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Austin et al. Rhinotermitidae Genetics

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**A Phylogenetic Analysis of the Subterranean Termite Family
Rhinotermitidae (Isoptera) using the mitochondrial cytochrome
oxidase (COII) gene**

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1 **ABSTRACT** Previous molecular phylogenetic studies have focused on
2 either the relationship between isopteran families or among
3 species within a given genus, but there are presently few
4 studies focusing on individual families and no known molecular
5 studies for Rhinotermitidae. We examined 38 Rhinotermitid
6 species representing 10 genera, relative to representatives of 4
7 other Isopteran families. Sequencing of a 667-bp region of the
8 mtDNA COII gene revealed 343 polymorphic sites within the
9 family. Tajima-Nei genetic distances ranged from 11 to 23% among
10 Rhinotermitid genera. Maximum parsimony and maximum likelihood
11 analysis of DNA sequence support existing hypotheses that
12 Mastotermitidae is the basal lineage among extant termites and
13 the family Rhinotermitidae is polyphyletic given the current
14 familial status of Serritermitidae. DNA sequence data suggest
15 that Serritermitidae should be relegated to the subfamily
16 Serritermitinae, as previously proposed by Emerson in 1965.

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18 **KEY WORDS** COII, DNA sequence, genetic variation, population
19 genetics, Rhinotermitidae, termite.

1 SUBTERRANEAN TERMITES in the family Rhinotermitidae
2 (Isoptera), as originally classified by Holmgren (1911 and 1912)
3 as part of the Mesotermitidae (modified later by Emerson (1941))
4 and recognized at the family level by Snyder (1949), contain
5 some of the most destructive and damaging termite species with
6 respect to their feeding preferences—namely wood and wood by-
7 products. In the United States, the National Pest Management
8 Association has estimated damage from subterranean termites to
9 exceed \$2.5 Billion (NPMA 2003). Similarly, in Europe and other
10 parts of the world, subterranean termites in the genus
11 *Reticulitermes* are the most expensive and damaging pest species.
12 The cost of treatment against termites in Europe is expected to
13 top 1 billion euros by 2005 (UNEP and FAO 2000). World-wide
14 damage caused by termites may account for greater than \$20
15 billion annually (Su 2002). Introductions of exotic
16 Rhinotermitids include: *Reticulitermes flavipes* in Germany
17 (Heisterberg 1958, 1959, Harris 1962, Becker 1970), Austria
18 (Hrdý 1961), the Bahamas (Scheffrahn et al. 1999), Santiago and
19 Valparaiso, Chile (Clement et al 2001); *R. lucifugus* in Uruguay
20 (Aber and Fontes 1993); *Coptotermes spp.* in the Gulf Coast
21 States (Jenkins et al. 2002, Messenger et al. 2002) and southern
22 California (Atkinson et al. 1993, Haagsma et al. 1995); and in
23 recent years *Heterotermes spp.* in Miami, Florida (Scheffrahn and
24 Su 1995).

1 Discoveries of *R. grassei* in southwestern England have
2 prompted studies to understand the risks associated with its
3 occurrence and associated threat to structural timbers in the UK
4 (Lainè 2002). Given its economic importance, it is remarkable
5 there has not been a more recent, comprehensive phylogenetic
6 analysis of Rhinotermitidae as a whole.

7 The family Rhinotermitidae Froggatt are believed to have
8 originated approximately 100 mya during the Cretaceous period
9 (Krishna and Grimaldi 2003). The shared characteristics of
10 Rhinotermitidae imagoes include: (1) Reticulate wings; (2) large
11 forewing scales, that overlap hindwing scales (a feature absent
12 only in the Psammotermitinae); (3) fontanelle and ocelli
13 present; (4) three marginal teeth on the left mandible; (5)
14 right mandible with a subsidiary tooth at the base of the upper
15 margin of the first marginal tooth; and (6) four-segmented tarsi
16 (Krishna and Grimaldi 2003).

17 Snyder (1949) subdivided the Rhinotermitidae into six
18 subfamilies. Emerson and Banks (1965) elevated the subfamily
19 Serritermitinae, previously included in the family
20 Rhinotermitidae, to full family status. Krishna (1969) later
21 reclassified the Rhinotermitidae into the subfamilies
22 Psammotermitinae, Heterotermitinae, Stylotermitinae,
23 Coptotermitinae, Termitogetoninae and Rhinotermitinae—a
24 classification still generally accepted to date. More recently,

1 the addition of another subfamily, Archeorhinotermitinae
2 (fossilized in preserved Burmese amber) has been suggested
3 (Krishna and Grimaldi 2003).

4 Recent studies of termite phylogeny have focused on higher
5 level (family-level or higher) determinations using both
6 morphological (Donovan et al. 2000) and molecular (Kambhampati
7 et al. 1996) characters. Molecular phylogenetic studies of
8 *Kalotermes* (Thompson et al. 2000a) and *Nasutitermes* (Miura et
9 al. 2000) have demonstrated the utility of the COII gene in
10 supporting previous morphology-based classification schemes and
11 clarifying relationships which have languished in uncertainty
12 for decades at the family-level. To date, there are currently no
13 molecular studies of Rhinotermitidae.

14 Some studies have focused on specific genera within the
15 Rhinotermitidae such as inter-specific genetic variation of
16 *Reticulitermes* (Austin et al. 2002, Clement et al. 2001, Jenkins
17 et al. 1998, 2001; Marini and Mantovani 2002), intra-specific
18 variation of *Coptotermes* (Husseneder and Grace 2001, Jenkins et
19 al. 2002) or using genetic profiling to determine the origin of
20 introduced, exotic *Heterotermes* spp. to the United States (ALS,
21 unpublished data). However, none have directly focused on the
22 classification of Rhinotermitidae with truly convincing or
23 robust datasets which confirm the currently accepted
24 relationships within the family Rhinotermitidae.

1 Information on how genetic variation is partitioned within
2 populations and among termite species can be useful for
3 determining the extent of gene flow, and for the development of
4 molecular diagnostics for identifying species (Szalanski et al.
5 2003). The usefulness of the cytochrome oxidase II (COII) region
6 of the mitochondrial DNA (mtDNA) genome has been well
7 demonstrated in studying the phylogenetic relationship of
8 termites (Austin et al. 2002; Jenkins et al. 1999, 2001; Lo et
9 al. 2000; Miura et al. 1998). Mitochondrial genes are known to
10 evolve more rapidly than nuclear genes and are therefore good
11 markers to analyze relatively close relationships, such as
12 species relationships within a genus (Miura et al. 2000). We
13 investigated the phylogenetic relationships among members of
14 Rhinotermitidae and determined the amount of genetic
15 differentiation among several disjunct populations using the
16 COII gene.

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Materials and Methods

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Termites were collected from North America and the
Caribbean (Table 1), and preserved in 100% ethanol. Specimens
were identified applying keys by Goellner et al (1931),
Scheffrahn and Su (1994), and Hostettler et al. (1995). Voucher
specimens, preserved in 100% ethanol, are maintained at the
Arthropod Museum, Department of Entomology, University of

1 Arkansas, Fayetteville, AR. DNA was extracted from whole
2 individual termites following Austin et al. (2002). Polymerase
3 chain reaction (PCR) was conducted using the primers TL2-J-3037
4 (5'-ATGGCAGATTAGTGCAATGG-3') designed by Liu and Beckenbach
5 (1992) and described by Simon et al. (1994) and Miura et al.
6 (1998), and primer TK-N-3785 (5'-GTTTAAGAGACCAGTACTTG-3') from
7 Simon et al. (1994). These primers amplify a 3' portion of the
8 mtDNA COI gene, tRNA-Leu, and a 5' section of the COII gene. PCR
9 reactions were conducted using 1 μ l of the extracted DNA
10 (Szalanski et al. 2000), with a profile consisting of 35 cycles
11 of 94°C for 45 s, 46°C for 45 s and 72°C for 60 s. Amplified DNA
12 from individual termites was subjected to DNA sequencing per
13 Austin et al. (2002). GenBank accession numbers for termite DNA
14 sequenced in this study are provided in Table 1 along with the
15 accession numbers for the DNA sequences of additional termite
16 taxa.

17 The distance matrix option of PAUP* 4.0b8 (Swofford 2001)
18 was used to calculate genetic distances according to the Kimura
19 2-parameter model (Kimura 1980) of sequence evolution.
20 Mitochondrial DNA COII sequences from representatives of four
21 other Isopteran families (Table 1) were added to the
22 Rhinotermitidae DNA sequences, as well as the Australian wood-
23 feeding cockroach, *Panesthia cribrata* Saussure (Table 1), to act
24 as the outgroup taxon. DNA sequences were aligned using the

1 PILEUP program in GCG (Genetics Computer Group, Madison, WI) and
2 adjusted manually. Maximum likelihood and unweighted parsimony
3 analysis on the alignments were conducted using PAUP* 4.0b10
4 (Swofford 2001). Gaps were treated as missing data and a random
5 addition sequence was used. A bootstrap test was used to test
6 the reliability of trees (Felsenstein 1985). Parsimony bootstrap
7 analysis included 1,000 resamplings using the Branch and Bound
8 algorithm of PAUP*. For maximum likelihood analysis, the default
9 likelihood parameter settings were used (HKY85 6-parameter model
10 of nucleotide substitution, empirical base frequencies, and
11 transition/transversion ratio set to 2:1). These parameters were
12 used to carry out a heuristic search using PAUP*, using either
13 the single most parsimonious tree as the starting tree, or step-
14 wise addition.

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Results

17 Average amplicon size resulting from DNA sequencing was 780
18 bp. To facilitate genetic comparisons with existing GenBank DNA
19 sequences, 103 bp from the 5' end of the amplicon was excluded
20 and the remaining 667 bp COII portion was used. The average base
21 frequencies were A = 0.39, C = 0.23, G = 0.14, and T = 0.24. The
22 mtDNA COII Rhinotermitidae sequences, along with other Isopteran
23 DNA sequences, were aligned using *P. cribrata* as the outgroup
24 taxon. The aligned DNA data matrix, including the outgroup taxon

1 (available at TreeBASE, <http://www.treebase.org>, study accession
2 number SN14616, reviewer PIN number 14616) resulted in a total
3 of 667 characters. Of these, 290 (43%) were fixed, 48 (7%) were
4 phylogenetically uninformative and 329 (49%) were
5 phylogenetically informative.

6 Pairwise Tajima-Nei distances (Tajima and Nei 1984) within
7 *Reticulitermes* spp. ranged from 0.9% between *R. labralis* and *R.*
8 *perilabralis*, to 10.3% between *R. speratus* and *R. flavipes*.
9 Among Rhinotermitidae, pairwise Tajima-Nei distances ranged from
10 12.3% between *Reticulitermes* and *Coptotermes*, to 22.7% between
11 *Reticulitermes* and *Dolichorhinotermes* (Table 2). Distances
12 ranged from 10.1% between *H. tenuir* and *C. lacteus*, to 21.6%
13 between *H. tenuir* and *Schedorhinotermes* sp. Genetic divergence
14 within *Coptotermes* spp. ranged from 0.0% between *C.*
15 *acinaciformis* and *Coptotermes* sp., to 8.0% between *C. lacteus*
16 and *C. formosanus* Galveston, TX. Pairwise Tajima-Nei distances
17 (Tajima and Nei 1984) within *Heterotermes* ranged from 9.6%
18 between *Heterotermes* sp. and *H. cardini*, to 14.0% between *H.*
19 *tenuir* and *H. cardini*. Distances within *Parrhinotermes* ranged
20 from 9.1% between *P. queenslandicus* and *P. buttel-reepeni*, to
21 10.7% between *P. aequalis* and *P. queenslandicus*.

22 This dataset produced only one most parsimonious tree (Fig.
23 1), (length = 2110, CI = 0.31, RI=0.52), as documented using the
24 Branch and Bound search algorithm of PAUP*. Bootstrap analysis

1 indicated by bootstrap analysis. The relationships among taxa
2 inferred from maximum parsimony and maximum likelihood analyses
3 were, for the most part, congruent with accepted classification
4 schemes. Distinct groups were established within the
5 Rhinotermitidae. For example, within the *Reticulitermes* clade
6 depicted in our Maximum likelihood tree, group VI consisted of
7 *R. labralis*, *R. perilabralis* Ping and Xu, *R. guangzhouensis*
8 Ping, *R. flaviceps* Oshima, *R. ampliceps*, *R. chinensis* Snyder,
9 and *R. speratus* Kolbe. This clade reflects a clear delimitation
10 of these eastern Asian *Reticulitermes* spp. from both Nearctic
11 and Palearctic *Reticulitermes* spp. (groups V, VIII, and IX) and
12 from the *Reticulitermes* originating from the eastern
13 Mediterranean (group VII). This relationship has previously been
14 demonstrated (Austin et al. 2002) and the subsequent addition of
15 specimens from other subfamilies within Rhinotermitidae only
16 reaffirms their respective alignments (Fig. 1). Molecular data
17 from this study along with Austin et al. (2002), and Jenkins et
18 al. (2001) supports the hypothesis that both *R. arenicola* and *R.*
19 *santonensis* are *R. flavipes*. It should be noted that *Coptotermes*
20 and *Heterotermes* (groups X and XI, respectively) (Fig. 2) were
21 clearly delimited from *Reticulitermes*.

22 While many currently accepted classifications have been
23 supported by our results, some taxa represented in this dataset
24 prompt as many questions as they answer. For example, there has

1 been some disagreement concerning the position of
2 *Prorhinotermes*. Quennedy and Deligne (1975) suggest placing
3 *Prorhinotermes* in the subfamily Prorhinotermitinae based on the
4 absence of a labral brush in its soldier caste. However, this
5 is generally accepted as a characteristic representative of
6 Rhinotermitinae. Grassé (1986) accepted Quennedy and Deligne's
7 classification but suggested that *Prohinotermes* is more similar
8 to *Coptotermes*. Our maximum Parsimony and maximum likelihood
9 analysis suggest that *Prohinotermes* is a sister group to
10 Rhinotermitidae with an intermediate position between
11 Rhinotermitidae and Termitidae (*Nasutitermes* clade) (group XII)
12 (Fig. 2).

13 Thompson et al. (2000b) found the Rhinotermitidae to be
14 polyphyletic, but stated that a topology with the
15 Rhinotermitidae constrained to monophyly is statistically just
16 as likely. Our results suggest polyphyly is more plausible.
17 Eggleton (2001) suggests a universal consensus exists throughout
18 the termite systematics literature with the Serritermitidae +
19 Rhinotermitidae + Termitidae forming a monophyletic group. Our
20 results only partially support this hypothesis with a
21 monophyletic grouping of Serritermitidae and Termitidae with
22 Rhinotermitidae. Based on COII mtDNA sequence data,
23 Serritermitidae is placed in the Serritermitinae, within the
24 family Rhinotermitidae. However, representatives from the

1 Nasutitermitinae used in this study form a sister group to the
2 Rhinotermitidae. Inclusion of additional taxa from the
3 Termitidae (e.g. Amitermitinae, Termitinae and Macrotermitinae)
4 would be desirable to clarify this relationship.

5 Future studies that focus on the family-level
6 classification of various groups within Isoptera are needed. A
7 more robust representation of the various species which comprise
8 taxa at the family-level is also needed. Some studies lack
9 sufficient representatives to accurately and reliably
10 demonstrate the true phylogenetic relationships within a family.
11 Termite researchers who use molecular techniques for termite
12 phylogenetic studies must collectively agree on what genetic
13 markers offer the best opportunity to clarify the relationships
14 between groups within Isoptera. Only then can we begin
15 eliminating synonymy at the species-level, ambiguity at the
16 family-level and eventually represent the true, overall
17 phylogeny of the Isoptera. We hope our data, have in part,
18 contributed to this effort. Contributions of more diverse and
19 wide-ranging taxa will undoubtedly enhance this study and help
20 answer some fundamental questions concerning the Rhinotermitidae
21 and its relationship to other families in Isoptera.

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6 lineages of drywood termites (Isoptera: Kalotermitidae).
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8 **Thompson, G.J., O. Kitade, N. Lo and R.H. Crozier. 2000b.**
9 Phylogenetic evidence for a single ancestral origin of a
10 "true" worker caste in termites. *J. Evol. Biol.* 13:869-881.

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12 (Geneva: UNEP), pp.60.

1 **Table 1.** Termite collection data.

Species	GenBank	Source
<i>Schedorhinotermes mediobscurus</i>	AF262602	Kitade et al. (unpub.)
<i>Schedorhinotermes</i> sp.	AF262603	Kitade et al. (unpub.)
<i>Dolichorhinotermes</i> sp.	AF262601	Kitade et al. (unpub.)
<i>Parrhinotermes buttel-reepeni</i>	AF262605	Kitade et al. (unpub.)
<i>P. queenslandicus</i>	AB005585	Miura et al. (1998)
<i>P. aequalis</i>	AF262604	Kitade et al. (unpub.)
<i>Reticulitermes grassei</i>	AF525327	Austin et al. (2002)
<i>Reticulitermes</i> n. sp.	AF525342	Austin et al. (2002)
<i>R. tibialis</i>	AF525355	Austin et al. (2002)
<i>R. arenicola</i>	AFXXXXX	This study
<i>R. flavipes</i>	AF525321	Austin et al. (2002)
<i>R. santonensis</i>	AF525343	Austin et al. (2002)
<i>R. labralis</i>	AB050711	Xing et al. (unpub.)
<i>R. perilabralis</i>	AB050710	Xing et al. (unpub.)
<i>R. guangzhouensis</i>	AB050709	Xing et al. (unpub.)
<i>R. flaviceps</i>	AB050708	Xing et al. (unpub.)
<i>R. ampliceps</i>	AB050704	Xing et al. (unpub.)
<i>R. chinensis</i>	AB050705	Xing et al. (unpub.)
<i>R. speratus</i>	AF525344	Austin et al. (2002)
<i>R. banyulensis</i>	AF525319	Austin et al. (2002)
<i>R. lucifugus</i>	AF291724	Marini et al. (2002)

<i>R. clypeatus</i>	AF525320	Austin et al. (2002)
<i>R. balkaensis</i>	AF525318	Austin et al. (2002)
<i>R. lucifugus</i>	AF525333	Austin et al. (2002)
<i>R. virginicus</i>	AF525357	Austin et al. (2002)
<i>R. hageni</i>	AF525328	Austin et al. (2002)
<i>R. hesperus</i>	AF525329	Austin et al. (2002)
<i>Coptotermes formosanus</i>	AF525317	Austin et al. (2002)
<i>C. formosanus</i>	AFXXXX	This study
<i>C. acinaciformis</i>	AF262610	Kitade et al. (unpub.)
<i>Coptotermes</i> sp.	AB005583	Miura et al. (1998)
<i>C. lacteus</i>	AF220600	Lo et al. (2000)
<i>Heterotermes cardini</i>	AFXXXX	This study
<i>Heterotermes</i> sp.	AB050715	Xing et al. (unpub.)
<i>H. tenuir</i>	AB050714	Xing et al. (unpub.)
<i>Psammotermes allocerus</i>	AF262597	Kitade et al. (unpub.)
<i>Serritermes serrifer</i>	AF220598	Lo et al. (2000)
<i>Prorhinotermes japonicus</i>	AF262599	Kitade et al. (unpub.)
<i>Termitogeton planus</i>	AF262598	Kitade et al. (unpub.)
<i>Nasutitermes pinocchio</i>	AB037336	Miura et al. (2000)
<i>N. walkeri</i>	AB037332	Miura et al. (2000)
<i>N. longinasus</i>	AB037339	Miura et al. (2000)
<i>Microhodotermes viator</i>	AF220599	Lo et al. (2000)
<i>Stolotermes</i> sp.	AF262596	Kitade et al. (unpub.)

Mastotermes darwiniensis AB014071 Maekawa & Matsumoto
(unpub.)

Panesthia cribrata (cockroach) AF220580 Lo et al. (2000)

Table 2. Tajima-Nei pairwise distances among 14 Isoptera taxa

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>Schedorhinotermes mediob.</i>	-													
2 <i>Parrhinotermes buttel-re.</i>	0.13	-												
3 <i>Dolichorhinotermes sp.</i>	0.13	0.16	-											
4 <i>Nasutitermes pinocchio</i>	0.22	0.23	0.23	-										
5 <i>Reticulitermes flavipes</i>	0.21	0.21	0.21	0.20	-									
6 <i>Coptotermes formosanus</i>	0.21	0.20	0.21	0.19	0.15	-								
7 <i>Heterotermes cardini</i>	0.20	0.21	0.21	0.18	0.17	0.11	-							
8 <i>Psammotermes allocerus</i>	0.21	0.22	0.23	0.21	0.19	0.18	0.20	-						
9 <i>Serritermes serrifer</i>	0.22	0.23	0.22	0.23	0.18	0.20	0.20	0.21	-					
10 <i>Prorhinotermes japonicus</i>	0.22	0.24	0.22	0.21	0.19	0.19	0.20	0.19	0.22	-				
11 <i>Termitogeton planus</i>	0.24	0.25	0.25	0.22	0.20	0.21	0.22	0.20	0.21	0.21	-			
12 <i>Microhodotermes viator</i>	0.25	0.25	0.26	0.24	0.22	0.23	0.23	0.25	0.22	0.26	0.25	-		
13 <i>Stolotermes sp.</i>	0.26	0.27	0.27	0.26	0.23	0.23	0.25	0.25	0.24	0.24	0.26	0.21	-	
14 <i>Mastotermes darwiniensis</i>	0.29	0.31	0.28	0.28	0.27	0.28	0.28	0.29	0.26	0.29	0.28	0.23	0.22	-

Figure Legend

Fig. 1. Single most parsimonious tree during a branch and bound search using PAUP*. Bootstrap values for 1,000 replicates are listed above the branches supported at $\geq 50\%$.

Fig. 2. Topology obtained by maximum likelihood analysis based on the HKY85 model (see text). Log L = - 10184.77443.



