Reconstructing the phylogeny of Blattodea: robust support for interfamilial relationships and major clades

Zongqing Wang¹, Yan Shi², Zhiwei Qiu¹, Yanli Che¹ & Nathan Lo²

Cockroaches are among the most recognizable of all insects. In addition to their role as pests, they play a key ecological role as decomposers. Despite numerous studies of cockroach phylogeny in recent decades, relationships among most major lineages are yet to be resolved. Here we examine phylogenetic relationships among cockroaches based on five genes (mitochondrial 12S rRNA, 16S rRNA, COII; nuclear 28S rRNA and histone H3), and infer divergence times on the basis of 8 fossils. We included in our analyses sequences from 52 new species collected in China, representing 7 families. These were combined with data from a recent study that examined these same genes from 49 species, resulting in a significant increase in taxa analysed. Three major lineages, Corydioidea, Blaberoidea, and Blattoidea were recovered, the latter comprising Blattidae, Tryonicidae, Lamproblattidae, Anaplectidae, Cryptocercidae and Isoptera. The estimated age of the split between Mantodea and Blattodea ranged from 204.3 Ma to 289.1 Ma. Corydioidea was estimated to have diverged 209.7 Ma (180.5–244.3 Ma 95% confidence interval [CI]) from the remaining Blattodea. The clade Blattoidea diverged from their sister group, Blaberoidea, around 198.3 Ma (173.1–229.1 Ma). The addition of the extra taxa in this study has resulted in significantly higher levels of support for a number of previously recognized groupings.

Cockroaches are considered to play a key role in terrestrial ecosystems, recycling dead plants, dead animals and excrement and contributing to ecosystem functioning via the breakdown of organic matter and the release of nutrients. The morphologically and ecologically diverse group Blattodea including Isoptera is widely accepted to be a monophyletic. In recent decades a number of studies have examined the phylogeny of Blattodea based on morphological characters, molecular data, or both. Taken together, these studies displayed some consistent relationships, including Ectobiidae (Blattellidae) being paraphyletic with respect to Blaberidae, and Isoptera being placed within Blattodea as sister to Cryptocercidae (morphological methods; molecular methods; combined data). The monophyly of termites and their closest relatives Cryptocercus is supported by strong synapomorphies, such as xylophagy, biparental care, proctodeal trophallaxis and a rich and highly specific hindgut fauna of flagellates.

Despite these advances, the evolutionary relationships among the main lineages of Blattodea have yet to be well resolved, and a number of other results from previous studies remain under discussion. These include: (i) the proposal that Tryonicidae and Lamproblattidae are given family status and excluded from Blattidae; (ii) the proposed sister grouping between Nocticolidae and Corydiidae (=Polyphagidae); (iii) the sister group relationships between Lamproblattidae and Blattidae; (iv) the sister group of Cryptocercidae + Isoptera, which may be either Tryonicidae, Anaplecta, or Tryonicidae + Anaplecta.

Although the Nocticolidae are generally accepted to be a monophyletic group, the placement of Nocticolidae and the relationships with Corydiidae have been debated over the last 20 years. Grandcolas proposed that Nocticolidae should be lowered to the subfamily level and be synonymised with Latindiinae. In most other

¹College of Plant Protection, Southwest University, Beibei, Chongqing, China. ²School of Life and Environmental Sciences, University of Sydney, Sydney, New South Wales, Australia. Correspondence and requests for materials should be addressed to N.L. (email: nathan.lo@sydney.edu.au)
studies, Nocticolidae were recovered as the sister group to Corydiidae\textsuperscript{7,8,11,19}. When additional Latindinae taxa were included, Nocticolidae was recovered to be the sister group to\textit{Latindia + Paralatindia}\textsuperscript{12,13}. In this study, we sequenced three mitochondrial (12S rRNA, 16S rRNA and COII) genes and two nuclear (28S rRNA and Histone H3) genes from 52 blattarian (mainly Ectobiidae, Blaberidae and Blattidae) species collected in China, including representatives of three important genera:\textit{Anaplecta, Nocticola} and\textit{Cryptocerus}. Combining these sequences with previously published sequences, and using 8 fossils, we performed phylogenetic and divergence date analyses, and inferred the biogeographic history and timescale of evolution within Blattodea.

### Material and Methods

**DNA extraction, amplification, purification and sequencing.** We sampled 5 genes of 52 species (Table S1) from Blattodea in this study: mitochondrial 12S rRNA, 16S rRNA, COII, nuclear 28S rRNA and Histone H3. Total DNA was extracted from hindleg tissues of samples preserved in 100% ethanol. The extraction procedure was according to the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing). Fragments of 12S rRNA, 16S rRNA, COII, 28S rRNA and H3 were amplified using PCR. Primers for the amplifications of these partial genes are given in Table 1.

For PCR amplification, a 25 μL cocktail of 1 μL DNA template, 15.25 μL double-distilled H\textsubscript{2}O (ddH\textsubscript{2}O), 2 μL MgCl\textsubscript{2} (25 mM), 2.5 μL 10×PCR Loading Buffer, 0.25 μL Taq DNA polymerase (TakaRa DNA kit; 100 mM Tris–HCl, pH 8.3, 500 mM KCl), 2 μL dNTP mixture (1 mM concentration of each dNTP) and 1 μL of each primer was used. The PCR conditions included are given in Table S2. The amplified products were electrophoresed in a 1% agarose gel. PCR products were used for sequencing. In the case where sequencing was not successful, purified PCR fragments were cloned and sequenced.

All new sequences were checked for contamination using unrestricted BLAST searches, and NJ trees were produced based on the alignment of each sequenced fragment to check for internal contamination and incorrectly identified GenBank sequences.

### Sequence alignment and phylogenetic analysis.

The taxon sample consists of 103 Blattodea taxa (ingroup) and 26 outgroup taxa (Table S3). The molecular data set consists of five genes: the mitochondrial 12S (390 nucleotides, nt), 16S (430nt), COII (730nt), nuclear 28S (600nt), H3 (330nt); the total length of the aligned molecular data set is 2831 nt. GenBank sequences were used when available from previous works (390 nucleotides, nt), 16S (430nt), COII (730nt), and the nuclear 28S (600nt), H3 (330nt); the total length of the aligned molecular data set is 2831 nt. GenBank sequences were used when available from previous works.

For the alignments, GenBank sequences were reverse complemented. The resulting aligned molecular dataset consisted of five genes: the mitochondrial 12S rRNA, 16S rRNA and COII genes and two nuclear genes (28S and H3). The aligned data was inspected visually and manually corrected in Mega6\textsuperscript{29} after translation into amino acids; few gaps were detected, and alignment was straightforward. Alignments of the ribosomal sequences (12S, 16S and 28S) were aligned visually and manually adjusted in Mega6\textsuperscript{29}. Poorly aligned characters were removed but these were limited.

Subsequent analyses were performed on the combined dataset utilizing Maximum likelihood (ML) and Bayesian inference (Bi). Bayesian inference (Bi) was performed using MrBayes 3.2\textsuperscript{10} and maximum likelihood (ML) was performed using RAxML 7.7.1\textsuperscript{11}.

### Table 1. Primers used to generate sequences.

| Genes | Forward/Reverse | Primer name | Sequence(5′-3′) | Reference |
|-------|-----------------|-------------|----------------|-----------|
| 12S   | F    | 12S forward   | ATCTATGTTACGACTTTAT | Inward et al\textsuperscript{7} |
|       | R    | 12S reverse   | AAACTAGGATTAGATAACC | Kambhampati\textsuperscript{12} |
| 12S   | F    | 12S F1 or 12S F2 | GATCATTTCTAGTTACACCTCC or GTCAACTAAGTGTGTTAGCT | N/A |
|       | R    | 12S reverse   | AAACTAGGATTAGATAACC | Kambhampati\textsuperscript{12} |
| 16S   | F    | 16S Forward   | CGCGCTGTTAACACAAACAT | Simon et al\textsuperscript{24} |
|       | R    | 16S Reverse   | TTAAATCCAAACAGCAGG | Cognato et al\textsuperscript{25} |
| 16S   | F    | 16S F1        | GGAAGGTGTTAACACTGAAATGATC | N/A |
|       | R    | 16S R1        | GATAGAAACCAACCTGCTCAC | N/A |
| COII  | F    | COII-F        | AGAGCWTCAACCTATATTAGAC | Park et al\textsuperscript{26} |
|       | R    | COII-R        | GTARWACCTCTGCTCCTGTTAC | Park et al\textsuperscript{26} |
| COII  | F    | Modified A-tLeu | CAGAATAAGTGCAATGGATT | Miura et al\textsuperscript{27} |
|       | R    | B-tLeys       | GTTAAAGACACAGACTTCTG | Simon et al\textsuperscript{24} |
| 28S   | F    | Hax           | ACACGGACCAAGGAGTCTAAC | Inward et al\textsuperscript{7} |
|       | R    | Win           | GTCCCTGCTGCTTAAGCAAC | Inward et al\textsuperscript{7} |
| H3    | F    | H3 AF         | ATGGCTGTACCAACAGACGAC | Inward et al\textsuperscript{7} |
|       | R    | H3 AR         | ATATCCTTRGGCGATRAATG | Inward et al\textsuperscript{7} |
| Species                          | Age (Ma) | Minimum Age Constraint for Group | Calibration Group | Soft Maximum Bound (97.5% probability) | Reference |
|---------------------------------|----------|----------------------------------|-------------------|----------------------------------------|-----------|
| Baissatermes lapideus           | 137      |                                 | Cryptocerus + Isoperta | 250                                   | Engel et al. |
| Baissomatites musculata         | 112.6    |                                  | mantids           | 250                                    | Grimaldi  |
| Prochaeradodis enigmaticus      | 60       |                                  | Hoplocorixa + S phidromantis + Mantid | 130                        | Nel & Roy  |
| Cretaholocompsa montsecana      | 125.5    |                                  | Tervinae + Holocorixa + Euthyrhaphinae + Corydinae | 250                      | Evangelista et al. |
| Cratomastotermes wolfsenningeri | 113      | termites excluding Mastotermes   | termites          | 200                                    | Makarkin & Menon |
| Mastotermes indet.              | 93.5     |                                  | Zootermopsis + Parotermes | 150                                   | Schluter |
| Zootermopsis coloradensis       | 33.9     |                                  | Zootermopsis      | 150                                    | James    |
| Cryptotermes sp.                | 16       |                                  | Cryptotermes + Termes + Rhinotermes | 150                              | Park & Downing |

Table 2. Fossils Used for Estimation of Divergence Time of Major Clades in the Analysis of Blattodea with 26 outgroup taxa.

The molecular data set was divided into 9 partitions (partitioned by gene: 12S, 16S, 28S, COII, H3; COII and H3 were divided by codon position (pos1–3)). For ML, the GTR+Gamma model was selected for the combined datasets and 1000 bootstrap replicates were performed. For BI, PartitionFinder v.1.1.18 was used to choose models and model selection was based on BIC. For the 9 partitions, PartitionFinder resulted in the following models: GTR+I+G: 12S, 16S, COII_pos1, COII_pos2, 28S, TVM+G: COII_pos3, GTR+I: H3_pos1, JC+I: H3_pos2, TVM+I+G: H3_pos3. Two independent sets of Markov chains were run, each with one cold and three heated chains for 1 × 10^6 generations, and every 1000th generation was sampled. Convergence was inferred when a standard deviation of split frequencies < 0.01 was completed. Sump and sumt burninfrac were set to 25% and contype was set to allcompat.

**Divergence dating analysis.** We performed divergence date analyses based on the combined mitochondrial, nuclear and histone dataset of Blattodea and 26 outgroups (see Table S3). For this analysis, the molecular clock was calibrated using eight minimum age constraints based on termite, cockroach and mantid fossils as shown in Table 2. Analyses were performed using a relaxed molecular-clock model with the Bayesian phylogenetic program BEAST 1.8.10. Rate variation was modeled among branches using uncorrelated lognormal relaxed clocks, with a single model for all genes. A Yule speciation process was used for the tree prior and posterior distributions of parameters, including the tree, were estimated using MCMC sampling. We performed two replicate MCMC runs, with the tree and parameter values sampled every 5000 steps over a total of 50 million generations. A maximum clade credibility tree was obtained using Tree Annotator within the BEAST software package with a burn-in of 1000 trees. Acceptable sample sizes and convergence to the stationary distribution were checked using Tracer 1.5.13.

**Results**

**Phylogenetic inference.** For the concatenated dataset (12S rRNA, 16S rRNA, 28S rRNA, COII and H3), phylogenetic analyses yielded essentially identical topologies with generally high support values across the topologies for the two methods utilized (ML and BI) (Figs 1 and S1). Three recognized major lineages of Blattodea from ML and BI inferences were recovered with high support: Corydioidea, Blattoidea and Blaberoidea. Corydioidea was recovered as sister to the remaining Blattodea (MLB 100, BPP = 100), and was the first clade within Blattodea. Nocticolidae was recovered as sister group to *Latindia* + *Paralatindia* (MLB = 100, BPP = 100).

Blaberoidea was comprised of Blaberidae and Ectobiidae. In our inferred trees, Ectobiidae was paraphyletic with respect to Blaberidae with high support. *All Ectobius* clustered together and were recovered as the sister to the remaining Blaberoidea (MLB = 100), or to the remaining Ectobiidae (BPP = 46). *Nyctiborinae* + *Latiblattella* was the sister group of Blaberidae in both methods (BPP = 64, MLB = 100). For four subfamilies of the Blaberidae, (Oxyhaloinae, Blaberinae, Panchlorinae and Panesthiinae) relationships were the same among the two trees; for the remaining subfamilies (Perisphaerinae, Pycnoscelinae, Eplamprinae, Paranauphoetaeinae and Diplopterinae) there was lower resolution.

Blattidae, Tryonicidae, Lamproblattidae, Anaplectidae, Cryptocercidae and Isoptera formed one large clade, Blattoidea (MLB = 100, BPP = 100). Blattoidea was the earliest branching lineage within this clade (MLB = 100, BPP = 100). The topology derived from ML analyses showed that Anaplectidae was the sister group of (Cryptocercidae + Isoptera), followed by Lamproblattidae + Tryonicidae (both MLB = 100). However, in BI analyses, Anaplectidae was recovered to be the sister group of Lamproblattidae + Tryonicidae (BPP = 79), followed by Cryptocercidae + Isoptera (BPP = 99). Cryptocercidae was recovered as the sister group of Isoptera (MLB = 100, BPP = 100). North American *Cryptocercus* species (*Cryptocercus punctulatus*) and Asian species were recovered as sister groups (MLB = 100, BPP = 100).

**Divergence time analysis.** The estimated age of the split between Mantodea and Blattoidea was 243.6 Ma (204.3 Ma to 289.1 Ma 95% confidence interval [CI]). Corydioidea was recovered as the earliest branching group within Blattoidea, having diverged 209.7 Ma (180.5–244.3 Ma 95% CI) from the remaining taxa. The clade comprising Blattidae, Tryonicidae, Lamproblattidae, Anaplectidae, Cryptocercidae and Isoptera diverged from its sister group, Blaberoidea, around 198.7 Ma (173.1–229.1 Ma). The divergence of Blattidae from the remaining...
A group of this clade occurred about 178.2 Ma (155.2–204.4 Ma). The divergence of the lineages leading to termites and *Cryptocercus* was estimated to have occurred 146.4 Ma (137–164.2 Ma 95% CI). American and Asian *Cryptocercus* were estimated to have diverged 67.2 Ma (44.1–96.3 Ma 95% CI). Anaplectidae + (Lamproblatidae + Tryonicidae), was estimated to have arisen 154.8 Ma (133.3–179.3 Ma 95% CI). *Latiblattella* sp.3 from *Pseudophyllodromiinae*, and *Nyctibora* sp.1 and *Paratropes* sp.1 from *Nyctiborinae* clustered together, and were recovered as the first clade in Blaberoida, emerging 183.6 Ma (158.4–214.9 Ma 95% CI) from the remaining Blaberoida. Blaberidae was found to be monophyletic in this analysis and began to diverge 134.7 Ma (110.6–162.0 Ma) from the remaining Ectobiidae. The lineages leading to most Blattoidea species diverged from their sister lineages around 100 Ma or less.
Discussion

Our analyses using Maximum likelihood (ML) and Bayesian inference (BI) showed that the backbones of the inferred trees were nearly identical, and partly in agreement with previous studies12. Three major blattodean lineages, Corydioidea, Blattoidea and Blaberoida, were recovered with high support values. Our result was markedly different from previous phylogenetic studies based only on morphological characters6,10-13. A number of previous molecular studies did not include Anaplectidae2,11,19, Lamproblattidae2,19 or Tryoniidae11 or combinations of these28. Legendre et al.13 included a large number of taxa in their analyses, however several molecular markers were missing for a number of taxa. Ware et al.10 combined molecular and morphological data of 59 taxa (12 taxa with both molecular and morphological data, and 15 taxa with only morphological data), and used doublet and MK models in MrBayes.

Placement and monophyly of members within Corydioidea. Corydioidea was found as the sister group to the remaining Blattodea and considered as the basal split within Blattodea with high support (BPP = 100, MLB = 100), consistent with previous studies7,10 but not congruent with a recent study12. We found Nocticola subfamilies to be monophyletic and firmly nested within Corydioidea with strong support values (BPP = 100, MLB = 100), partially consistent with the results of Djernæs et al.15 (morphological analyses; molecular and combined analyses). The placement of Nocticola subfamilies found here was not consistent with the proposal that Nocticola was the sister group of Corydioidea12-13. In Djernæs et al.12, N. babindaensis formed an exceedingly long branch. Similarly, very long branches were found in the Noctiola clade in the study of Legendre et al.13. In our study, N. babindaensis (epigean, from Australia) and one Chinese Noticola species (termitephilous, Zhao Tiexiong, pers. obs., from China), were well grouped together and formed two short terminal branches (Figs 1 and S1), also with N. australiensis (cavernicolous, from Australia) and Noticola sp. (Cryptocercus baltae, em. from Australia) as their sister group. The inclusion of our Noticola specimen, the first from outside Australia, provides molecular support for the monophyly of this family. That N. babindaensis and N. australiensis are placed in different clades is consistent with the notion that N. babindaensis and N. australiensis are from two different species groups based on the presence or absence of the male tergal gland44.

The family Nocticola subfamilies consists of 8 genera, mainly distributed in Madagascar, Australia, Africa and southeastern Asia. It contains representatives with depigmentation and thinning of cuticle, the reduction or loss of eyes, the reduction or loss of tegmina and wings, the elongation and attenuation of appendages, and a more slender body form45. Although Noticola representatives show broad morphological similarities to ectobiid cockroaches, the complex and highly variable nature of their genitalia indicates a closer relationship with Corydoididae44.

Currently the subfamily Latindiniini is composed of three genera, the type genus Latindia with 9 species, Buboblattia with 2 species and Sinolatindia with 1 species46-47. Latindiniini are gracile, delicate, small bodied cockroaches with a number of features similar to ectobiid cockroaches. These include legs weakly covered with spines, long cerci, both sexes winged, and very complex male genitalia46-49. In both our study and that of Djernæs et al.12, the placement of Nocticola + Paralatindia as the sister group of Nocitoida subfamilies indicates that Latindiniini should be upgraded to the family Latindiniidae.

Placement of Anaplectidae. Consistent with results from a previous molecular study12, in our study Anaplectidae had a close genetic relationship with Blattoidea (Blattidae, Tryoniidae, Lamproblattidae, Cryptocercidae and Isotera) and together formed one large clade, similar to the results of Djernæs et al.12. Species of Anaplecta have a small body size and brown color, and are very similar to ectobiid cockroaches, however they don’t rotate their ootheca before producing them, and the subgenital plate of females is bilobed. Moreover, the male genitalia are more complicated than that of other ectobiids and similar to Blattidae (Fig. S2). It would therefore appear reasonable that Anaplecta is closer to Blattoidea than to Ectobiidae.

Placement and monophyly of Blaberoida. Within Blaberoida, Blaberidae is strongly supported to be monophyletic, but Ectobiidae was paraphyletic. This confirmed the results of previous studies6,11-13. Compared with Djernæs et al.12, more ectobiids and blabrids (52 species vs 12 species) were included in our analysis, and our results were quite different. Species of Blaberidae and Ectobiidae each formed monophyletic groups, with the exception of the three ectobiid genera Nyctibora, Paratropes and Latiblattella, which clustered together as the sister group of Blaberidae (BI = 64, MLB = 100). Our finding from ML analyses that Ectobiinae was the earliest branch within the clade Blaberidae (MLB = 100) is inconsistent with Djernæs et al.11,12 and Che et al.50, but, to some extent, similar to the results of Murienne19 (Fig. 1). However, in BI analysis, Ectobius was recovered as the sister of Pseudophyllodromiinae and Blattellinae, similar to other recent molecular studies6,11-13. Grandcolas15 proposed that Blaberidae was the sister-group of Pseudophyllodromiinae based on morpho-anatomical characters. In contrast, we found Pseudophyllodromiinae (Latiblattella) and Nyctiboridae (Nyctibora and Paratropes) as sister to Blaberidae, similar to Kluss19. Some Pseudophyllodromiinae representatives (Supella, Balta and Marginata were included) were placed as the sister of Blaberidae44, but support values were low.

Divergence times. The estimated age of the split between Mantodea and Blattoidea (243.6 Ma (204.3–289.1 Ma 95% CI) shown in Fig. 2) is older than some recent estimates around the Triassic-Jurassic boundary (~200 Ma)51-53, although much younger than others (Djernæs et al.12, 273 ± 15 Ma; Legendre et al.12; ~ 300 Ma). However, the divergence time is much older than that of Che et al.50 (2017: 155.41 Ma (145.0–185.09 Ma)), which was based on only a single mitochondrial marker. The divergence of the lineages leading to termites and Cryptocercus was estimated to have occurred 146.4 Ma (137–164.2 Ma 95% CI), similar to previous studies (Misof et al.22; 145 Ma; Tong et al.33; 140 Ma; Che et al.54; 145.8 Ma) but younger than others (Djernæs et al.12; 185 ± 19 Ma; Legendre et al.13; 195 Ma). The 67.2 Ma
(44.1–96.3 Ma 95%CI; Fig. 2) divergence time of the Asian and American *Cryptocercus* lineages is consistent with recent estimates (Che et al.54: 55.09 Ma (41.55–72.28 Ma); Maekawa et al.18 58.7–77.8 Ma). The divergence times of Corydioidea from the remaining Blattodea, and Blattidae from the remaining Blattoidea were estimated beyond 250 Ma and 220 Ma by Djernæs et al.12, somewhat older than our 209.7 Ma (180.5–244.3 Ma 95% CI) and 178.2 Ma (155.2–204.4 Ma).

Overall our estimated divergence times are younger than those of Djernæs et al.12 and Legendre et al.13. One possible reason for this is the selection of fossils for node calibration. In the aforementioned studies, the following were used: 1) a divergence event within Mantodea; 2) the basal split between Mantodea and Blattodea; 3) splits within termites. Calibrating evolutionary rates on the basis of fossils closely related to the taxa under investigation is thought to increase the accuracy of inferred evolutionary timescales55.
Conclusions
This study is a comprehensive analysis of Blattodea phylogeny based on mitochondrial and nuclear genes. Although some deeper nodes are not well resolved, the recovery of major nodal support for the proposed interfamily relationships is an advance over the majority of previous studies. Perhaps the most instructive finding of the present study is the strong effect of additional sampling on Blattodea molecular analyses. For instance, the inclusion of additional Ectobiidae and Blaberidae representatives appears to greatly influence the resulting Blaberoidea topology. In future reconstructions of cockroach phylogeny, the introduction of samples that better represent the full diversity of the group is therefore recommended.

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Author Contributions

Z.W.Q. and Y.S. generated data. Z.Q.W., Y.L.C., and N.L. conceived the project, analyzed data, interpreted the results, and wrote the paper. All authors read and approved the final manuscript.

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