Phylogenetic Analysis of the Subterranean Termite Family Rhinotermitidae (Isoptera) by Using the Mitochondrial Cytochrome Oxidase II Gene

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ABSTRACT   Previous molecular phylogenetic studies have focused on either the relationship between isopteran families or among species within a given genus, but there are presently few studies focusing on individual families and no known molecular studies for Rhinotermitidae. We examined 38 rhinotermitid species representing 10 genera, relative to representatives of four other isopteran families. Sequencing of a 667-base pair region of the mitochondrial DNA cytochrome oxidase II gene revealed 343 polymorphic sites within the family. Tajima-Nei genetic distances ranged from 11 to 23% among rhinotermitid genera. Maximum parsimony and maximum likelihood analysis of DNA sequences support existing hypotheses that Mastotermitidae is the basal lineage among extant termites, and the family Rhinotermitidae is polyphyletic given the current familial status of Serritermitidae. DNA sequence data suggest that Serritermitidae should be relegated to the subfamily Serritermitinae, as proposed by Emerson in 1965.

KEY WORDS   COII, DNA sequence, genetic variation, Rhinotermitidae, termite

SUBTERRANEAN TERMITES IN THE family Rhinotermitidae (Isoptera), as originally classified by Holmgren (1911, 1912) as part of the Mesotermidae [modified later by Emerson (1941)] and recognized at the family level by Snyder (1949), contain some of the most destructive and damaging termite species with respect to their feeding preferences, namely, wood and wood by-products. In the United States, the National Pest Management Association has estimated damage from subterranean termites to exceed $2.5 billion (NPMA 2003). Similarly, in Europe and other parts of the world, subterranean termites in the genus *Reticulitermes* are the most expensive and damaging pest species. The cost of treatment against termites in Europe is expected to top 1 billion euros by 2005 (UNEP and FAO 2000). Worldwide damage caused by termites may account for >$20 billion annually (Su 2002). Introductions of exotic rhinotermitids include *Reticulitermes flavipes* in Germany (Heisterberg 1958, 1959; Harris 1962; Becker 1970), Austria (Hrdỳ 1961), the Bahamas (Scheffrahn et al. 1999), and Santiago and Valparaiso, Chile (Clément et al. 2001); *R. lucifugus* in Uruguay (Aber and Fontes 1993); *Coptotermes* spp. in the Gulf Coast states (Jenkins et al. 2002, Messenger et al. 2002) and southern California (Atkinson et al. 1993, Haagsma et al. 1995); and in recent years, *Heterotermes* spp. in Miami, FL (Scheffrahn and Su 1995).

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

The family Rhinotermitidae Froggatt is believed to have originated ≈100 mya during the Cretaceous period (Krishna and Grimaldi 2003). The shared characteristics of Rhinotermitidae imagoes include 1) reticulate wings, 2) large forewing scales that overlap hindwing scales (a feature absent only in the Psammotermitinae), 3) fontanelle and ocelli present, 4) three marginal teeth on the left mandible, 5) right mandible with a subsidiary tooth at the base of the upper margin of the first marginal tooth, and 6) four-segmented tarsi (Krishna and Grimaldi 2003).

Snyder (1949) subdivided the Rhinotermitidae into six subfamilies. Emerson and Banks (1965) elevated the subfamily Serritermitinae, previously included in the family Rhinotermitidae, to full family status. Krishna and Weesner (1969) later reclassified the Rhinotermitidae into the subfamilies Psammotermitinae, Heterotermitinae, Stylotermitinae, Coptotermitinae, Termitogetoninae, and Rhinotermitinae, a classification still generally accepted to date. More recently, the addition of another subfamily, Archeorhinotermitinae (fossilized in preserved Burmese amber) has been suggested (Krishna and Grimaldi 2003).

Recent studies of termite phylogeny have focused on higher level (family level or higher) determina-

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tions by using both morphological (Donovan et al. 2000) and molecular (Kambhampati et al. 1996) characters. Molecular phylogenetic studies of *Kalotermes* (Thompson et al. 2000a) and *Nasutitermes* (Miura et al. 2000) have demonstrated the utility of the COII gene in supporting previous morphology-based classification schemes and clarifying relationships that have languished in uncertainty for decades at the family level. To date, there are currently no molecular studies of Rhinotermitidae.

Some studies have focused on specific genera within the Rhinotermitidae such as interspecific genetic variation of *Reticulitermes* (Jenkins et al. 1998, 2001; Clément et al. 2001; Austin et al. 2002; Marini and Mantovani 2002), intraspecific variation of *Coptotermes* (Husseneder and Grace 2001, Jenkins et al. 2002), or by using genetic profiling to determine the origin of introduced, exotic *Heterotermes* spp. into the United States (A.L.S., unpublished data). However, none have directly focused on the classification of Rhinotermitidae with truly convincing or robust data sets that confirm the currently accepted relationships within the family Rhinotermitidae.

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

Materials and Methods

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 Arkansas, Fayetteville, AR). DNA was extracted from whole individual termites following Austin et al. (2002). Polymerase chain reaction (PCR) was conducted using the primers TL2-J-3037 (5′-ATGGCAGATTAGTGCAATGG-3′) designed by Liu and Beckenbach (1992) and described by Simon et al. (1994) and Miura et al. (1998) and primer TK-N-3785 (5′-GTTTAAGAGACCAGTACTTG-3′) from Simon et al. (1994). These primers amplify a 3′ portion of the mtDNA COI gene, tRNA-Leu, and a 5′ section of the COII gene. PCR reactions were conducted using 1 μl

of the extracted DNA (Szalanski et al. 2000), with a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 60 s. Amplified DNA from individual termites was subjected to DNA sequencing per Austin et al. (2002). GenBank accession numbers for termite DNA sequenced in this study are provided in Table 1 along with the accession numbers for the DNA sequences of additional termite taxa.

The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution. Mitochondrial DNA COII sequences from representatives of four other isopteran families (Table 1) were added to the Rhinotermitidae DNA sequences, as well as the Australian wood-feeding cockroach *Panesthia cribrata* Saussure (Table 1) to act as the outgroup taxon. DNA sequences were aligned using the PILEUP program in Genetics Computer Group (Madison, WI) and adjusted manually. Maximum likelihood and unweighted parsimony analysis on the alignments were conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing data and a random addition sequence was used. A bootstrap test was used to test the reliability of trees (Felsenstein 1985). Parsimony bootstrap analysis included 1000 resamplings by using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the default likelihood parameter settings were used (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies, and transition/transversion ratio set to 2:1). These parameters were used to carry out a heuristic search using PAUP*, by using either the single most parsimonious tree as the starting tree, or stepwise addition.

Results

Average amplicon size resulting form DNA sequencing was 780 base pairs (bp). To facilitate genetic comparisons with existing GenBank DNA sequences, 113 bp from the 5′ end of the amplicon was excluded, and the remaining 667-bp COII portion was used. The average base frequencies were A = 0.39, C = 0.23, G = 0.14, and T = 0.24. The mtDNA COII Rhinotermitidae sequences, along with other isopteran DNA sequences, were aligned using *P. cribrata* as the outgroup taxon. The aligned DNA data matrix, including the outgroup taxon (available at TreeBASE, http://www.treebase.org, study accession number SN14616) resulted in a total of 667 characters. Of these, 290 (43%) were fixed, 48 (7%) were phylogenetically uninformative and 329 (49%) were phylogenetically informative.

Pairwise Tajima-Nei distances (Tajima and Nei 1984) within *Reticulitermes* spp. ranged from 0.9% between *R. labralis* and *R. perilabralis* to 10.3% between *R. speratus* and *R. flavipes.* Among Rhinotermitidae, pairwise Tajima-Nei distances ranged from 12.3% between *Reticulitermes* and *Coptotermes* to 22.7% between *Reticulitermes* and *Dolichorhinotermes* (Table 2). Distances ranged from 10.1% between *H. tenuir* and

Table 1. Termite sample data

Species	GenBank	Source
Schedorhinotermes mediobscurus	AF262602	Kitade et al. (unpubl.)
Schedorhinotermes sp.	AF262603	Kitade et al. (unpubl.)
Dolichorhinotermes sp.	AF262601	Kitade et al. (unpubl.)
Parrhinotermes buttel-reepeni	AF262605	Kitade et al. (unpubl.)
P. queenslandicus	AB005585	Miura et al. (1998)
P. aequalis	AF262604	Kitade et al. (unpubl.)
Reticulitermes grassei	AF525327	Austin et al. (2002)
Reticulitermes n. sp.	AF525342	Austin et al. (2002)
R. tibialis	AF525355	Austin et al. (2002)
R. arenicola	AY453589	This study
R. flavipes	AF525321	Austin et al. (2002)
R. santonensis	AF525343	Austin et al. (2002)
R. labralis	AB050711	Xing et al. (unpubl.)
R. perilabralis	AB050710	Xing et al. (unpubl.)
R. guangzhouensis	AB050709	Xing et al. (unpubl.)
R. flaviceps	AB050708	Xing et al. (unpubl.)
R. ampliceps	AB050704	Xing et al. (unpubl.)
R. chinensis	AB050705	Xing et al. (unpubl.)
R. speratus	AF525344	Austin et al. (2002)
R. banyulensis	AF525319	Austin et al. (2002)
R. lucifugus	AF291724	Marini et al. (2002)
R. clypeatus	AF525320	Austin et al. (2002)
R. balkaensis	AF525318	Austin et al. (2002)
R. lucifugus	AF525333	Austin et al. (2002)
R. virginicus	AF525357	Austin et al. (2002)
R. hageni	AF525328	Austin et al. (2002)
R. hesperus	AF525329	Austin et al. (2002)
Coptotermes formosanus	AF525317	Austin et al. (2002)
C. formosanus	AY453588	This study
C. acinaciformis	AF262610	Kitade et al. (unpubl.)
Coptotermes sp.	AB005583	Miura et al. (1998)
C. lacteus	AF220600	Lo et al. (2000)
Heterotermes cardini	AY453590	This study
Heterotermes sp.	AB050715	Xing et al. (unpubl.)
H. tenuir	AB050714	Xing et al. (unpubl.)
Psammotermes allocerus	AF262597	Kitade et al. (unpubl.)
Serritermes serrifer	AF220598	Lo et al. (2000)
Prorhinotermes japonicus	AF262599	Kitade et al. (unpubl.)
Termitogeton planus	AF262598	Kitade et al. (unpubl.)
Nasutitermes pinocchio	AB037336	Miura et al. (2000)
N. walkeri	AB037332	Miura et al. (2000)
N. longinasus	AB037339	Miura et al. (2000)
Microhodotermes viator	AF220599	Lo et al. (2000)
Stolotermes sp.	AF262596	Kitade et al. (unpubl.)
Mastotermes darwiniensis	AB014071	Maekawa & Matsumoto (unpubl.)
Panesthia cribrata (cockroach)	AF220580	Lo et al. (2000)

C. lacteus to 21.6% between *H. tenuir* and *Schedorhinotermes* sp. Genetic divergence within *Coptotermes* spp. ranged from 0.0% between *C. acinaciformis* and *Coptotermes* sp., to 8.0% between *C. lacteus* and *C. formosanus* Galveston, TX. Pairwise Tajima-Nei distances (Tajima and Nei 1984) within *Heterotermes*

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 *Schedorhinotermes mediob.*	—													
2 *Parrhinotermes buttel-re.*	0.13	—												
3 *Dolichorhinotermes* sp.	0.13	0.16	—											
4 *Nasutitermes pinocchio*	0.22	0.23	0.23	—										
5 *Reticulitermes flavipes*	0.21	0.21	0.21	0.20	—									
6 *Coptotermes formosanus*	0.21	0.20	0.21	0.19	0.15	—								
7 *Heterotermes cardini*	0.20	0.21	0.21	0.18	0.17	0.11	—							
8 *Psammotermes allocerus*	0.21	0.22	0.23	0.21	0.19	0.18	0.20	—						
9 *Serritermes serrifer*	0.22	0.23	0.22	0.23	0.18	0.20	0.20	0.21	—					
10 *Prorhinotermes japonicus*	0.22	0.24	0.22	0.21	0.19	0.19	0.20	0.19	0.22	—				
11 *Termitogeton planus*	0.24	0.25	0.25	0.22	0.20	0.21	0.22	0.20	0.21	0.21	—			
12 *Microhodotermes viator*	0.25	0.25	0.26	0.24	0.22	0.23	0.23	0.25	0.22	0.26	0.25	—		
13 *Stolotermes* sp.	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 *Mastotermes darwiniensis*	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	—
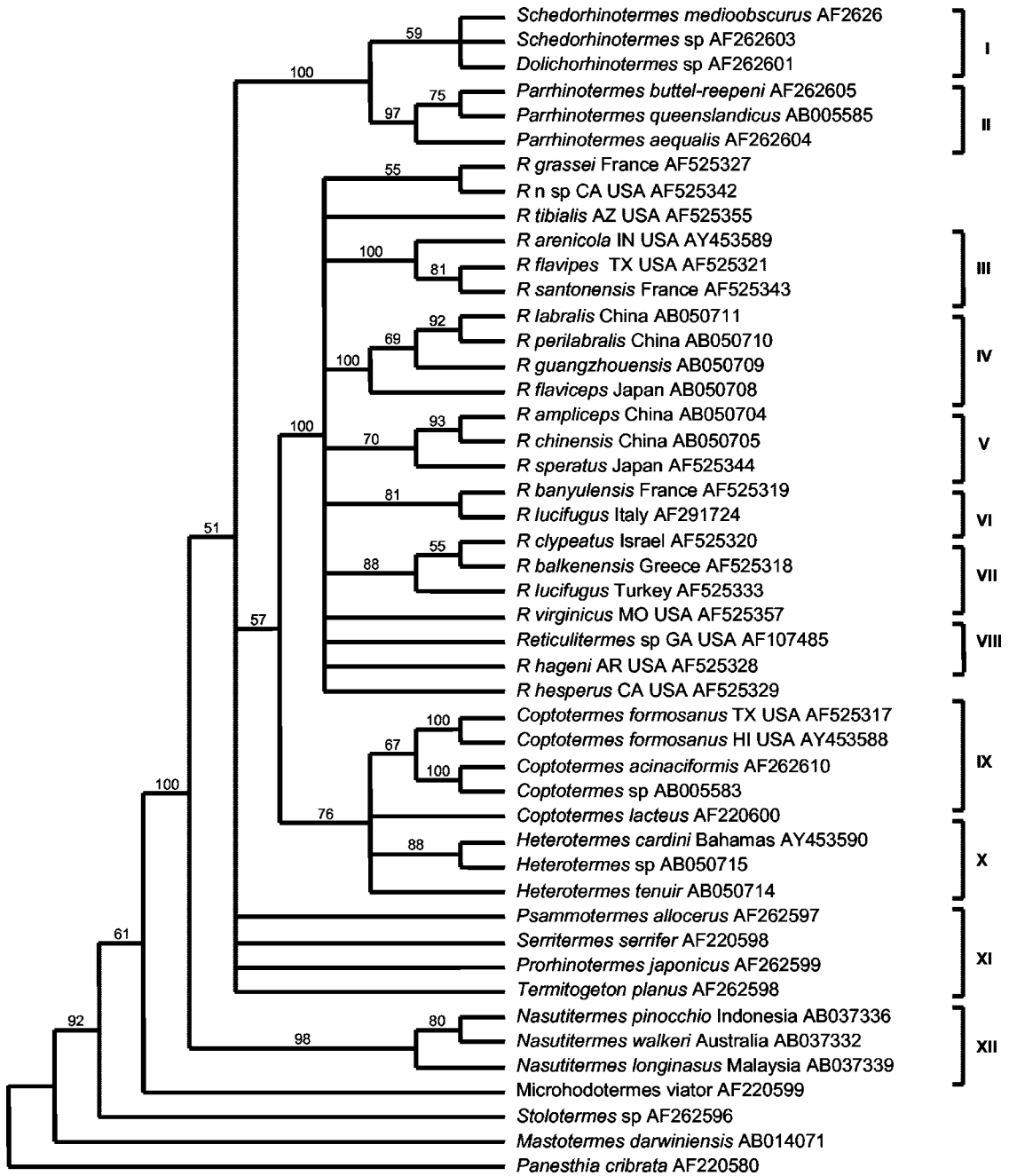
Fig. 1. Single most parsimonious tree during a branch and bound search using PAUP*. Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%. Roman numerals refer to the clades discussed in *Results and Discussion*.

ranged from 9.6% between *Heterotermes* sp. and *H. cardini* to 14.0% between *H. tenuir* and *H. cardini*. Distances within *Parrhinotermes* ranged from 9.1% between *P. queenslandicus* and *P. buttel-reepeni* to 10.7% between *P. aequalis* and *P. queenslandicus.*

This data set produced only one most parsimonious tree (Fig. 1) (length = 2110, CI = 0.31, RI = 0.52), as documented using the Branch and Bound search al-

gorithm of PAUP*. Bootstrap analysis of the aligned rhinotermitid species and the outgroup taxon resulted in a consensus tree with several distinct branches.

Among the more distinct clades, there was a clear delimitation between *Reticulitermes* and its sister group containing both *Coptotermes* and *Heterotermes* species (Fig. 1). Regardless of whether the starting tree was the most parsimonious or was obtained via
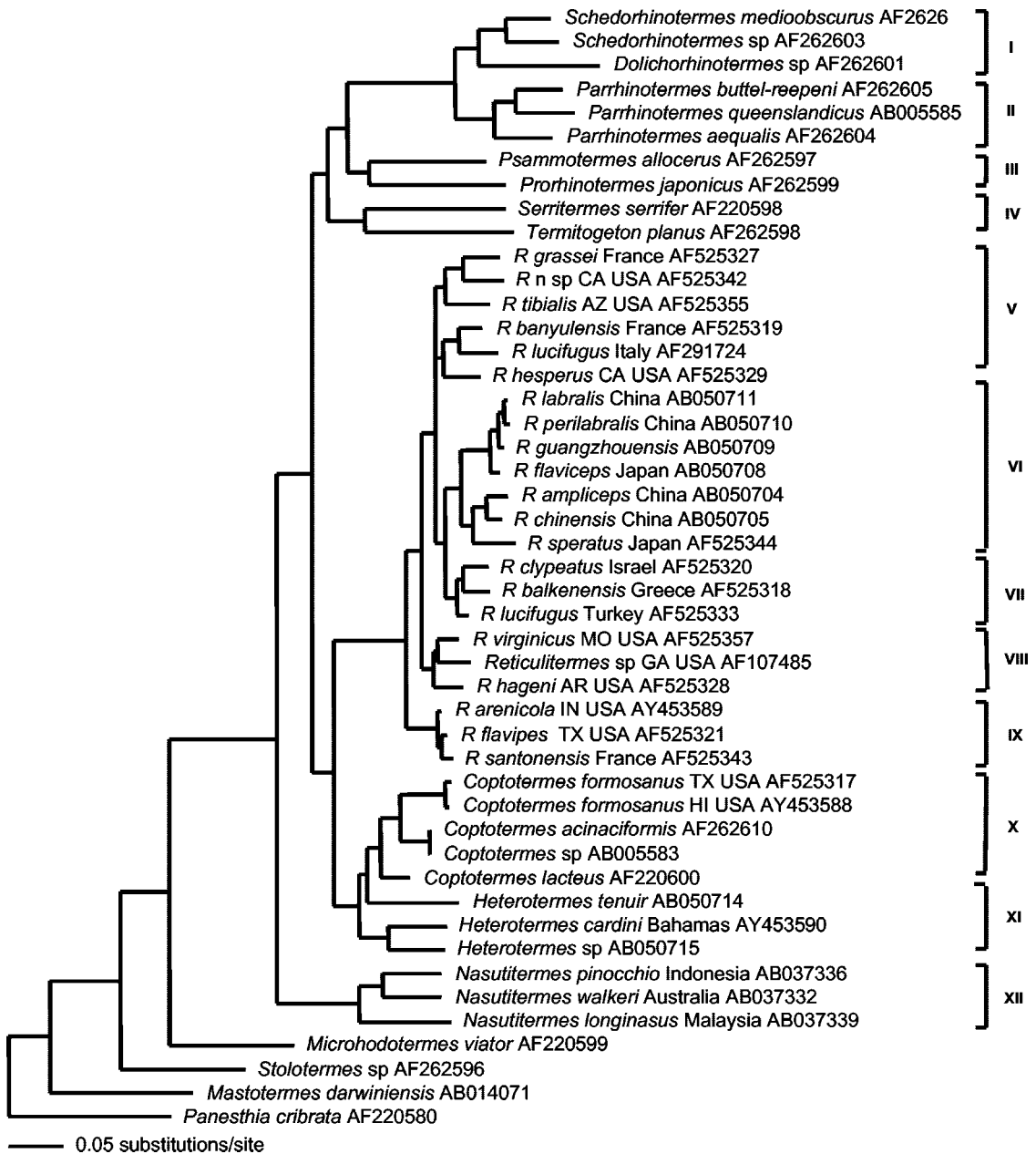
Fig. 2. Topology obtained by maximum likelihood analysis based on the HKY85 model (see text). Log L = −10184.77443. Roman numerals refer to the clades discussed in *Results and Discussion*.

stepwise addition, the maximum likelihood search found only one tree (Fig. 2). One difference more clearly revealed in the maximum likelihood tree was the relationship of the *Psammotermes* + *Prorhinotermes* clade (group XI) to both the *Serritermes* + *Termitogeton* and *Parrhinotermes* + *Schedorhinotermes/Dolichorhinotermes* clades (groups I and II), each forming distinct groupings within the Rhinotermitidae (Fig. 2). Based on our analysis, *Serritermes* falls within the Rhinotermitidae clade. Both the maximum likelihood tree and the maximum parsimony tree for

this study were congruent—the family Rhinotermitidae seems to be polyphyletic, given the more popular classification of *Serritermes*.

Discussion

By using the DNA sequence of a portion of the mitochondrial COII gene, this study represents the first attempt to address the phylogenetic relationships within the subterranean termite family Rhinotermitidae at the molecular level. Most of the inferred rela-

tionships had strong quantitative support as indicated by bootstrap analysis. The relationships among taxa inferred from maximum parsimony and maximum likelihood analyses were, for the most part, congruent with accepted classification schemes. Distinct groups were established within the Rhinotermitidae. For example, within the *Reticulitermes* clade depicted in our maximum likelihood tree, group VI consisted of *Reticulitermes labralis*, *Reticulitermes perilabralis* Ping & Xu, *Reticulitermes guangzhouensis* Ping, *Reticulitermes flaviceps* Oshima, *Reticulitermes ampliceps* Wang & Li, *Reticulitermes chinensis* Snyder, and *Reticulitermes speratus* Kolbe. This clade reflects a clear delimitation of these eastern Asian *Reticulitermes* spp. from both Nearctic and Palearctic *Reticulitermes* spp. (groups V, VIII, and IX) and from the *Reticulitermes* originating from the eastern Mediterranean (group VII). This relationship has previously been demonstrated (Austin et al. 2002), and the subsequent addition of specimens from other subfamilies within Rhinotermitidae reaffirms their respective alignments (Fig. 1). Molecular data from this study along with Austin et al. (2002) and Jenkins et al. (2001) support the hypothesis that both *R. arenicola* and *R. santonensis* are *R. flavipes*. It should be noted that *Coptotermes* and *Heterotermes* (groups X and XI, respectively) (Fig. 2) were clearly delimited from *Reticulitermes*.

Although many currently accepted classifications have been supported by our results, some taxa represented in this data set prompt as many questions as they answer. For example, there has been some disagreement concerning the position of *Prorhinotermes*. Quennedy and Deligne (1975) suggest placing *Prorhinotermes* in the subfamily Prorhinotermitinae based on the absence of a labral brush in its soldier caste. However, this is generally accepted as a characteristic representative of Rhinotermitinae. Grassé (1986) accepted Quennedy and Deligne's classification but suggested that *Prohinotermes* is more similar to *Coptotermes*. Our maximum parsimony and maximum likelihood analysis suggest that *Prohinotermes* is a sister group to Rhinotermitidae with an intermediate position between Rhinotermitidae and Termitidae (*Nasutitermes* clade) (group XII) (Fig. 2).

Thompson et al. (2000b) found the Rhinotermitidae to be polyphyletic, but they stated that a topology with the Rhinotermitidae constrained to monophyly is statistically just as likely. Our results suggest polyphyly is more plausible. Eggleton (2001) suggests a universal consensus exists throughout the termite systematics literature with the Serritermitidae + Rhinotermitidae + Termitidae forming a monophyletic group. Our results only partially support this hypothesis with a monophyletic grouping of Serritermitidae and Termitidae with Rhinotermitidae. Based on COII mtDNA sequence data, Serritermitidae is placed in the Serritermitinae, within the family Rhinotermitidae. However, representatives from the Nasutitermitinae used in this study form a sister group to the Rhinotermitidae. Inclusion of additional taxa from the Termitidae (e.g., Amitermitinae, Termitinae, and Macrotermitinae) would be desirable to clarify this relationship.

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

Acknowledgments

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