of *Cyrtotria* (Fig. S5.3) that disagrees with the ASTRAL-III tree. Strong morphological evidence (Princis, 1955, 1964; Roth, 1973a) indicates that the coalescent placement is correct (*Cyrtotria* + *Derocalymma*). When ignoring the erroneous placement of *Cyrtotria*, AM-"Rhabdoblatta" spp. as sister to all other Blaberidae is recovered in all relevant tiers with varying support (Tier 2 = 60 %, Tier 3 = 93 %, Tier 4 = 44 %; local posterior probability = 1.0; RAxML-NG TBE = 99 %). Low support in the Tier 2 analysis is concerning (condition ii). DeltaGLS values strongly support AM-"Rhabdoblatta" spp. as sister to all other Blaberidae. Support for the alternative, AM-"Rhabdoblatta" spp. in a polytomy with all other Blaberidae, was strongly correlated with high sequence distance alignments. Recently, Wang et al. (2023) proposed Rhabdoblattellinae, represented by *Rhabdoblattella,* was sister to all other Blaberidae. If Rhabdoblattellinae and AM-*Rhabdoblatta* are monophyletic further study is needed to ascertain this.

What comprises the Blaberidae "backbone"? Legendre et al. (2017) recovered a clade containing Epilamprinae *s.s.*, Diplopterinae and Panchlorinae as sister to all remaining Blaberidae. Djernæs et al. (2020) recovered (Panchlorinae, (Oxyhaloinae, remaining Blaberidae)). Evangelista et al. (2020) obtained Epilamprinae *s.s.* as sister to all remaining Blaberidae. Liu et al. (2023) recovered (Diplopterinae, (Oxyhaloinae, remaining Blaberidae)), while Wang et al. (2023) found ((Diplopterinae, Oxyhaloinae), remaining Blaberidae). We tested these relationships, but omitted the monogeneric Diplopterinae that has previously been considered a long branch taxon (Evangelista et al., 2018). Also, its placement is so volatile that it required too many tests to resolve. However, coalescent analyses on a small sample of extensively vetted gene tree topologies suggest that Diplopterinae and *Paraplecta* spp. may be sister to Oxyhaloinae, and these sister to most other Blaberidae lineages (excluding AM-"Rhabdoblatta") (Evangelista et al., in review 2024).

The Blaberidae backbone was volatile in the tiered analysis (Supplementary data; see above). Tiered topologies also differed strongly with the RAxML-NG and ASTRAL-III trees. Across tiers, sCF's are mostly 0–33 % (Supplementary data). Comparison of all tiers with past studies (Legendre et al., 2017; Djernæs et al., 2020; Evangelista et al., 2020; Liu et al., 2023; Wang et al., 2023) informed follow-up topological tests which yielded some positive results. FcLM showed strong support for Oxyhaloinae (Liu et al., 2023; Wang et al., 2023; Evangelista et al., in review 2024), not Epilamprinae *s.s.* (Evangelista et al., 2020), as sister to the remaining Blaberidae. DeltaGLS tests corroborated the position of Oxyhaloinae, but only weakly over Panchlorinae, as sister to the remaining Blaberidae (Djernæs et al., 2020). Within the clade sister to Oxyhaloinae, there was support for Panchlorinae as sister to all the "Blaberidae clade *X*" (FcLM). Tests also showed support for Epilamprinae *s.s.* as sister to a clade comprising *Aptera*, Gyninae, Blaberinae, and Zetoborinae (FcLM, AU Test, deltaGLS). These issues do not appear to derive from a lack of data (see Supplementary data for details). Thus, the original observation of poor support values appears to be a true case of lacking signal (condition viii; Table 2). More work should be done to resolve the timing and reconstruct the ancestral conditions of a possible hard polytomy. Future tests could target Diplopterinae, *Paraplecta* spp., *Pronauphoeta* spp., and *Pseudocalolampra* spp. as potential close relatives of Oxyhaloinae, or other possible positions along the blaberid "backbone".

What is the position of Gyninae? Previous studies have not agreed about the position of Gyninae (Bourguignon et al., 2018; Evangelista et al., 2020; but see Wang et al., 2023). The RAxML-NG and ASTRAL trees differ in the position of Gyninae relative to the B-Z clade (-*Parasphaeria*), *Parasphaeria,* and *Aptera*. Here, the tiered analysis provides some clarity. In Tier 2, we recover Gyninae monophyletic with the B-Z clade (including *Parasphaeria*) with 100 % support. Therefore, we can perhaps consider previous hypotheses about Gyninae to be overturned (condition v).

Who is related to Pycnoscelinae? Pycnoscelinae are a morphologically unique (Roth, 1973b; Anisyutkin, 2002) group of Blaberidae native to Asia with one cosmopolitan species, *P. surinamensis*. There is no strong hypothesis for which subgroup of blaberids is its closest relative. Perisphaeriinae (Djernæs et al., 2020; Evangelista et al., 2020; Liu et al., 2023) and Paranauphoetinae (Wang et al., 2023) are two recently proposed suggestions. A single Pycnoscelinae was included in the Tier 2 analysis and that was sister to Asian "Epilamprinae", but with low support (Fig. 2C). This was also found in the RAxML-NG tree (RaXML-NG TBE = 67 %). In Tier 3, a different relationship was recovered, but with no support. Surprisingly, we recovered the same relationship in Tier 4 as in Tier 2 with moderate support.

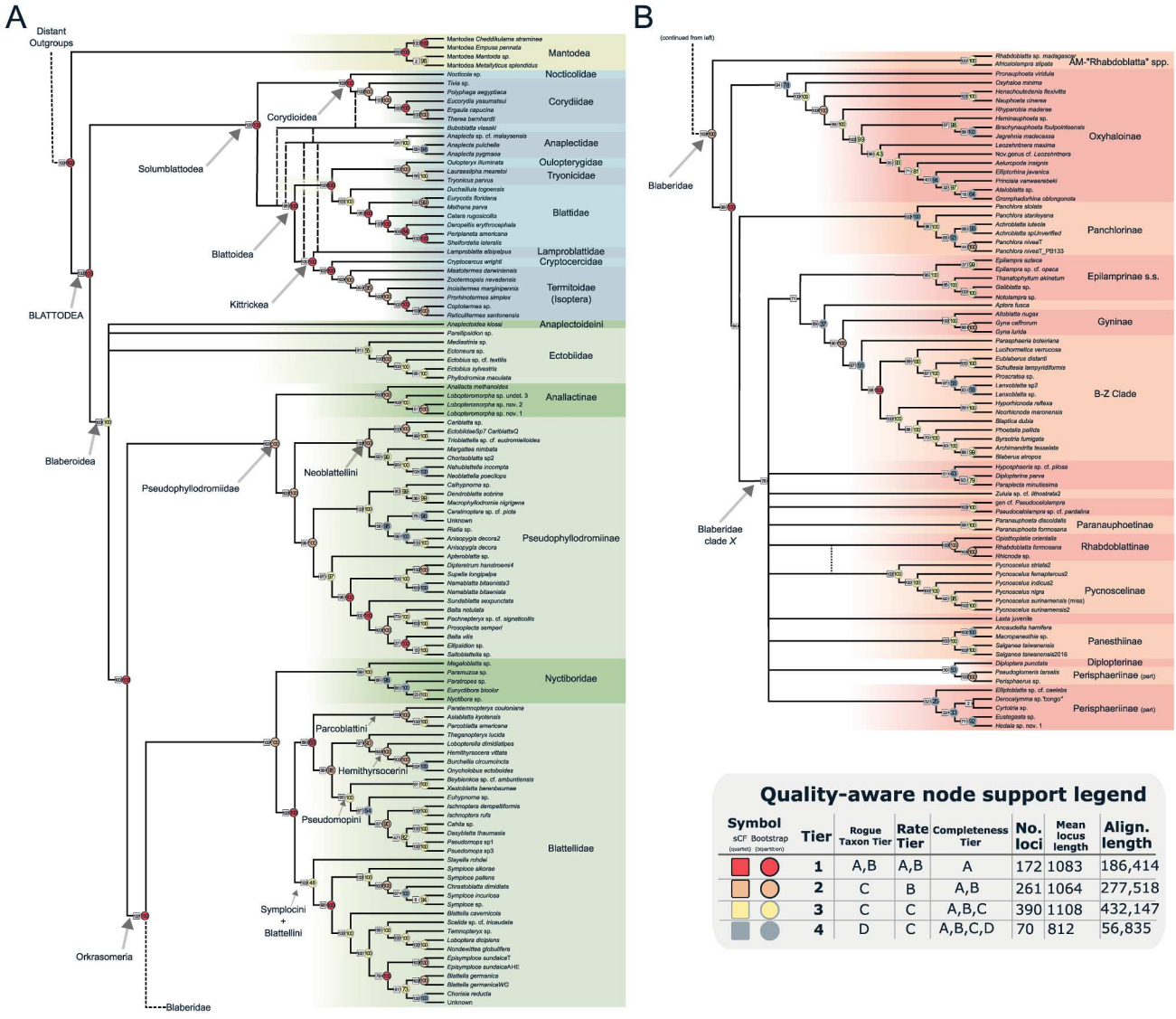
We compared the two hypotheses recovered using deltaGLS. There was stronger support for Pycnoscelinae as sister to Asian "Epilamprinae" but this was correlated with evolutionary rate. This could suggest the relationship is artefactual. In this case, however, since the radiation likely occurred in a very short period, we might expect only rapidly substituting sites to be informative about these relationships (Townsend, 2007). This is a catch-22, since rapidly evolving sites are more likely to be saturated (Townsend, 2007; Evangelista et al., 2020). It is therefore unclear how to interpret these results. Perhaps the only clear conclusion is that there is little information to inform this relationship. Indeed, Evangelista et al. (in review 2024) placed this in a polytomy with Asian "Epilamprinae" and Asian-Perisphaerinae, and demonstrated that this was an anomalous node. The ASTRAL-III tree placed Pycnoscelinae in a clade with Asian "Epilamprinae", *Hedaia*, *Elliptoblatta,* and *Eustegasta*.

3.4. Further systematic advances

Neoblattellini are recovered as paraphyletic with respect to *Chorisoblatta*. Evangelista et al. (2020) recovered *Chorisoblatta* in Blattellinae as sister to *Chromatonotus* but discussed the problematic nature of this placement. Here, we have re-sequenced this taxon and recover its position with more confidence. While the paraphyly of Neoblattellini is unexpected, this tribe has huge diversity in secondary sexual morphology (e.g., male genitalia, subgenital plate morphology, and external tergal gland modifications; (Bruijning, 1959)) and may have been lumped together by symplesiomorphic pronotum and gestalt. The disjunct biogeography of this recovered clade (Neoblattellini is Neotropical; *Chorisoblatta* is Afrotropical and Malagasy; *Margattea* is Paleotropical) does cast further doubt on their placement.

We recover *Anisopygia decora* within Pseudphyllodromiinae as a close relative of *Riatia*. Anisyutkin (2008) gave a compelling argument that *Anisopygia* is nested within *Ischnoptera* (Blattellinae: Pseudomopini) based on shared secondary sexual characteristics (e.g., tergal glanad, subgenital plate). Anisyutkin (2008) illustrated the genitalia of *A. latisecta* and A. *profundisecta* as having the hooked phallomere on the left (consistent with Blattellinae). Estrada-Álvarez et al. (2020) shows A. *saussurei* also having the hook on the left and the external male morphology of A. *jocosicluna* being strongly consistent with A. *latisecta* and *A. profundisecta*. The male of A. *decora* (our included taxon) is not known, but we suspect its morphology would be more consistent with Pseudophyllodromiinae (e.g., its hooked phallomere would be on the right).

Ovoviviparity is a key innovation among cockroaches, being a synapomorphy for Blaberidae. Roth (1982) also reported ovoviviparity (type A) in *Stayella bimaculata* (Blattellinae) and other ovoviviparous



Quality-aware node support legend

Symbol (sCF Bootstrap (lowest) / (quartiles))	Tier	Rogue Taxon Tier	Rate Tier	Completeness Tier	No. loci	Mean locus length	Align. length
	1	A,B	A,B	A	172	1083	186,414
	2	C	B	A,B	261	1064	277,518
	3	C	C	A,B,C	390	1108	432,147
	4	D	C	A,B,C,D	70	812	56,835

Fig. 3. Complete phylogenetic tree incorporating changes suggested by all support tests (Grafted tree). (A) Solumblattodea and Blaberoidea (part), and (B) Blaberidae. Values in colored circles are quality aware bootstrap support (GENESITE UF-bootstraps). Red corresponds to the analysis of most highly vetted dataset, with the highest mean information content and tree-likeness. Orange, and yellow correspond to analyses of datasets that are increasingly more inclusive to data with lower mean information content and taxa with more missing data. The final tier (grey) includes all remaining taxa and any data relevant to their placement. Values in (squares) are split support from RAxML-NG bootstrapping summarized as transfer bootstrap expectations (TBE). Nodes missing support values are relationships manually grafted post-analyses or multi-furcations with no relevant support value. Grafts are based on signal in follow-up support tests (FcLM, deltaGLS, AU Test) or comparisons among tree inference methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

blaberoids have since been identified (see Djernæs et al., 2020 for summary). We recover *Stayella rohdei* within Blattellinae as sister to other Symplocini and Blattellini. It should be noted, though, that Roth (1982) did not observe ootheca laying in any *Stayella* other than *S. bimaculata*. Assuming *Stayella* is monophyletic, our results suggest at least three independent gains of ovoviviparity: at the origin of Blaberidae, in *Stayella*, and possibly in Anaplectoideini. Roth (1995) reported ovoviviparity A from *Pseudoanaplectinia* and Yanagisawa et al. (in press, 2022) reported oviparity A from *Anaplectella*. Both genera are hypothesized to be closely related to *Anaplectoidea* (and *Malaccina*) in Anaplectoideini (Wang et al., 2023).

Other findings in Blattellinae clarify some systematic issues. First, Roth (1982) recognized *Blattella* spp. and *Chorisia* sp. carrying ootheca externally for the entire embryonic period (oviparity B). We indeed recovered *Chorisia reducta* as sister to *Blattella germanica*. Another case of oviparity-B was described in *Lophoblatta brevis* (Roth, 1968), which is placed within Neoblattellini (Pseudophyllodromiidae) and has never been included in phylogenetic studies. Next, *Blattella cavernicola* was placed in *Blattella* (Roth, 1985) because of similarities in oviposition behavior and ootheca morphology, but also says the hindwing cubitus vein and tergal modification are more like *Symploce*. Our results exclude it from *Blattella*. Clearly, further investigation into ootheca and parental care phenotypes is warranted. Finally, Anisyutkin (2020) corroborated placement of *Chrastoblatta* in Blattellinae. We included this unusual Malagasy taxon in our analysis and can further refine its placement as a close relative of *Symploce* spp.

4. Conclusions

We combined three tree inferences methods and follow-up tests to resolve a new understanding of Blattodea evolutionary history (Fig. 3). One of our inference methods, tiered phylogenetic inference, is a novel approach to phylogenomics that seeks to maximize data inclusion but minimize errors from low quality data. This method of hierarchical inferences incrementally increases taxon sampling while mean information content of the data decreases. This tree inference design allowed us to annotate the final tree with data-quality information (i.e., quality-aware support) that directed us in follow-up support tests. The magnitude of quality-aware support values (derived from UF-bootstraps) were more comparable to TBE values than UF-bootstrap values from an analysis of the whole alignment. Like other super-tree methods, tiered inference has different taxon sampling among constituent trees, which prevents straightforward interpretation of support values. Final support values must be interpreted in the context of the individual tiers. While this process is difficult in practice it does assist in identifying possible estimation errors. The tiered inference did not perform as well as RAxML-NG in placement of poorly sequenced taxa, but it did perform better than ASTRAL-III.

These three inference methods, and tiered tree inference in particular, led us towards a number of empirical findings. We saw various controversial relationships receiving high support values from low-information content data (e.g., *Zootermopsis* as sister to all other termites, the placement of *Anaplectoidea* Shelford, 1906, *Lamproblatta* and Anaplectidae, and the backbone of Blaberidae). Further investigation sometimes revealed the relationships were tenuously supported (e.g., *Anaplectoidea* sister to all other Blaberoidea, and Anaplectidae sister to *Lamproblatta*) or that they were not at all supported (e.g., the placement of Epilamprinae *s.s.*, *Cyrtotria* Stål, 1871 and Oxyhaloinae, *Zootermopsis* as sister to all other termites). In these cases, errors derived from outlier genes, long branch effects, and incomplete lineage sorting. We were also able to contrast lack of signal with analytical ambiguity in the radiation of Blaberidae.

CRediT authorship contribution statement

Dominic A. Evangelista: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dvorah Nelson:** Data curation. **Zuzana Kotyková Varadínová:** Writing – review & editing, Validation, Resources. **Michael Kotyk:** Resources. **Nicolas Rousseaux:** Resources. **Tristan Shanahan:** Resources. **Phillippe Grandcolas:** Resources. **Frédéric Legendre:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

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.

Data availability

All new sequence data is available on the NCBI sequence read archive under project number PRJNA482916. Code used to combine support values is available on GitHub: https://github.com/dominicev/Roach_brain_Phylos/tree/main/AwareSupport. Supplementary data (trees, taxon lists, and support test results) can be found on Dryad at https://doi.org/10.5061/dryad.wstqjq2rw.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108177.

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Glossary

Support value terminology

ASTRAL support: support values unique to ASTRAL-III tree inference, which identify support for quartets.
TBE: transfer bootstrap expectation from CITE. In our study, we calculated TBE support values on our RAxML-NG tree using the full concatenated dataset (RAxML-NG TBE), and on the Tier 4 tree from the full concatenated dataset in IQ-TREE2 (IQ-TREE2 TBE).
UF-bootstrap: ultra-fast bootstrap support values calculated in IQ-TREE2 (CITE). In our study, we used UF-bootstrap values in the Tiered tree inference
sCF: site concordance factor is a measure of character support for a bipartition from CITE
QA-support: Quality-aware support comprises a magnitude and a quality annotation. In this study, the magnitudes represent UF-bootstrap and the quality annotation corresponds to the tier the UF-bootstrap value was calculated in.

Named phylogenetic (species) trees

ASTRAL-III tree: a phylogenetic tree of species inferred in ASTRAL-III from 1183 gene trees, which were each estimated in IQ-TREE2.
RAxML-NG tree: a phylogenetic tree of species inferred in RAxML-NG from the concatenation of 1183 gene trees
Tiered tree: a phylogenetic tree of species inferred using an incremental tree building protocol described in the text. This utilizes multiple concatenation (IQ-TREE2) tree inferences, with each subsequent analysis constrained to the results of the prior analysis. Our analysis used 4 tiers, and we interpret the results by looking at the final tree (Tier 4), and each subsequent tree (Tier 3, Tier 2, and Tier 1). Tier 1 had the smallest taxon set, but the highest quality alignment.
Grafted tree: the Tiered tree with manual modifications that reflect the results from comparison of all tree inferences and follow-up support tests.