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DNA Sequences from a Fossil Termite in Oligo-Miocene Amber and Their Phylogenetic Implications

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DNA was extracted from the fossil termite *Mastotermes electrodominicus* preserved in Oligo-Miocene amber (25 million to 30 million years old). Fragments of mitochondrial [16*S* ribosomal DNA (rDNA)] and nuclear (18*S* rDNA) genes were amplified by polymerase chain reaction. Phylogenetic analysis of fossil and extant 18*S* rDNA confirmed morphological cladistic analyses of living dictyopterans (termites, cockroaches, and mantids). The fossil termite shares several sequence attributes with *Mastotermes darwiniensis.* Addition of this fossil to living-species phylogeny is required to substantiate *Mastotermes* monophyly and affects molecular phylogenetic hypotheses of termites in this, the oldest DNA yet characterized.

The tropical northern half of Australia is home to *Mastotermes darwiniensis*, one of the most intriguing of the 2000 or so described species of termites (Isoptera). This species has been a popular candidate for the most primitive isopteran and an apparent "missing link" between cockroaches and termites (*1*, *2*). It is the sole living species in the family Mastotermitidae, classified as such to reflect its primitive phylogenetic position. The features on which this classification is based are (i) an egg mass or pod resembling a rudimentary form of the ootheca in cockroaches, (ii) presence of gut symbionts in certain cockroaches such as the apterous colonial *Cryptocercus*, and (iii) a host of primitive morphological features (*3*). A cladogram of this traditional view of termite phylogeny appears in Fig. 1A.

Established views on the primitive nature of *Mastotermes* have been challenged by a cladistic analysis of dictyopteran insects, based on a review of morphological, behavioral, chromosomal, and cell ultrastructural characters (*4*) (see Fig. 1B). In the scheme of Thorne and Carpenter (*4*) *Mastotermes* is the sister group to the family Kalotermitidae, with Termopsidae as the primitive sister group of the remaining termites. The Isoptera's unquestionable monophyly is based on eusociality (with its associated behavioral and morphological caste polytypism), deciduous wings, and other morphological features. In the relationships proposed by Thorne and Carpenter, termites are the sister group to the mantids and cockroaches (the latter also includes *Cryptocercus*). Thorne and Carpenter do not address what appear to be pleisomorphic features that define *Mastotermes* and its family. Although they cite many derived features, it is unclear whether these attributes pertain to the Mastotermitidae as a whole. The plesiomorphies include, for example, a large anal lobe on the wings seen in mantids, cockroaches, and many orthopteroids (*2*). Thus, the monophyly of the one living species and of fossil mastotermitids is questionable and has implications for the interpretation of other evolutionary aspects of this "group."

Thorne and Carpenter dismiss *Mastotermes* as a "living fossil" because it possesses a large number of derived features and, in their scheme, is not the most primitive of termites. The fossil record of the Mastotermitidae indicates that, if the group is monophyletic, the present distribution is narrowly restricted and relict. Rock fossils indicate that there were extinct genera from the Mio-Pliocene of Brazil, the Eocene of Tennessee, and the early Cretaceous of England; the genus *Mastotermes* occurs from the Eocene to the Miocene (20 million to 40 million years ago) of Europe (*5*). The genus has been found only recently in amber as the extinct and closely related species *M. electromexicus* from Chiapas, southern Mexico (*6*), and *M. electrodominicus* from the Dominican Republic (*7*). Oddly, the genus has been unknown from huge, diverse collections of Baltic amber fossils (*8*). Clearly, a great deal of mastotermitid evolution has been obscured by extinction, glimpses of which are seen in the fossil record.

It is not surprising that *M. electromexicus* and *M. electrodominicus* are closely related, given the paleontology of the ambers in which they are preserved. Both Mexican and Dominican deposits have the same botanical source, the tropical canopy legume *Hymenaea* (*9*). Stratigraphy indi-

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cates that the two deposits are roughly contemporaneous, formed in the late Oligocene and early Miocene (*10*). Obvious to the naked eye is the similarity in the ambers, which are renowned for the clarity of microscopic details of insect cuticle and for fine preservation of inclusions (*11*). Discovery of cells with intact organelles from amber fossil flies (*12*) has inspired many efforts to extract DNA from amber fossil insects. We have sequenced the oldest DNA extracted from a fossil (in 25-million-year-old amber) (*13*) and have used analyses of this DNA in phylogenetic reconstruction.

A large piece of clear yellow amber with three-winged *M. electrodominicus* was used (Fig. 2). Exact origin of the amber in the Dominican Republic is unknown, but it is assumed to come from lower Miocene deposits in the Cordillera Septentrional (*14*). Morphology of the termites, aspects of the preservation, and pyrolysis–gas chromatography profiles of a barren fragment of the piece indicate that the amber is authentic (*15*). Any suggestion that it is a much younger copal, which occurs in the Cordillera Oriental, can be dismissed (*16*).

The amber surrounding one specimen was sliced open with a razor blade under sterile conditions (*17*) and small pieces of gummy, brown material were removed. DNA extraction yielded an extremely small quantity of nucleic acid that was highly degraded [in general, smaller than 250 base pairs (bps)]. The 18*S* nuclear rRNA and 16*S* mitochondrial rRNA genes were targeted for amplification because of their high copy number and highly conserved segments that can be used as primers (*18*) (Figs. 3 and 4). Previous analyses of insect 18*S* sequences indicated that this locus was likely to be informative (*19*). Two 200-bp regions were chosen for amplification. Because of their high levels of variation, the two 150-bp regions from the 16*S* rRNA gene sequence are not as well suited for phylogenetic analysis as the 18*S* rDNA. They are, however, effective for distinguishing among the closely related isopteran taxa.

While the nuclear sequences helped place *M. electrodominicus* in the framework of insect relations, the 16*S* rDNA sequences were used to assess the relative divergence of the fossil taxon from its extant sister taxon and to assess the possibility of contamination. These analyses revealed that 16*S* sequences had accumulated nine substitutions between the extant *M. darwiniensis* and the amber *M. electrodomininicus* and nearly twice that number for the cockroach *Blaberus*. Comparison of the same 100-bp region in drosophilid genera (*20*) revealed seven sequence differences between the most distantly related pair of genera in the Drosophilinae. The level of variation in the genus *Mastotermes* is then greater than this entire subfamily of flies, which is consistent with the greater age of the Mastotermitidae. Moreover, there were 17 substitutions between *M. darwiniensis* and

Fig. 2. Winged adult specimen of the extinct termite *M. electrodominicus* Krishna and Grimaldi in a piece of amber from the Oligo-Miocene (25 million to 30 million years ago) of the Dominican Republic. DNA was extracted and sequenced from similar specimens of this species.

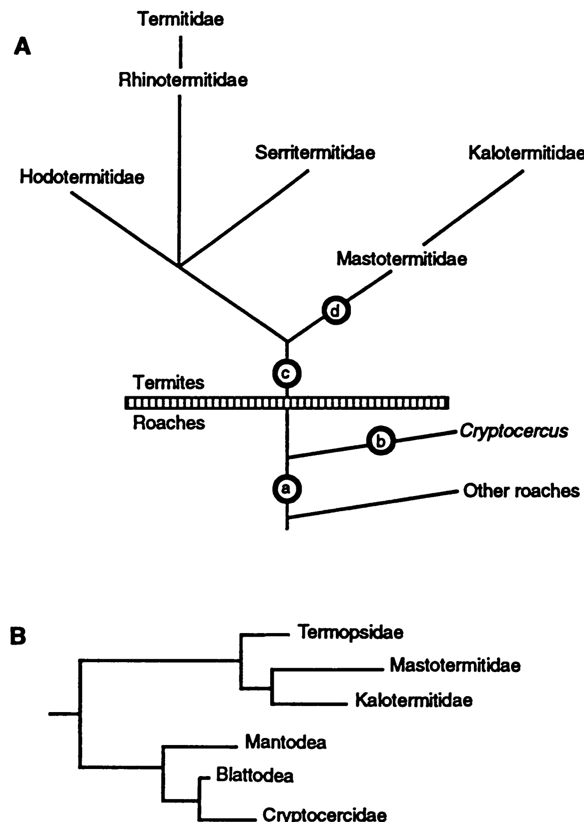

Fig. 1. (**A**) Traditional termite-roach phylogeny [adapted from findings in (*3*)]. Not all characters in the original tree are shown. Note that roaches are presented as a grade (paraphyletic group) leading to termites, and that Mastotermitidae is paraphyletic (with respect to Kalotermitidae). The striped line across the base of the tree indicates the break in the grade from termites to cockroaches. Circled letters on branches refer to the following characters that define these branches: a, symbiotic gut flagellates; b, coloniality and loss of wings; c, eusociality and deciduous wings; d, loss of mandibular tooth and *Metadevescovina* gut flagellates. (**B**) Phylogeny of Thorne and Carpenter (*4*) based on 70 morphological and behavioral characters. The cladogram was rooted with the use of inferred ancestral states.

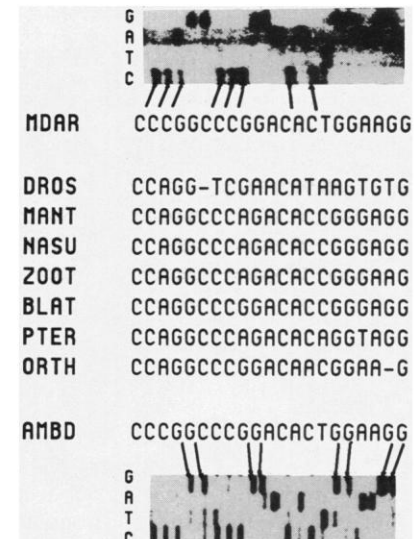

Fig. 3. Sequence gel of amber-preserved *M. electrodominicus* (AMBD, Mastotermitidae) and extant *M. darwiniensis* (MDAR, Mastotermitidae) for a short region of the 18*S* "C" fragment. The figure shows the sequences of the insect taxa used here and illustrates one of the synapomorphies uncovered in this study. The lines to the MDAR sequence show Cs and those from AMBD show Gs. Abbreviations are: BLAT = *Blaberus* sp. (Blattodea); MANT = *Mantis religiosa* (Mantodea); NASU = *Nasutitermes costalis* (Termitidae); ZOOT = *Zootermopsis nevadensis* (Termopsidae); DROS = *Drosophila melanogaster* (Drosophilidae); ORTH = *Warramaba picta* (Orthoptera); and PTER = *Pteronarcys* sp. The sequence shown here corresponds to bases 38 to 58 in Fig. 4B and is read from left to right. Double-stranded PCR amplifications were done with template DNA from *M. electrodominicus* (amber) and *M. darwiniensis* with standard protocols (*25*).

Drosophila and 14 between *M. darwiniensis* and fellow dictyopteran *Blaberus* (Fig. 4). The nuclear 18*S* rDNA data showed 55 and 39 substitutions, respectively, for identical comparisons. Because the 16*S* rDNA sequence cannot discriminate between such large differences in kinship (due, no doubt, to multiple substitutions) its effectiveness in checking for contamination is limited.

As a result, we concentrated our efforts on obtaining dictyopteran sequence information for the 18*S* rRNA gene. We partitioned the analysis to address four questions: What are the phylogenetic relations in the Dictyoptera? How does the extinct *M. electrodominicus* relate to the extant *M. darwiniensis* and the other Isoptera? Because fossils can be crucial for elucidating evolutionary pattern (*21*), what is the effect of adding the fossil taxon to the DNA phylogeny of both the Dictyoptera and the Isoptera? And, how do the molecular and morphological data interact in a "global parsimony" analysis after the addition of molecular data from the fossil taxon?

18*S*A fragment

```
DROS   TTACCCACTCCCAGCTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACTCATATCCGAGGCCCTGTAATTGGAATGAGTACACTTTAAATCCTTTAACAAGGAC-CAATTGGAGGG
MANT   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGTACACTTTAAATCCTTTAACGAGTAT-CCATTGGAGGG
NASU   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGTACACTCTAAATACTTTAACGAGTAT-CCATTGGAGGG
AMBD   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--TTCCGAGGCCCCGTAATCGGAATGAGTACACTCTAAATCCTTTAACGAGGAT-CCATTGGAGGG
ZOOT   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACAATACGGCACTC--ATCCGAGGCCCCGTAATCGGAATGAGTACACTCTAAATCCTTTAACGAGGAT-CCATTGGAAGG
MDAR   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGTACACTCTAAATCCTTTAACGAGGAT-CTATTGGAGGG
BLAT   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGCACACTTTAAATCCTTTAACGAGTAT-CCATTGGAGGG
PTER   TTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGCACACTTTAAATCCTTTAACGAGTAT-CCATTGGAGGG
ORTH   TTACCCACTCCCGGCAC-GGGAGGTAGTGACGAAAAATAACGATACGGGACTC--ATCCGAGGCCCCGTAATCGGAATGAGTACACTTTAAATCATTTAACGAGTATACTATTGGAGGG
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18*S*C fragment

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DROS   GCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGG-TCGAACATAAGTGTGTAAGACAGATTGATAGCTCTTTCTCGAATCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTCG
MANT   GCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCAGACACCGGGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
NASU   GCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCAGACACCGGGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
AMBD   GCCTGC-GCTTAATTTGACTCAACACGGGAAAACTCACCCGGCCCGGACACTGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
ZOOT   GCCTGC-GCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCAGACACCGGGAAGATTGACAGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
MDAR   GCCTGC-GCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACTGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
BLAT   GCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCGGACACCGGGAGGATTGACAGATTGATAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
PTER   GCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCAGACACAGGTAGGATTGACAGATTGATAGCTCTTTCTCGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
ORTH   GCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCGGACAACGGAA-GATTGACAGATTGATAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGG
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16*S* fragment

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AMBD   AATAAAATTTTAATAAATATAAAGATTTATAGGGTCTTCTCGGCCTTTAAAAATATTTTAGCCTTTTGACAAAAAAAAAAAAAATCTACAAAAAA
MDAR   AATTAAAATTTACAAAATATAAAGGTTTATAGGGTCTTCTCGGCTTTTAAAAATATTTTTGCCTTTTGGCTAAAAAAAAAAAAATCTACAAAAAA
DROS   AATTAAAATAAATAAAATATAAAGATTTATAGGGTCTTCTCGTCTTTTAAATTAATTTTAGCTTTTTGACTAAAAAATAAAATTCTATTTTAAA
BLAT   AATTAAAATAAACAAAATATAAAGATTTATAGGGTCTTCTAGAATTTTAAATATGCTTTAGCTTTTTGACTAAAAAAAAAAAAATCTATAAAAGA
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Fig. 4. DNA sequences from several insect taxa (see Fig. 3 for abbreviations of species names) used in this study for the 18*S* A, 18*S* C, and 16*S* fragments. The 18*S* A fragment corresponds to bases 444 to 561 in (*26*). The 18*S* C fragment corresponds to bases 1260 to 1382 in (*26*). The 16*S* fragment corresponds to bases 13,146 to 13,240, as in Fig. 3. Sequences were aligned using MALIGN (*27*), a computer program that accomplishes multiple alignment. A single set of two primers was used to generate the 225-bp-long 18*S* A fragment [18sai (5'CCTGAGAAACGGCTACCACATC) and 18sbi5.0 (5'TAACCGCAACAACTTTAAT)]. Two primers were used to generate the 215-bp-long 18*S* C fragment [18sbi (5'GAGTCTCGTTCGTTATCGGA) and 18sai2.0 (5'ATGGTGCAAAGCTGAAAC)]. Two sets of primers were used to generate the sequences for the 16*S* fragment. The first pair used [16*S*1 (5'AAGGCTGGAATGAATGGTTGG) and 16*S*2 (5'GATTTATAGGGTCTTCTCGTC] generated a 150-bp fragment. The second pair [16*S*3 (5'TTTAAAAGACGAGAAGACCC) and 16*S*4 (5'TTTAAATTTTAATATCACCCC] generated a 150-bp fragment immediately adjacent to the first. The primers used in the control experiments were 18sai1.0 (5'GGTGAAATTCTTGGACCGTC) as well as those for 18sai and 18sbi listed above. The extant isopteran sequences were generated in a physically separated lab at the American Museum of Natural History and well after all of the amber termite sequences were generated. We ruled out contamination from the extant taxa in our fossil taxon samples by working on the extant samples after generation of the fossil sequences. Because most of the sequences from these taxa were from ethanol-preserved museum specimens, sterile techniques and special care were also taken in handling these specimens.

The 18*S* rDNA sequences of three insects outside the Dictyoptera were used to root the cladogram: *Pteronarcys* (stone fly), *Drosophila* (fruit fly), and *Warramaba* (grasshopper). These taxa include representatives of both holometabolous and hemimetabolous insect groups to discriminate between character transformations. Although the most parsimonious solution (*22*) (Fig. 5A) to the molecular data alone differs in detail from that of Thorne and Carpenter, their overall scheme of relations has support. Removal of the fossil *M. electrodominicus* from the analysis resulted in the extant *M. darwiniensis* showing sister group affinity either to other termites or to the sample orthopteran (*Warramaba*) (Fig. 5B). Hence, only through the inclusion of this extinct taxon are termites seen as monophyletic by these data. When the data of Thorne and Carpenter are included to determine the best-supported interpretation of both morphological and molecular information, the rejection of an Isoptera-Blattaria clade is upheld (Fig. 6).

The sister group status of the extinct and extant *Mastotermes* is compelling evidence that authentic fossil DNA has been amplified, especially because their sequences are not identical. The major pitfall of this type of investigation is contamination from some outside source of DNA. We are confident that the amber fossil DNA is not a contamination product for the following reasons: (i) extraction of the

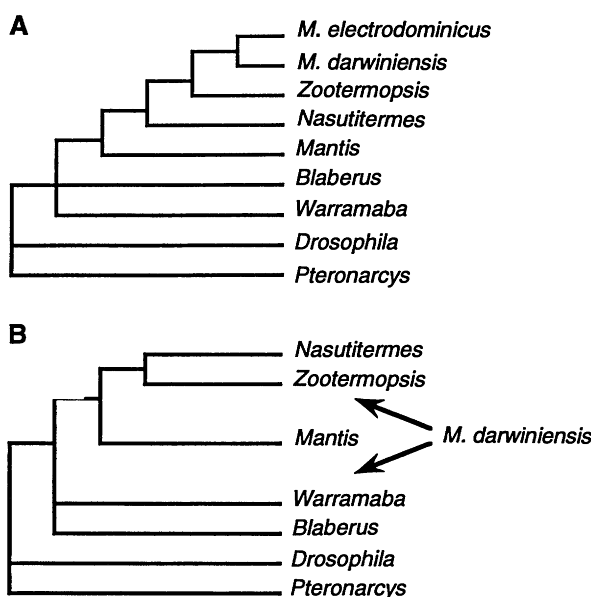

Fig. 5. (**A**) Molecular phylogeny of the Dictyoptera including the extinct *M. electrodominicus*. This cladogram requires 51 steps and has a consistency index of 0.667 (excluding uninformative characters) and a retention index also of 0.667 (*22*). (**B**) Molecular phylogeny of the Dictyoptera excluding the extinct *M. electrodominicus*. Exact analysis produced two cladograms, each with a length of 49 steps, consistency index (excluding uninformative characters) of 0.667, and a retention index of 0.611. In the two cladograms the position of *M. darwiniensis* switches between the orthopteran *Warramaba* and the termite *Zootermopsis*. Cladograms were constructed with the exact solution algorithm (ie*) in HENNIG86 (*22*).

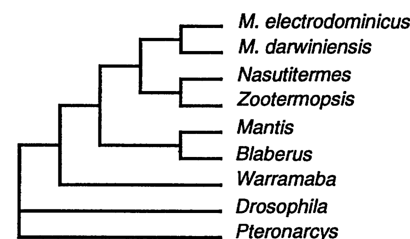

Fig. 6. Phylogeny of the Dictyoptera including morphological and molecular information [morphological characters from the matrix (*4*) and (*28*)]. This cladogram requires 143 steps and has a consistency index (excluding uninformative characters) of 0.833 and a retention index of 0.884 (*22*).

DNA from amber was done in a part of a new facility in the American Museum of Natural History where insect DNA had not been previously isolated, and extreme care was taken with positive displacement pipettes and tips; (ii) negative control amplifications did not occur; and (iii) prior attempts to extract DNA from amber-preserved woodgnats (Diptera: Anisopodidae) revealed obvious contaminants. Certain cloned polymerase chain reaction (PCR) products from amber *Mastotermes* amplifications were also easily confirmed as contaminants. They were almost exclusively dipteran in general and drosophilid in particular. The sequence differences and similarities between fossil and living *Mastotermes* were inconsistent with contamination but consistent with close relation.

Among all modes of fossilization, it can be reasonably assumed that preservation in amber will more consistently yield fossil DNA from Tertiary and perhaps older geological periods. So far, most ancient DNA has been recovered from human, mammoth, and other Holocene–late Pleistocene remains (*23*). The common factor among these remains is thorough dehydration, which also occurs in "amberization." The polymerization that results from the linking of isoprene units in resinous sap (and diterpenoids in *Hymenaea* sap, in particular) (*24*) causes inert dehydration of organic inclusions. This process, as well as flowing resin's ability to encapsulate an organism entirely and the bactericidal action of terpenes, lends amber its natural embalming features.

The isolation and characterization of DNA from this fossilized specimen allow us to uncover variation previously hidden from molecular systematic analysis. This affords direct comparison of both organisms and molecules with their forebears. In the history of the Isoptera, the great diversity of some of the most important groups has vanished through extinction. Without analysis of this and similar specimens we would be forced to use depauperate samples to represent large and (formerly) diverse groups, which has been shown to be an extremely error-prone method (*21*). The case of *M. electrodominicus* shows again how the inclusion of fossil taxa alters our understanding of the relations among living creatures. In the past, these comparisons have been limited, for the most part, to hard components of large-scale anatomy. Here is an illuminating glimpse into the DNA of 25 million to 30 million years ago.

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25. Amplification by PCR and direct sequencing of the products were conducted under standard conditions to avoid contamination: all manipulations were done in an area of the American Museum of Natural History lab designated solely for amber work, and positive displacement technology was used at all times. In addition, several controls were run during the PCR experiments. These included a negative water control, or "no DNA," reaction and a "large DNA" control. In both, primers were added to a reaction with amber template DNA that was meant to generate PCR products larger than 250 bases. We used primers that can produce 550-bp (18sbi and 18sai1.0) and 1100-bp (18sai and 18sbi in combination) fragments for this purpose. There is such a high degree of degradation to the amber genomic DNA that, if a large fragment is produced in a reaction, that amplification product can almost certainly be attributed to high molecular weight DNA from some contaminant. We used only amber-amplified DNAs that were generated in experiments where the "no DNA" and "large DNA" controls gave no signal. When proper amplification did occur for the amber sample, enough DNA was produced for double-strand sequencing, and so no asymmetric reamplification step was performed on any of these sequences. Annealing was at 50°C for 1 min in each cycle and 30 to 35 cycles were performed to obtain an ample product for sequencing. The double-stranded amplified DNA was treated with GENECLEAN (BIO 101, Vista, CA) and sequenced as a double-stranded template with ^{35}S and the SEQUENASE (U.S. Biochemicals) protocol. The sequencing products were run on a 6% acrylamide gel for 2 hours, dried under vacuum, and exposed to film from 24 to 120 hours. Film was developed in an X-OMAT automatic film processor. Sequence was determined for both strands of the PCR product.
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