# A Comparative Genetic Analysis of the Subterranean Termite Genus *Reticulitermes* (Isoptera: Rhinotermitidae)

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**ABSTRACT** DNA sequencing analysis of the mitochondrial DNA cytochrome oxidase II (COII) region was used to examine genetic variation in the termite genus *Reticulitermes* Holmgren. We examined 21 species and subspecies from three continents. Sequencing of a 677-bp region of a 780-bp amplicon from 41 individuals and from 17 sequences obtained from GenBank revealed 221 polymorphic sites within the genus. Tajima–Nei distances from species ranged from 0.9 to 12.7%, and parsimony and maximum likelihood analysis revealed several clades within the genus. *Reticulitermes flavipes* (Kollar) formed a distinct clade along with *R. santonensis* De Feytaud. European *R. lucifugus* (Rossi) formed a distinct clade with *R. banyulensis* (Béziers). Turkish *R. lucifugus* was distinct relative to European *R. lucifugus*, and along with *R. clypeatus* Lash from Israel formed a sister group with *R. balkanensis* Clément from Greece. This study provides support for the separation of Turkish *R. lucifugus* from European members of the species. This mitochondrial DNA marker was also able to identify several *Reticulitermes* specimens from Oklahoma, Texas, Missouri, and South Korea to *R. flavipes*, *R. hageni* Banks, *R. virginicus* (Banks), and *R. speratus* Shimizu.

**KEY WORDS** COII, DNA sequence, genetic variation, population genetics, *Reticulitermes*, termite

SPECIES OF THE genera *Reticulitermes* Holmgren (Isoptera: Rhinotermitidae) are the major termite pests infesting wooden structures in the United States and other countries. It has been estimated that more than $1.5 billion is spent annually for termite control in the United States, of which 80% is spent to control subterranean termites (Su 1993). A breakdown of damage caused by termite species reveals that the five principal subterranean termite species in the United States are *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes hesperus* (Banks), *Reticulitermes tibialis* (Banks), and *Coptotermes formosanus* (Shiraki). Ninety percent of the termite control industry involves these five subterranean termite species (Forschler and Lewis 1997).

*Reticulitermes* spp. are the most abundant naturally residing termites in Europe, with six described phenotypes that have been identified on the basis of morphological, chemical (cuticular hydrocarbons and soldier defensive secretions), and molecular (enzymatic alleles and mitochondrial ND1 sequence) characters (Clément et al. 2001). *Reticulitermes* spp. in Europe are known pests of urban structures and frequently pose threats to various agricultural crops.

Unlike in Europe, the distribution of termites and their subsequent impact as an economic pest in developing countries such as Turkey have not been reported. The Mediterranean termite *Reticulitermes lucifugus* (Rossi) was first described by Weber (1954) as occurring in the Zubair desert region, Iraq, and it has been hypothesized by Weidner (1972) that *R. lucifugus* may in fact belong to *R. clypeatus* Lash. *R. lucifugus* (Rossi, 1792) has been the only documented species of the genera *Reticulitermes* to occur in Turkey (Bodenheimer 1958, Weidner 1972, Lodos 1982, Karaat and Göven 1983).

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 developing molecular diagnostics for identifying species. Previous studies (Jenkins et al. 1998, 2001; Marini and Mantovani 2002) have focused on *Reticulitermes* spp. from the southeastern United States or Western Europe, but have not included populations from other areas of the world where these termites occur.

The cytochrome oxidase II (COII) region of the mitochondrial DNA (mtDNA) genome has proved useful for the phylogenetic relationship of termites (Miura et al. 1998; Jenkins et al. 1999, 2001; Lo et al. 2000). We investigated the phylogenetic relationships among *Reticulitermes* spp. from three continents and determined the amount of genetic differentiation among several disjunct *R. lucifugus* populations.

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**Table 1. Termite collection data**

| Species | Collection Site | Country | GenBank |
|---|---|---|---|
| *R. balkanensis* | Shinias | Greece | AF525318 |
| *R. banyulensis* | Béziers | France | AF525319 |
| *R. chinensis* | Beijing | China | AB050705 |
| *R. clypeatus* | Ben Shemen | Israel | AF525320 |
| *R. flaviceps* | Tokunoshima | Japan | AB050708 |
| *R. flavipes* | Grand Bahamas | Bahamas | AF525322 |
| | Toronto, ON | Canada | AF525326 |
| | Hamburg strain "A" | Germany | AF525324 |
| | Hamburg strain "B" | Germany | AF525323 |
| | Alachua Co., FL | USA | AF525321 |
| | Lincoln, NE | USA | AF525325 |
| | Sapelo Island, GA | USA | AF107484 |
| *R. grassei* | Charente | France | AF525327 |
| | | Italy | AF291744 |
| *R. guangzhouensis* | Guangzhou | China | AB050709 |
| *R. hageni* | Barnesville, GA | USA | AF107486 |
| | Cumberland Island, GA | USA | AF525328 |
| *R. hesperus* | Los Angeles, CA | USA | AF525329 |
| *R. labralis* | | China | AB050711 |
| *R. lucifugus* | Corsica | France | AF525332 |
| | Narbonne | France | AB050707 |
| | Sardegna | Italy | AF291730 |
| | Castel | Italy | AF291724 |
| | Chieti | Italy | AF291738 |
| | Palermo | Italy | AF291741 |
| | Patanella | Italy | AF525341 |
| | Dış Kapı, (Ankara) | Turkey | AF525333 |
| | Antayla | Turkey | AF525330 |
| | Fethiye | Turkey | AF525334 |
| | K. Maraş | Turkey | AF525338 |
| | Kaş | Turkey | AF525336 |
| | Konya | Turkey | AF525337 |
| | Izmir | Turkey | AF525335 |
| | Mersin | Turkey | AF525339 |
| | Muğla | Turkey | AF525340 |
| *R. 1. corcicus* | Corsica | France | AF525331 |
| *R. n. sp.* | Catalina Island, CA | USA | AF525342 |
| *R. perilabralis* | | China | AB050710 |
| *R. santonensis* | Charente | France | AF525343 |
| | | France | AF262607 |
| *R.* sp. | Conway, AR | USA | AF525349 |
| | Columbia, MO | USA | AF525348 |
| | Oklahoma City, OK | USA | AF525353 |
| | Arlington, TX | USA | AF525345 |
| | Bryan, TX | USA | AF525346 |
| | College Station, TX | USA | AF525347 |
| | Ft. Worth, TX | USA | AF525351 |
| | Mansfield, TX | USA | AF525352 |
| | Domene | France | AF525350 |
| | Taejon | S. Korea | AF525354 |
| *R. speratus* | Mito City | Japan | AF525344 |
| | | Japan | AB005584 |
| *R. tibialis* | Cochise Co., AZ | USA | AF525355 |
| *R. virginicus* | Pinellas Co., FL | USA | AF525356 |
| | Roanoke, VA | USA | AF525357 |
| *Coptotermes formosanus* | Galveston, TX | USA | AF525317 |
| *Heterotermes tenuior* | Borneo Island | Indonesia | AB050714 |
| *Panesthia cribrata* | | | AF220580 |

GenBank DNA sequences from this study are italicized.
*R.*, *Reticulitermes.*

## Materials and Methods

Termites were collected from various locations in North America, Europe, and Asia and preserved in 70% ethanol (Table 1). For morphological identification of *R. lucifugus*, we used (1) a modified biometric analysis method using winged, immature and neotenic termites belonging to French, Turkish, and American populations of the genus *Reticulitermes* (Clemént 1978, 1979a); and (2) discriminate function analysis (Mayr and Ashlock 1991) of worker and soldier castes from other available *R. lucifugus* specimens previously collected. *R. lucifugus* from Turkey was validated using a cuticular hydrocarbon analysis by gas chromatography-mass spectrometry (GC-MS) and quantified

**Table 2. Tajima–Nei pairwise distances among 19 *Reticulitermes* taxa**

| No. | Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *R. ampliceps* CHN | – | 8.8 | 8.5 | 3.5 | 8.5 | 11.2 | 10.4 | 7.0 | 8.8 | 8.2 | 7.5 | 6.6 | 8.7 | 11.0 | 7.8 | 10.3 | 6.8 | 10.1 | 9.5 |
| 2 | *R. balkanensis* Shinias, GRC | | – | 8.0 | 8.6 | 4.5 | 10.4 | 9.3 | 8.9 | 6.7 | 7.0 | 9.1 | 4.0 | 8.7 | 9.9 | 9.4 | 9.9 | 8.0 | 6.8 | 7.3 |
| 3 | *R. banyulensis* Beziers, FRA | | | – | 8.0 | 8.5 | 9.4 | 7.4 | 7.5 | 5.5 | 7.2 | 7.5 | 7.0 | 6.0 | 8.7 | 7.8 | 8.5 | 8.9 | 8.2 | 7.7 |
| 4 | *R. chinensis* CHN | | | | – | 8.8 | 10.2 | 10.4 | 6.1 | 9.5 | 7.3 | 7.0 | 6.6 | 8.9 | 10.4 | 7.0 | 9.4 | 6.3 | 9.8 | 8.4 |
| 5 | *R. clypeatus* Ben Shemen, ISR | | | | | – | 10.5 | 10.1 | 8.0 | 6.7 | 8.2 | 8.9 | 3.7 | 9.1 | 9.0 | 8.9 | 9.8 | 8.7 | 7.8 | 7.9 |
| 6 | *R. flavipes* NE, USA | | | | | | – | 11.6 | 8.8 | 8.2 | 9.4 | 9.0 | 9.9 | 11.4 | 12.7 | 9.2 | 1.7 | 12.7 | 9.9 | 9.8 |
| 7 | *R. grassei* Charente, FRA | | | | | | | – | 9.9 | 8.7 | 7.8 | 10.8 | 8.5 | 8.3 | 6.8 | 10.8 | 10.6 | 9.3 | 10.3 | 8.0 |
| 8 | *R. guangzhouensis* Guangzhou, CHN | | | | | | | | – | 8.1 | 7.7 | 1.7 | 7.3 | 8.9 | 10.1 | 1.7 | 8.0 | 8.0 | 8.2 | 8.1 |
| 9 | *R. hageni* GA, USA | | | | | | | | | – | 5.7 | 8.1 | 6.2 | 7.5 | 8.9 | 8.4 | 7.5 | 9.1 | 5.0 | 8.3 |
| 10 | *R. hesperus* CA, USA | | | | | | | | | | – | 8.9 | 6.5 | 7.9 | 9.0 | 8.6 | 8.5 | 7.1 | 7.9 | 7.2 |
| 11 | *R. labralis* CHN | | | | | | | | | | | – | 7.5 | 8.7 | 10.7 | 0.9 | 8.2 | 8.7 | 8.0 | 8.6 |
| 12 | *R. lucifugus* Antalya, TUR | | | | | | | | | | | | – | 7.5 | 7.6 | 7.8 | 9.0 | 7.5 | 6.5 | 6.5 |
| 13 | *R. lucifugus* Corsica, FRA | | | | | | | | | | | | | – | 11.7 | 8.7 | 10.1 | 9.0 | 9.0 | 8.6 |
| 14 | *R. n. sp.* CA, USA | | | | | | | | | | | | | | – | 10.5 | 11.2 | 10.5 | 10.3 | 7.1 |
| 15 | *R. perilabralis* CHN | | | | | | | | | | | | | | | – | 8.3 | 8.9 | 8.4 | 8.7 |
| 16 | *R. santonensis* Charente, FRA | | | | | | | | | | | | | | | | – | 11.8 | 9.1 | 8.4 |
| 17 | *R. speratus* Mito City, JPN | | | | | | | | | | | | | | | | | – | 10.5 | 8.0 |
| 18 | *R. virginicus* FL. USA | | | | | | | | | | | | | | | | | | – | 7.4 |
| 19 | *R. tibialis* AZ, USA | | | | | | | | | | | | | | | | | | | – |

by gas chromatography using an internal standard for each caste and all colonies (PU, unpublished data). GC-MS was only used for the Turkish samples because the samples must be preserved in pure pentane, and at least 20–30 individuals are needed for each sample. Identification of other *Reticulitermes* spp. collected in this study was done using keys by Weidner (1959, 1960, 1972), Krishna and Weesner (1969), Clément (1978), Scheffrahn and Su (1994), and Donovan et al. (2000).

Voucher specimens, preserved in 70% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville.

Alcohol-preserved specimens were allowed to dry on filter paper, and DNA was extracted from individual heads using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μl of Tris:EDTA and stored at –20°C. 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 purified and concentrated using Microcon-PCR Filter Units (Millipore, Bedford, MA).

Samples were sent to the University of Arkansas DNA Sequencing Facility (Fayetteville) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer. GenBank accession numbers for the termites subjected to DNA sequencing in this study are AF525317 to AF525357 (Table 1. The accession numbers for the DNA sequences of additional *Reticulitermes* spp. termites obtained from GenBank are also provided in Table 1.

The distance matrix option of PAUP* 4.0b8 (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 the Australian wood feeding cockroach, *Panesthia cribrata* Saussure (GenBank AF220580); Formosan termites, *Coptotermes formosanus* Shiraki and *Heterotermes tenuior* (Haviland) (GenBank AB050714) were added to the *Reticulitermes* DNA sequences to act as outgroup taxa. DNA sequences were aligned using the PILEUP program in GCG (Genetics Computer Group, Madison, WI) and adjusted manually. Maximum-likelihood and unweighted parsimony analyses on the alignments were conducted using PAUP*. Gaps were treated as missing data. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum-likelihood analysis, the default likelihood parameter settings were used (HKY85 6-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*, using either the single most parsimonious tree as the starting tree or step-wise addition.

## Results

DNA sequencing of the amplicon revealed that it averaged 780 bp in size. To facilitate genetic comparisons with existing GenBank DNA sequences, 103 bp from the 5′ end of the amplicon was excluded and the remaining 677 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 *Reticulitermes* sequences were aligned using *P. cribrata*, *C. formosanus*, and *H. tenuior*, as the outgroup taxa. The aligned DNA data matrix, including the outgroup taxa (available at TreeBASE, http://www.treebase.org, study accession

**Table 3. Tajima–Nei pairwise distances in *Reticulitermes lucifugus* and *R. grassei***

| No. | Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *R. lucifugus* Antalya, TUR | – | 8.4 | 8.5 | 7.5 | 3.5 | 2.9 | 4.2 | 3.5 | 2.3 | 1.2 | 3.5 | 4.6 | 7.1 | 8.0 | 8.7 | 6.6 | 7.5 | 6.5 |
| 2 | *R. l.* Castel, ITA | | – | 1.1 | 2.1 | 7.8 | 10.0 | 8.2 | 9.0 | 8.5 | 8.6 | 9.1 | 9.8 | 6.0 | 4.0 | 0.3 | 3.4 | 1.2 | 5.8 |
| 3 | *R. l.* Chieti, ITA | | | – | 3.2 | 8.0 | 10.1 | 8.4 | 9.2 | 8.7 | 8.4 | 9.2 | 9.9 | 5.8 | 3.5 | 1.4 | 3.2 | 1.8 | 5.6 |
| 4 | *R. l.* Corsica, FRA | | | | – | 7.3 | 9.4 | 8.0 | 8.5 | 8.0 | 8.0 | 8.5 | 8.8 | 6.1 | 6.0 | 2.4 | 4.7 | 2.1 | 6.1 |
| 5 | *R. l.* Ankara–Dış Kapı, TUR | | | | | – | 5.0 | 1.2 | 4.0 | 4.0 | 3.9 | 4.0 | 4.2 | 7.0 | 7.3 | 7.8 | 6.0 | 7.7 | 6.1 |
| 6 | *R. l.* Fethiye, TUR | | | | | | – | 5.3 | 5.0 | 1.5 | 2.6 | 5.0 | 5.8 | 9.2 | 9.6 | 10.3 | 8.2 | 9.4 | 8.2 |
| 7 | *R. l.* Izmir, TUR | | | | | | | – | 4.0 | 4.7 | 4.2 | 4.3 | 3.5 | 7.6 | 7.5 | 8.2 | 6.3 | 8.0 | 6.8 |
| 8 | *R. l.* K. Maras, TUR | | | | | | | | – | 4.0 | 3.7 | 0.6 | 4.5 | 7.5 | 8.0 | 9.4 | 7.5 | 9.2 | 7.6 |
| 9 | *R. l.* Kas, TUR | | | | | | | | | – | 2.0 | 4.0 | 5.5 | 8.0 | 8.2 | 8.9 | 6.8 | 8.4 | 7.0 |
| 10 | *R. l.* Konya, TUR | | | | | | | | | | – | 3.5 | 5.0 | 7.7 | 8.2 | 8.9 | 7.0 | 8.0 | 7.1 |
| 11 | *R. l.* Mersin, TUR | | | | | | | | | | | – | 4.8 | 7.5 | 8.2 | 9.4 | 7.5 | 9.2 | 7.6 |
| 12 | *R. l.* Muğla, TUR | | | | | | | | | | | | – | 8.1 | 8.7 | 9.8 | 7.8 | 9.2 | 7.6 |
| 13 | *R. l.* Narbonne, FRA | | | | | | | | | | | | | – | 6.0 | 6.3 | 5.0 | 5.6 | 4.7 |
| 14 | *R. l.* Palermo, ITA | | | | | | | | | | | | | | – | 4.3 | 3.1 | 4.5 | 5.5 |
| 15 | *R. l.* Patanella, ITA | | | | | | | | | | | | | | | – | 3.7 | 1.5 | 6.1 |
| 16 | *R. l.* Sardegna, ITA | | | | | | | | | | | | | | | | – | 3.7 | 4.2 |
| 17 | *R. grassei*, FRA | | | | | | | | | | | | | | | | | – | 5.1 |
| 18 | *R. grassei*, ITA | | | | | | | | | | | | | | | | | | – |

number S769) resulted in a total of 677 characters. Of these characters, 332 (49%) were variable, and 208 (31%) were phylogenetically informative. Pairwise Tajima–Nei distances (Tajima and Nei 1984) among *Reticulitermes* taxa ranged from 0.9% between *R. labralis* and *R. perilabralis*, to 12.7% between *R. flavipes* to *Reticulitermes* n. sp. (Table 2). Within *R. lucifugus*, pairwise Tajima–Nei distances ranged from 0.3% between Castel and Patanella, Italy, to 10.3% between Fethiye, Turkey, and Patanella, Italy (Table 3).

This data set had only one most parsimonious tree (Fig. 1), (length = 1,003, CI = 0.44), as documented using the Branch and Bound search algorithm of PAUP*. Bootstrap analysis of the aligned *Reticuli-*

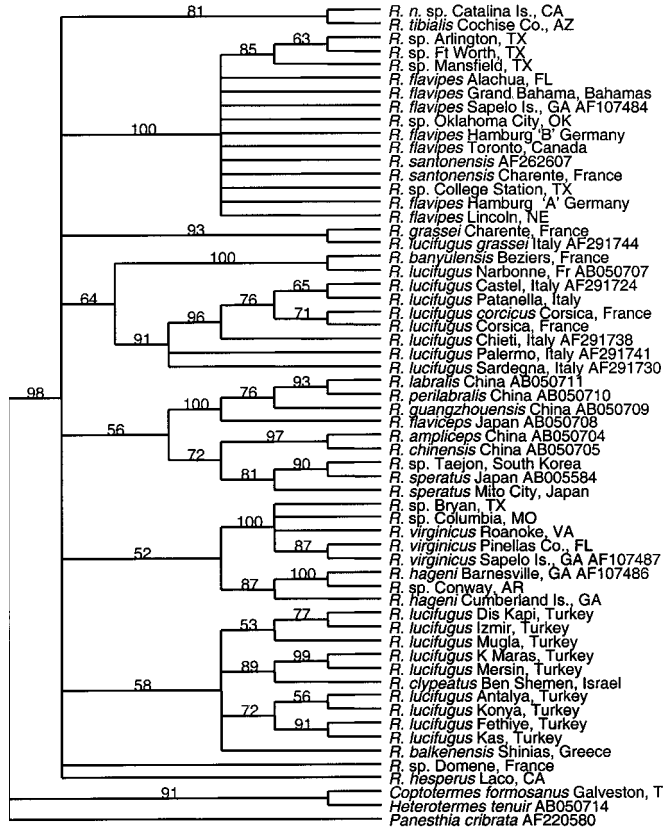


**Fig. 1.** Single most parsimonious tree during a branch and bound search using PAUP* (Swofford 2001). Bootstrap values for 1,000 replicates are listed above the branches supported at ≥50%.

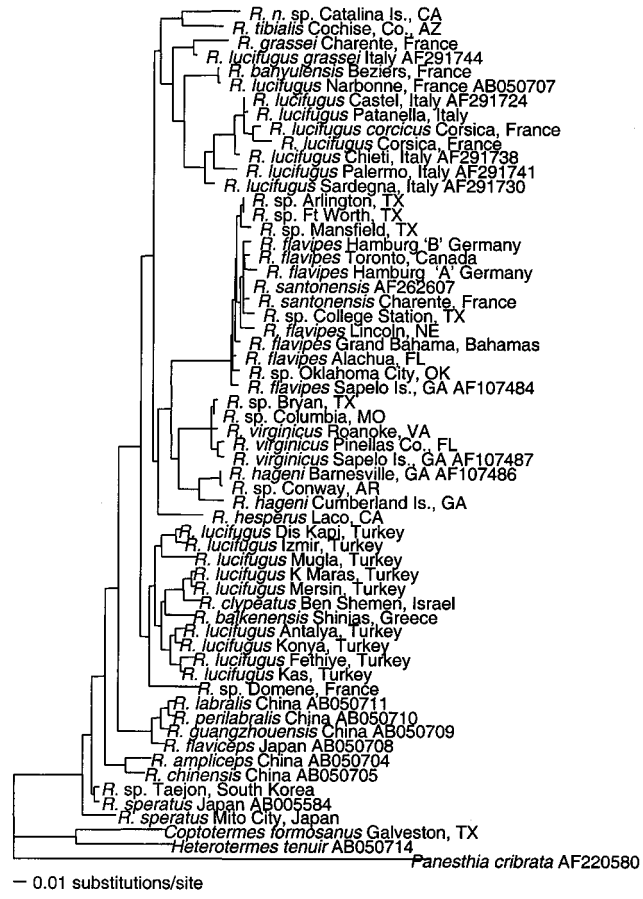


**Fig. 2.** Topology obtained by maximum-likelihood analysis based on the HKY85 model (see text). Log L = –5851.71969.

*termes* spp. and the outgroup taxa resulted in a consensus tree with several distinct branches. These distinct clades included: *R. flavipes* and *R. santonensis* De Feytaud; *R. banyulensis* (Béziers, France) and European *R. lucifugus; R. virginicus* and *R. hageni; R. clypeatus, R. balkanensis* and Turkish *R. lucifugus; Reticulitermes* n. sp. Scheffrahn, and *R. tibialis;* and the *Reticulitermes* spp. from China and Japan. *R. clypeatus* from Israel formed a distinct clade with *R. lucifugus* from K. Maraş and Mersin, Turkey. Based on the maximum parsimony analysis, we were unable to determine the relationship of *Reticulitermes* sp. Domene, France, and *R. hesperus* with the other taxa. Of the six *Reticulitermes* spp. from the United States that were not classified to species morphologically, four were members of the *R. lucifugus*/*R. santonensis* clade, and the other two belonged to the *R. virginicus* clade (Fig. 1).

Regardless of whether the starting tree was the most parsimonious tree or was obtained via step-wise addition, the maximum-likelihood search found only one tree (Fig. 2). The maximum-likelihood tree differed from the maximum-parsimony tree for two samples: the two *R. hageni* samples did not form a sister group with *R. virginicus;* and *Reticulitermes* sp. France formed a sister group with the samples from Turkey and Greece.

## Discussion

In this study, a phylogenetic analysis of *Reticulitermes* belonging to 21 species and subspecies from three continents, based on the DNA sequence of a portion of the mitochondrial COII gene, is presented. The mtDNA COII marker was used to allow the incorporation of *Reticulitermes* spp. DNA sequences submitted to GenBank. Most of the inferred relationships 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 currently accepted groupings. For example, the grouping of *R. labralis, R. perilabralis* Ping and Xu, *R. guangzhouensis* Ping, *R. flaviceps* (Oshima), *R. ampliceps, R. chinensis* Snyder, and *R. speratus* (Kolbe) reflects a clear delimitation of eastern Asian *Reticulitermes* spp., when compared with their more western counterparts in Europe and North America (Fig. 1).

However, the addition of previously unsampled populations of *R. lucifugus* from Turkey in combina-

tion with various other unsampled populations from throughout the world resulted in some notable differences in their classification. The most notable difference was observed in the *lucifugus* complex, as previously described by Plateaux and Clément (1984), which included one ponto-Mediterranean probable species (*balkanensis*), one Mediterranean species (*lucifugus*) with a Corsican probable subspecies (*corsicus*), and an atlanto-Mediterranean species. The last two forms, Aquitanian (*grassei*) and Catalan (*banyulensis*), are naturally intersterile just like two different species, but they are connected by a range of interfertile Iberian forms that constitute a large zone of primary intergradation. Our results clustered *R. banyulensis* (Béziers) (with both *R. lucifugus* France, Narbonne and Corsica), *R. lucifugus corsicus* France (Corsica) and *R. lucifugus* Italy (Castel, Patanella, Palermo, and Sardinia) (Fig. 3).

The large zones of sympatric occupation of *Reticulitermes* spp., as previously described by Plateaux and Clément (1984), suggest the likelihood of hybridization in numerous instances (examples include, *R. grassei* and *R. santonensis* in southwestern France, and *R. banyulensis* and *R. grassei* stretching from northwestern Spain through Portugal to southeastern Spain on the Iberian peninsula). By modifying the breeding temperature, Clément (1979b) demonstrated that artificial constitution of heterospecific pairs allowed hybridization between sympatric *R. santonensis* and *R. lucifugus.* However, because these termites possessed different flagellates, suggesting they do not naturally intermingle, there was no support for natural hybridization to occur. Clément et al. (2001) suggest that this demonstrates the efficacy of the species' isolation mechanism between them. It might also demonstrate, with respect to *Reticulitermes* spp. in other locations, that large sympatric zones may (1) provide the framework for natural hybridization to occur or (2) be a function of environmental similarities that potentially induce clinal variations in *Reticulitermes* spp., where otherwise strong species isolation mechanisms (whether behavioral, chemical, chronological with respect to mating time, or genetic) are rendered inadequate to prevent hybridized mating.

More recent discoveries of *R. grassei* in southwestern England (Fig. 3), where it was previously believed that termites were incapable of persisting, support the concept that some isolation mechanisms may be rendered inactive in the presence of anthropogenic interference. The behavioral adaptation, whereby termites migrate to structures for warmth emitted from buildings in the winter months, permits the survival of the respective species outside of its believed normal habitation range. A similar scenario has been described for the occurrence of *R. flavipes* in Toronto, Canada, and Hamburg, Germany (Weidner 1970).

In our study, the clade containing *R. grassei* was clearly distinct from *R. lucifugus* in France and from *R. lucifugus* in Turkey (Fig. 1). These results are supported by phylogenetic analyses of DNA sequences of the mtDNA 16s rRNA gene and NADH dehydrogenase one genes, and GC-MS analyses of cuticular hydrocarbons (unpublished data). The clade containing *R. lucifugus* (France and Italy), *R. banyulensis* (which occurs in the southern Mediterranean coastal areas of France and Spain), and *R. lucifugus corcicus*, likewise formed a distinct group. Plateaux and Clément (1984) describe *R. banyulensis* as being intersterile, like a different species, but connected by a range of interfertile Iberian forms that constitute a large zone of primary intergradation. Although *R. lucifugus corcicus* is considered morphologically similar to certain populations of *R. grassei* (Clément 1982), in our study, *R. lucifugus corcicus* formed a sister clade with other *R. lucifugus* populations from France and Italy (Fig. 1). Our results suggest that *Reticulitermes* sp. from Domene France is either the result of hybridization of two sympatric species within the zones of their occurrence, or most probably an introduced *Reticulitermes* sp. from one of the neighboring countries or regions.

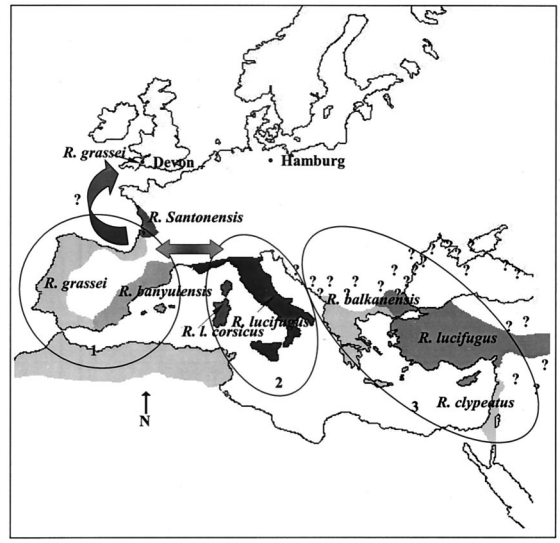


**Fig. 3.** Natural geographical distribution of *Reticulitermes* species in Europe. 1, the *R. grassei–R. banyulensis* group; 2, *R. lucifugus* in Italy; 3, the *R. balkanensis* GRE–*R. lucifugus* TUR–*R. clypeatus* ISR group. The arrow between groups 1 and 2 reflects the pairing of *R. banyulensis* with *R. lucifugus* Italy as was observed in the phylogenetic analysis.

Turkish *R. lucifugus* formed sister clades with *R. balkanensis* (Greece) and *R. clypeatus* (Israel) that were clearly distinct from French and Italian *R. lucifugus* (Figs. 1 and 2). It would appear that the differences observed from the common clade containing *R. balkanensis*, *R. lucifugus* (Turkey), and *R. clypeatus* (Israel) with taxa from more northerly locations support geographic isolation that is clinal in nature (Fig. 3). Weidner (1960) supports this hypothesis using morphological analysis of a limited number of samples. He concluded that termite morphology is influenced by geography. An increase in body size occurs in an east-to-west direction. The gula was broader at the northern edge of the circulation area (La Rochelle,

France, and Dojran, Macedonia) compared with a slimmer gula in the more southern areas (Madeira, Israel, and Iraq). Likewise, the theory about more recent speciation due to glacial movements seems as likely as any explanation for the variation that is observed in *Reticulitermes* spp. of the Mediterranean region and provides support for Turkish *R. lucifugus* being a different species than European *R. lucifugus.*

The relationship of *R. flavipes* with *R. santonensis* is not surprising. It has long been believed that *R. flavipes* was accidentally introduced from the United States into France and subsequent locations in Europe, including locations in Germany (Weidner 1937, 1951Becker 1970; Harris 1962) and Austria (Heisterberg, 1958, 1959; Hrdý 1961). Recently, Clément et al. (2001) found *R. santonensis* to occur in a sympatric zone with *R. grassei;* and based on its limited distribution (southwestern France), it would appear to be geographically isolated there. Also, on the basis of DNA sequencing analysis of two mtDNA genes and rDNA ITS2, Jenkins et al. (2001) found *R. santonensis* from France to form a close genetic relationship with *R. flavipes* from the United States. The *R. santonensis* analyzed in our study formed a sister clade with samples of *R. flavipes* from a wide geographic range including Texas, Nebraska, Florida, Canada, Germany, and the Bahamas. Our larger geographic range of *R. flavipes,* paired with its similar genetic sequence data obtained in this research lends further support to the aforementioned suppositions that *R. santonensis* may be the result of some limited hybridization event in France from introduced *R. flavipes.*

According to maximum-parsimony and maximum-likelihood analysis, *R. virginicus* and two unidentified groups (Columbia, MO, and Bryan, TX) formed a sister clade to *R. hageni* (Barnesville and Cumberland Island, GA). This relationship between these two species was also observed by Jenkins et al. (2000). Using cuticular hydrocarbon and DNA sequencing of 400 bp of the mtDNA A+T rich region, they found *R. virginicus* to form a sister clade to *R. hageni*, relative to *R. flavipes.* With the maximum-likelihood analysis, *R. hesperus*, *R. hageni*, *R. virginicus*, *R. santonensis*, and *R. flavipes* all formed a common clade (Fig. 2). The common relationship between *Reticulitermes* n. sp. (collected by R. Scheffrahn from Catalina Island, CA), and *R. tibialis* is of interest. Genetic divergence between these two taxa and the inclusion of additional specimens from within its native range may provide more insight into the classification of these taxa.

Although these results suggest that genetic differentiation exists among many *Reticulitermes* spp., by no means do we anticipate that this research will alleviate all of the confusion that has led to the various descriptions of *Reticulitermes,* in particular, the various subspecies of the *lucifugus* complex. This study should assist with a greater knowledge of *Reticulitermes* as a whole, and our results should serve as a baseline for further studies in *Reticulitermes* systematics. Also, the ability of this marker to identify unclassified *Reticulitermes* to species, has potential for the development of PCR-restriction fragment-length polymorphism (RFLP) diagnostics for economically important *Reticulitermes.*

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