A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes

(termites/mitochondrial DNA/molecular phylogenetics)

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ABSTRACT

Cockroaches are among the most ancient winged insects, the earliest fossils dating back to about 400 million years. Several conflicting phylogenies for cockroach families, subfamilies, and genera have been proposed in the past. In addition, the relationship of Cryptocercidae to other cockroach families and the relationship between the cockroach, Cryptocercus punctulatus, and the termite, Mastotermes darwiniensis, have generated debate. In this paper, a phylogeny for cockroaches, mantids, and termites based on DNA sequence of the mitochondrial ribosomal RNA genes is presented. The results indicated that cockroaches are a monophyletic group, whose sister group is Mantodea. The inferred relationship among cockroach families was in agreement with the presently accepted phylogeny. However, there was only partial congruence at the subfamily and the generic levels. The phylogeny inferred here does not support a close relationship between C. punctulatus and M. darwiniensis. The apparent synapomorphies of these two species are likely a manifestation of convergent evolution because there are similarities in biology and habitat.

In addition to the overall phylogenetic relationships among cockroaches, two other issues have generated debate. The first is the relationship of Cryptocercidae to other cockroach families. Cryptocercidae consists of one genus (Cryptocercus) and three species (6) and is generally considered a sister group of Blattidae (1). However, it was recently proposed that Cryptocercidae be merged with Polyphagidae (7). The second issue concerns the relationship among cockroaches, mantids, and termites. Three major schemes have been proposed: Blattaria and Mantodea are sister groups and Isoptera is a sister group to the Blattaria–Mantodea complex (8), cockroaches and termites belong to the order Blattodea and mantids are a sister group to that order (9), and all three groups belong to Dictyoptera (1, 10). Of particular interest is the presumed close phylogenetic relationship between Cryptocercus and the termite, Mastotermes darwiniensis. M. darwiniensis has been considered the most archaic living termite species and the “missing link” between cockroaches and termites (11). It is the sole living representative of the family Mastotermidae and is found in northern Australia (12). The close relationship between M. darwiniensis and Cryptocercus is based on several apparent synapomorphies (13–16).

A phylogeny of mantids (one species), cockroaches (two species), and termites (three species) based on previously published morphological characters has recently been proposed (6). A conclusion of that study was that Cryptocercus punctulatus is not closely related to M. darwiniensis but is a part of Blattodea (= Blattaria), which is a sister group to Mantodea. A phylogenetic study (12) that included four termite species and one each of cockroaches and mantids, and was based on DNA sequence of a portion of the nuclear 18S rRNA gene, indicated a sister group affinity of M. darwiniensis to other termites. In contrast, a study (17) that included C. punctulatus, M. darwiniensis, Blatta orientalis, and Reticulotermes flavipes and was based on DNA sequence of the entire 18S rRNA gene suggested that C. punctulatus and M. darwiniensis are closely related. This led the author to conclude that “Mastotermidae is considered to belong to Blattodea, instead of Isoptera” (ref. 17, p. 132). The conflicting conclusions of the above studies (6, 12, 17) suggest a need to verify their findings by including a more diverse range of cockroach taxa and employing a DNA sequence from a different gene because of the issue of gene trees and species trees (18, 19). Thus, the primary objective of this study was to infer a phylogeny for cockroaches, mantids, and termites based on the DNA sequence of mitochondrial large (16S rRNA) and small ribosomal (12S rRNA) subunit genes. The specific objectives were to (i) compare the molecular phylogeny with that proposed by McKittrick (1), (ii) infer the relationship between Cryptocercidae and other cockroach families, and (iii) infer the relationship between C. punctulatus and M. darwiniensis.*

MATERIALS AND METHODS

Insects. The species included in this study are as follows:

Blaberidae: Archimandrita tessellata, Blaberus atropos, Blaberus craniifer, Blaberus discoïdalis, Blaberus giganteus, Byrsotria fumigata, Diploptera punctata, Epilampra azteca, Eublaberus posticus, Gromphadorhina portentosa, Nauphoeta cinerea, Panclara nivea, Phoetalia pallida, Phortoeca phoraspoides, Pycnoscelus surinamensis, Rhyparobia maderae, Schistocerca lampyridiformis; Blattidae: Blattella vaga, Nahublatalla fratera, Nahublatalla nahu, Nycitibora azteca, Nycitibora latzi, Paroblatta pensylvanica, Symphoecellus pallens; Blattidae: Blatta orientalis, Melanoloxasteria soror, Periplaneta americana, Periplaneta australasiae, Periplaneta brunnea, Periplaneta fuligiosa, Selsondella lateralis; Cryptocercidae: C. punctulatus; Mantidae: Mantis religiosa; Rhinotermitidae: Cotoptermes formosanus, Reticulotermites flavipes; Mastotermidae: M. darwiniensis. One or more live specimens or DNA of the organism (M. darwiniensis and M. religiosa) were obtained from colleagues. In most

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U17761–U17832).
cases, at least one individual was preserved as a voucher specimen.

DNA Extraction, PCR, and DNA Sequencing. DNA was extracted from a small portion of the fat body of frozen specimens and PCR was set up as described (20). The PCR conditions were an initial denaturation step of 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 50°C for 1 min, and 72°C for 1.5 min. The amplification product was electrophoresed on a 2% low-melting-point agarose gel and purified using minicolumns (Wizard PCRpreps, Promega). DNA sequence was obtained directly from 3 μl of the purified PCR product using the cycle sequencing method ( fluorescent System, Promega). The reaction mixtures were electrophoresed on 6% polyacrylamide denaturing gels. Both strands of the PCR product were sequenced.

Oligonucleotide Primers. The primers for the amplification of a 415-bp fragment of the 16S rRNA gene were forward 5'-TTA CGC TGT TAT CCC TTA-3' (positions 13,000-13,017 of Drosophila yakuba), and reverse 5'-CGC CGA TTT ATC AAA AAC AT-3' (13,396-13,415 of D. yakuba). The primers for amplification of the 431-bp fragment of the 12S rRNA gene were forward, 5'-TAC TAT GTT ACG ACT TAT-3' (14,182-14,199 of D. yakuba), and reverse, 5'-AAA CTA GGA TTA GAT ACC C-3' (14,594-14,612 of D. yakuba). The primers were derived from previously published insect mitochondrial sequences (21–24). Both sets of primers result in the amplification of a homologous fragment from a wide range of insects. Internal primers (16S rRNA: 5'-TCT ATA GGG TCT TCT CGT C-3' and its reverse complement; 12S rRNA: 5'-TGC ACC TTG ACC TGA A-3' and its reverse complement) were used to obtain the sequence on the ends of the fragments.

Sequence Alignments and Phylogenetic Inference. The sequences were read manually from autoradiograms into a computer. They were aligned using CLUSTAL V (25) and then optimally aligned manually. The alignment parameters were 8-tuple score = 1, gap penalty = 3, and window size = 5 (pairwise alignments); fixed and floating gap penalties = 10 (multiple alignments). Phylogenetic analysis was carried out in PAUP 3.1.1 (26) using the multiple equally parsimonious heuristic search option with tree bisection–reconnection. The data set was too large to be used with the exhaustive or the branch and bound algorithms. Gaps were treated as a fifth base. The sequences of the two genes were analyzed as a single data set (27) without character weighting. The data set was bootstrapped for 1000 replications using PAUP. The DNA sequence of the 16S rRNA gene of Locusta migratoria (24) was included as the outgroup. The DNA sequence of the small ribosomal subunit gene of L. migratoria was not available; thus the 12S rRNA data for this taxon were designated as missing.

RESULTS

DNA Sequences of rRNA Genes. The sequences for taxon in this study can be obtained directly from GenBank or from the author. The average size of the sequenced portion of the 16S rRNA gene was 415.3 ± 0.75 bp (mean ± SE) and that of the 12S rRNA gene was 431.4 ± 0.72 bp. Summary statistics for the two genes are presented in Table 1.

Phylogenetic Inference. The alignment of the sequences resulted in a total of 923 characters, including gaps. Unambiguous alignment was possible for most regions; six regions totaling 140 characters were relatively more difficult to align. When the data were analyzed without these regions, the topology of the tree identified by PAUP was nearly identical to that of the tree generated with the full data set. Therefore, all further analyses were carried out with the full data set.

PAUP identified a single most parsimonious tree of 3750 steps (Fig. 1). The taxa in the various orders, suborders, and families formed distinct clades. Four major clades were identified among cockroach taxa corresponding to the four families in this study. Taxa within Blaberidae were subdivided into two subclades. One consisted of genera in Blaberinae, Oxyhaloinae, Panchlorininae, and Diplopterinae and the second consisted of genera in Zetoborinae, Epilamprinae, and Pycnoscelinae. Within Blattellidae, Bla. vaga and Par. pensylvanica were shown to be sister taxa and joined to Sy. pallens, followed by the joining of Nyctibora spp. and Nahublattella spp. to the above three genera. Within Blattidae, the four species of Periplaneta were found to be paraphyletic. The Periplaneta–Shefordella clade was first joined to B. orientalis followed by Me. soror.

At the family level, Blattellidae and Blaberidae were shown to be more closely related to each other than either was to Blattidae. The sole representative of Cryptocercidae, C. punctulatus, formed a separate clade that was joined to the above three families. This was followed by the joining of M. religiosa to the cockroach clade. Among the three species of termites included in this study, C. formosanus and R. flavipes were shown to be sister taxa. Next, M. darwiniensis was joined to the above two taxa. Most of the relationships shown in Fig. 1 were supported in 70–100% of the replications in a tree derived from bootstrap analysis (Fig. 2).

DISCUSSION

A phylogeny of cockroaches inferred from DNA sequence of the mitochondrial rRNA genes indicated that cockroaches are monophyletic. This is in contrast to the suggestion (17) that the group is perhaps paraphyletic. The relationship among families inferred here was in agreement with the presently accepted phylogeny (1). However, there were significant differences at the subfamily and generic levels between McKittrick’s (1) and the molecular phylogenies. Of particular note were taxa in Blaberidae, within which relationships among genera did not always reflect the subfamily designations of McKittrick. For example, D. punctata and Pho. pallida were placed in Diplopterinae by McKittrick (1) and in Diplopterinae and Epilamprinae, respectively, by Princis (4). In this study, Pho. pallida was shown to be more closely related to Sc. lampyridiformis of Zetoborinae than to D. punctata.

Two subfamilies of Blattellidae, Blattellinae and Nyctiborinae, were represented in this study. Blattella, Parcoblatta, and

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<th>Table 1. Summary statistics for the DNA sequences of the mitochondrial rRNA genes</th>
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The statistics represent the means for 36 taxa, excluding L. migratoria.
Fig. 1. Single most parsimonious tree for DNA sequence of mitochondrial 16S rRNA and 12S rRNA genes of cockroaches, termites, and mantid, rooted by the outgroup, L. migratoria. Tree length, 3750 steps; consistency index, 0.35; retention index, 0.54. Numbers above the branches are branch lengths. Numbers accompanying scientific names are family or subfamily designations: 1, Zetoborinae; 2, Epilamprinae; 3, Pycnoscelinae; 4, Blaberinae; 5, Diplop- terinae; 6, Oxyhaloinae; 7, Panclorhininae; 8, Blattellinae; 9, Nycitoborinae; 10, Blattinae; 11, Polyzosterini; 12, Cryptocercidae; 13, Mantidae; 14, Heterotermitinae; 15, Copiotermitinae; 16, Mastotermitidae. The roman numerals indicate ordinal, subordinal, or family designations: I, Blaberidae; II, Blattidae; III, Blattidae; IV, Cryptocercidae; V, Mantodea; VI, Isoptera; OG, outgroup.

Symptloe, all Blattellinae, were inferred to be closely related to one another as previously suggested (1). However, because of the placement of Nahubblattella relative to the above genera, Blattellinae as presently recognized is paraphyletic. Nycitoborinae has been inferred to be a sister group of Blattellinae (1), a relationship confirmed by this analysis.

Two subfamilies of Blattidae were included in this study: Blattinae and Polyzosterini. As expected, all genera in Blattinae were sister taxa to one another and Polyzosterini was shown to be the sister group of Blattinae. Within Blattinae, Sh. lateralis was found to be a sister taxon of Pe. brunnea and thus the genus Periplaneta is paraphyletic according to my analysis. No consensus on the generic status of Sh. lateralis is apparent. Historically, this species has been successively placed in the genera Periplaneta, Blatta, and Shelfordella. Walker (28) originally described this species as Periplaneta lateralis. The relationship inferred here suggests that this species should perhaps be placed in the genus Periplaneta as originally proposed rather than Blatta (4, 29) or Shelfordella as presently recognized.

The results of my analysis indicated that Cryptocercidae is most closely related to Blattidae. As mentioned, however, a proposal to merge Cryptocercidae and Polyphagidae has been made (7). Unfortunately, I could not include Polyphagidae to test its relatedness to Cryptocercidae. Further work is necessary to infer the relationship between Polyphagidae and Cryptocercidae.

The results of this study suggested that Mantoidea is a sister group of Blattaria and that termites are a sister group of the cockroach–mantid complex. Although it seems appropriate that cockroaches and mantids be retained in Dictoptera with each group being assigned a subordinal status (8), future studies that include a broader range of mantids and termites are needed to confirm the relationships inferred here.

The results of my analysis indicated that M. darwiensis and C. punctulatus are not closely related. The sister group affinity of M. darwiensis was clearly with other termites and that of C. punctulatus was with other cockroaches. At present, two opposing views exist concerning the phylogenetic relationship of these two taxa. One states that they are no more closely related to each other than cockroaches in general are related to termites and any apparent synapomorphies of Cryptocercus and Mastotermes together are the result of convergent evolution (6, 9). The alternate view (1, 17) holds that the synapomorphies of the two taxa are a result of common ancestry. Specifically, the conclusions of morphological analysis (6) and earlier analyses of DNA sequences (17) are in odds concerning the relationship of C. punctulatus with M. darwiensis. The present study, also based on DNA sequence analysis, but of different genes and with an extensive sampling of cockroach taxa, clearly supported the conclusions of the morphological analysis (6). In both trees (Figs. 1 and 2), M. darwiensis was a sister taxon of the other termites. In the parsimony tree (Fig 1) the affinity of C. punctulatus was clearly with other cockroaches.
A significant synapomorphy on which a close phylogenetic relationship of Cryptocercus with Mastotermes has been proposed is the cellulolytic gut fauna that is present in both taxa but not in other cockroaches (1, 13–16). Previous studies (1, 30–33) and the present study indicate that C. punctulatus belongs to a primitive phyletic line from which the other cockroaches have descended. This suggests two alternate scenarios for the presence of cellulolytic symbionts in Cryptocercus but not in other cockroaches. First, *M. darwinianus* and Cryptocercus share gut fauna due to common ancestry. In this case, it must be assumed that all other cockroaches (and mantids) that descended from a Cryptocercus-like ancestor have secondarily lost gut fauna (6). Alternatively, Cryptocercus acquired the symbionts through some mechanism (see below) after other cockroaches arose. In this scenario, a secondary loss need not be invoked for the non-cryptocercid cockroaches.

If secondary loss is not invoked, the shared gut fauna of Cryptocercus and Mastotermes implies convergent evolution (9) and perhaps interspecific transfaunation (34). It is not known which group first acquired the cellulolytic gut fauna. However, a scenario in which transfaunation could occur through interspecific predation has been proposed (34). Since Cryptocercus and Mastotermes were sympatric in the Americas before the latter became extinct there (9, 12), the possibility exists that the gut fauna was obtained through interspecific predation. Thorne (34) concluded that “Cryptocercus shares no unambiguous morphological, behavioral or symbiotic synapomorphies with the Isoperta that are not shared with other cockroaches as well. Cryptocercidae are clearly primitive roaches, but it is more likely that the family is a sister group to other cockroach taxa rather than a sister group to modern termites.”

Cogen arguments against the scenario in which transfaunation could have occurred are presented (35). Thus, the question of how Cryptocercus and Mastotermes came to share gut symbions is yet to be resolved. Further studies, directly on the DNA of the gut fauna, may help resolve the issue of whether the gut symbionts of Cryptocercus and Mastotermes are more closely related to each other than a random pair of symbionts from xylaphagous insects. With PCR, it is feasible to study symbiont-specific genes as has been done for other insect symbionts (36, 37).

As with other insect mtDNA studied to date (21–24), the base composition of sequences in this study was strongly biased toward adenine and thymine, which comprised 72% of the total. The observed transition and transversion rates were comparable to those reported for other insect groups and the rate of transversions was significantly greater than that of transitions. A greater transversion rate relative to transition rate has also been observed in these mtDNA genes: 16S rRNA of leafhoppers (38) and black flies (39), cytochrome oxidase II of 10 insect orders (40), and NADH 1 and 16S rRNA of Drosophila spp. (41). The bias toward transversions in insect mtDNA is in contrast to the mtDNA of primates in which 92% of the substitutions were transitions (42). It has been suggested that the bias may be due to deficient mtDNA repair mechanisms and a tautomeric base pairing chemistry (43). As with other insects (references above), >70% of transversions in cockroaches, termites, and mantids were A → T transversions.

In summary, the significant findings of this study are as follows. The molecular phylogeny was congruent at the family level with the morphological phylogeny proposed by McKittrick (1). However, significant differences were observed at the subfamily and generic levels. DNA sequences from more species need to be obtained to further delineate the relationships at the subfamily level. The results of this study suggested that Cryptocercidae is most closely related to Blattidae. Whether it should be placed in the Polyphaga needs to be resolved using appropriate taxa. My analysis did not lend support to the hypothesis that *C. punctulatus* and *M. darwiniensis* are closely related. Thus, the suggestion that *M. darwini-