

Genetic Evidence for the Synonymy of Two *Reticulitermes* Species: *Reticulitermes flavipes* and *Reticulitermes santonensis*

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ABSTRACT By applying the 16S rRNA mitochondrial marker to 434 populations of *Reticulitermes* termites from North, Central, and South America; France; and Germany with other locations around the world that we have analyzed, identical DNA sequence data were obtained from *Reticulitermes flavipes* (Kollar) in North America and Germany and for *Reticulitermes santonensis* Feytaud from undisturbed (nonurban) forested locations in western France. We also discovered identical DNA sequence data from previously unidentified *Reticulitermes* specimens from South America and Easter Island. Haplotypes F, M, and GG were observed in France; haplotype F in Germany; and haplotype GG was found on Easter Island, Santiago, Chile, and Montevideo, Uruguay. All of these haplotypes are found in numerous states within the continental United States. In light of their well documented morphological, chemical, and phylogenetic relationships, coupled with this new data that directly link these disjunct groups, *R. flavipes* and *R. santonensis* should be synonymized. Compared with other studies that largely suggest phyletic similarities, this is the first study that specifically matches haplotypes from North America (where populations of *R. flavipes* are endemic) with populations in Europe (where *R. flavipes*, described as *R. santonensis*, is presumed exotic).

KEY WORDS *Reticulitermes*, DNA sequence, genetic variation, termite

AMBIGUITY FROM CHARACTER-POOR and morphologically variable termite species, such as those in the genus *Reticulitermes* Holmgren, prompted other termite identification methods in conjunction with morphology. Nonmorphological methods for species identification include ethological, biochemical, chemotaxonomic, and molecular measures (Clément et al. 2001). In France, the genus *Reticulitermes* is composed of approximately six species/subspecies (Clément et al. 2001). One of these species, *Reticulitermes santonensis* Feytaud, occurs in sympatric zones in southwestern France with *Reticulitermes grassei* Clément. The current distribution of *R. santonensis* is north of the Gironde, Charentes, and Vendée; is prevalent in the Loire Valley, Normandy, Centre, Paris and its suburbs (Guillaume 2001); and is random in other northern districts (Bagnères 2003). In Paris, *R. santonensis* has become a significant pest to structures and trees (Lohou et al. 1997), more so than when previously discussed by Jacquot (1955).

Feytaud (1924) first described Charente, France, populations of *Reticulitermes lucifugus* as a subspecies, *R. lucifugus santonensis*. Feytaud (1925) and Jucci (1924) compared this subspecies to the American species *R. flavipes* and concluded that they were similar, if not the same species. Feytaud (1925) later supported the hypothesis of its synonymy with the North American *Reticulitermes flavipes* (Kollar), suggesting an introduction through La Rochelle harbor. Subsequent investigations by Feytaud (1966), Lash (1952), and Grassé (1954) collectively defined *R. santonensis* as a different species with a small geographic localization (in southwestern France). Considered as part of the *R. lucifugus* complex in Europe (Buchli 1958), morphological studies applying sophisticated biometric or multivariate analyses (Clément 1978, 1979, 1982a) in combination with sexual pheromones (Clément 1982b), alloenzyme analyses (Clément 1984), recognition and aggressiveness behaviors (Clément 1981, 1986) provided the impetus to elevate *R. santonensis* to species level (Plateaux and Clément 1984). Evaluating cuticular hydrocarbons and defensive compounds of soldiers, Bagnères et al. (1990) suggested that *R. santonensis* was most likely *R. flavipes* introduced sometime during the 18th or 19th century as supposed by Feytaud (1925). More recent genetic analyses that use mitochondrial DNA (mtDNA) have demonstrated that *R. santonensis* was phylogenetically most similar to North American *R. flavipes* (Clément et al. 2001, Jenkins et al. 2001, Austin et al. 2002, Marini

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and Mantovani 2002, Uva et al. 2004, and Ye et al. 2004). Further supporting this, Austin et al. 2005 have demonstrated the monophyly of *R. flavipes* relative to all other known species in North America. Vieau (1999) suggest that a closer examination of North American *Reticulitermes* species, particularly *R. flavipes*, might reveal a relationship with the European species (*R. santonensis*).

A comparison between *R. santonensis* and *R. flavipes* was recently approached using microsatellite markers showing a lower level of heterozygosity in *R. santonensis* than in the North American species (Dronnet et al. 2004). This finding could be consistent with some introduction events leading to a genetic bottleneck that needs to be corroborated (S.D. et al., unpublished data).

In Germany, *R. flavipes* was believed to have been introduced into Hamburg on pine (*Pinus* spp.) trunks imported from the United States on multiple occasions (Weidner 1937, 1970, 1978a; Harris 1962), with significant damage, repair, and control costs (Weidner 1978b). Becker (1970) suggests that *R. flavipes* had been introduced into Europe from North America many times. Becker's supposition seems likely, because the original description of *R. flavipes* occurred much earlier in Vienna, Austria (Kollar 1837), where this species has been described as a considerable pest (Heisterberg 1958, 1959; Hrdy 1961). Unfortunately, we do not possess any current samples for evaluation. More recently, additional introductions of *R. flavipes*/*R. santonensis* have been documented in the Bahamas (Scheffrahn et al. 1999) and in South America (Santiago, Chile) (Ripa and Castro 2000, Constantino 2002). Aber (1998) described an introduced *Reticulitermes* in Uruguay as being *R. lucifugus*, the only important pest species there. Although it has long been speculated that *R. lucifugus* had been introduced to Uruguay from Europe, the identification was only tentative and no subsequent investigations have been available (original assignation was *Heterotermes* spp.). In North America, the distribution of *R. flavipes* has been described to occur widely from Ontario, Canada, south through the entire eastern United States to both northern Mexico and Guatemala, west to Arizona and Utah (Snyder 1954, Weesner 1965, Nutting 1990).

In arguably one the most comprehensive evaluations of *Reticulitermes* in Europe, Clément et al. (2001) could not resolve the taxonomic status of *R. santonensis*. They were unable to find any American biotypes that possessed the same chemotaxonomic features of *R. santonensis*. Thus, no argument for the distribution of *R. santonensis* could be given. Clément et al. (2001) speculated that it could be due to an unknown genotype introduced from America (*R. flavipes* subspecies or sibling species) or a native European species. Furthermore, the only "naturally" occurring colonies of *R. santonensis* were found in the western forests of France. By observing populations of introduced *Reticulitermes* globally, particularly those of dubious identity, accidental establishment of *R. flavipes* can more easily be interpreted. This requires a historical account of their introductions, a comprehensive eval-

uation of the genus (Austin et al. 2002), and a thorough evaluation of the species *R. flavipes* (J.W.A., unpublished data), to approximate possible points of origin to these exotic locations.

The objective of this study was to clarify the relationship of *R. flavipes* to *R. santonensis* by providing genetic evidence (applying the 16S rRNA gene) of their synonymy, to determine whether *R. flavipes* haplotypes that occur in Europe and South America also occur in North America and to identify possible introduction routes from the United States.

Materials and Methods

Termites that were subjected to DNA sequencing in this study were collected from France, Germany, Chile, Uruguay, Easter Island, the Bahamas, and North America and preserved in 100% ethanol (Table 1). Voucher specimens preserved in 100% ethanol are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR. From alcohol-preserved specimens dried on filter paper, the DNA was extracted according to Liu and Beckenbach (1992) from individual worker termites with the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μ l of Tris-EDTA and stored at -20°C . Polymerase chain reaction (PCR) was conducted with the primers LR-J-13007 (5'-TTACGCTGTTATCCTAA-3') (Kambhampati and Smith 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al. 1994). These PCR primers amplify an \approx 428-bp region of the mtDNA 16S rRNA gene. The PCR reactions were conducted with 1 μ l of the extracted DNA (Szalanski et al. 2000), having a thermocycler 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 with minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega, Madison, WI). Samples were sent to The University of Arkansas Medical School DNA Sequencing Facility, Little Rock, AR, for direct sequencing in both directions. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999), and sequences were aligned using ClustalW (Thompson et al. 1994). Mitochondrial DNA haplotypes were aligned by MacClade version 4 (Sinauer Associates, Sunderland, MA). Mitochondrial DNA 16S sequences of *R. santonensis* from Biscarrosse, France (GenBank accession no. AF292025) (located 40 km south of Arcachon), and Île d'Oléron (maintained in Marseille laboratory), France (GenBank accession no. AF292026), also were compared with other *R. flavipes* haplotypes from North America and Germany (Szalanski et al. 2003; Austin et al. 2004a, b, c; Ye et al. 2004; J.W.A. et al., unpublished data).

Results and Discussion

From 434 *R. flavipes* DNA sequences analyzed, three haplotypes, F, M, and GG (GenBank accession

Table 1. Collection data and haplotypes (Hap) for *R. flavipes* (= *R. santonensis*)

Location	Country	Hap	n	Source
Hamburg	Germany	F	2	Szalanski et al. (2003)
Biscarrosse	France	F	1	Marini and Mantovani (2002)
Texas	USA	F	9	Austin et al. (2004a)
Oklahoma	USA	F	2	Austin et al. (2004b)
Louisiana	USA	F	1	Austin et al. (2004c)
Arkansas	USA	F	2	Austin et al. (2004c)
Tennessee	USA	F	4	This study
South Carolina	USA	F	1	This study
Nebraska	USA	F	1	This study
Mississippi	USA	F	3	This study
Kansas	USA	F	4	This study
Texas	USA	M	2	Austin et al. (2004a)
Tennessee	USA	M	1	This study
Mississippi	USA	M	4	This study
Kansas	USA	M	2	This study
Florida	USA	M	1	This study
Kentucky	USA	M	1	This study
Iowa	USA	M	1	This study
Louisiana	USA	M	2	Austin et al. (2004c)
Arkansas	USA	M	5	Austin et al. (2004c)
Wisconsin	USA	M	1	This study
Indiana	USA	M	1	This study
Arcachon (Gironde)	France	M	1	This study
La Teste	France	M	1	This study
Ile d'Oleron Sables	France	M	1	This study
Charentes, La Coubre forest	France	M	2	This study
Albi	France	GG	1	This study
Ile d'Oléron (from Marseilles laboratory)	France	GG	1	Marini and Mantovani (2002)
Ile d'Oléron	France	GG	2	This study
Ile d'Oléron Nord	France	GG	2	This study
La Rochelle	France	GG	1	This study
Arkansas	USA	GG	1	This study
Iowa	USA	GG	1	This study
Louisiana	USA	GG	2	This study
Easter Island	Chile	GG	1	This study
Santiago	Chile	GG	1	This study
Montevideo	Uruguay	GG	1	This study

nos. AY441980, AY441987, and AY702099, respectively), were found in the United States that also occur in Europe and South America (Table 1; Fig. 1). Five nucleotide sites were variable among haplotypes F, M, and GG (Table 2). A 402-bp portion of the mtDNA 16S sequence of two *R. santonensis* sequences from Biscarrosse and Oléron (Marini and Mantovani 2002) also was compared with sequences obtained from the aforementioned material. Haplotype F was observed in Hamburg, Germany; Biscarrosse, France; and nine U.S. states (Table 1). Haplotype M was observed in four locations in France: Arcachon, La Teste (Gironde), Ile d'Oleron and La Coubre forest (Charente-maritime), and Charentes, with 12 U.S. states. Haplotype GG was observed in three locations from France: Ile d'Oleron (three sites, including Marseilles laboratory material), Albi (Tarn), and La Rochelle (Charente-maritime); two locations from South America: Santiago, Chile, and Montevideo, Uruguay; and on Easter Island (Pacific Region, Chile), with three U.S. locations: Arkansas, Louisiana, and Iowa (Fig. 1).

The utility of mtDNA sequencing data to clarifying relationships between and among *Reticulitermes* has most recently been observed in Austin et al. (2002), Marini and Mantovani (2002), Szalanski et al. (2003), Kutnik et al. (2004), Luchetti et al. (2004), Uva et al. (2004a, b, and Ye et al. 2004. The application of 16S

rRNA has proven to be reliable and easy to use for clarification between numerous *Reticulitermes* groups, particularly within *R. flavipes* from North America (Austin et al. 2004a, b, c; J.W.A. et al., unpublished data) and from Europe (Marini and Mantovani 2002, Luchetti et al. 2004).

When applying molecular markers, both the phylogenetic relationships of the family Rhinotermitidae (Austin et al. 2004d) and the genus *Reticulitermes* (Austin et al. 2002) are more resolute than nongenetic-based methods of identification. Evaluation of the genetic variation of *R. flavipes* (applying the 16S rRNA gene) from North America support the monophyletic nature of this species (Austin et al. 2004a, b, c; J.W.A., unpublished data) in general.

Additional samples from South America would be desirable to validate descriptions of *Reticulitermes* spp. found there. There have been reports of *R. hesperus* in Chile (Cabrera and Camousseight 1997, 1998; Fontes and Milano 2002) and *R. lucifugus* in Uruguay [Aber and Fontes 1993, originally identified as *Heterotermes* sp. by Aber (1989)]. There is no record of *R. flavipes* from Uruguay. Based on genetic evidence, we only find evidence of one species in South America, *R. flavipes/R. santonensis*.

Phylogenetic evidence from numerous researchers using multiple genetic markers (nuclear and mito-

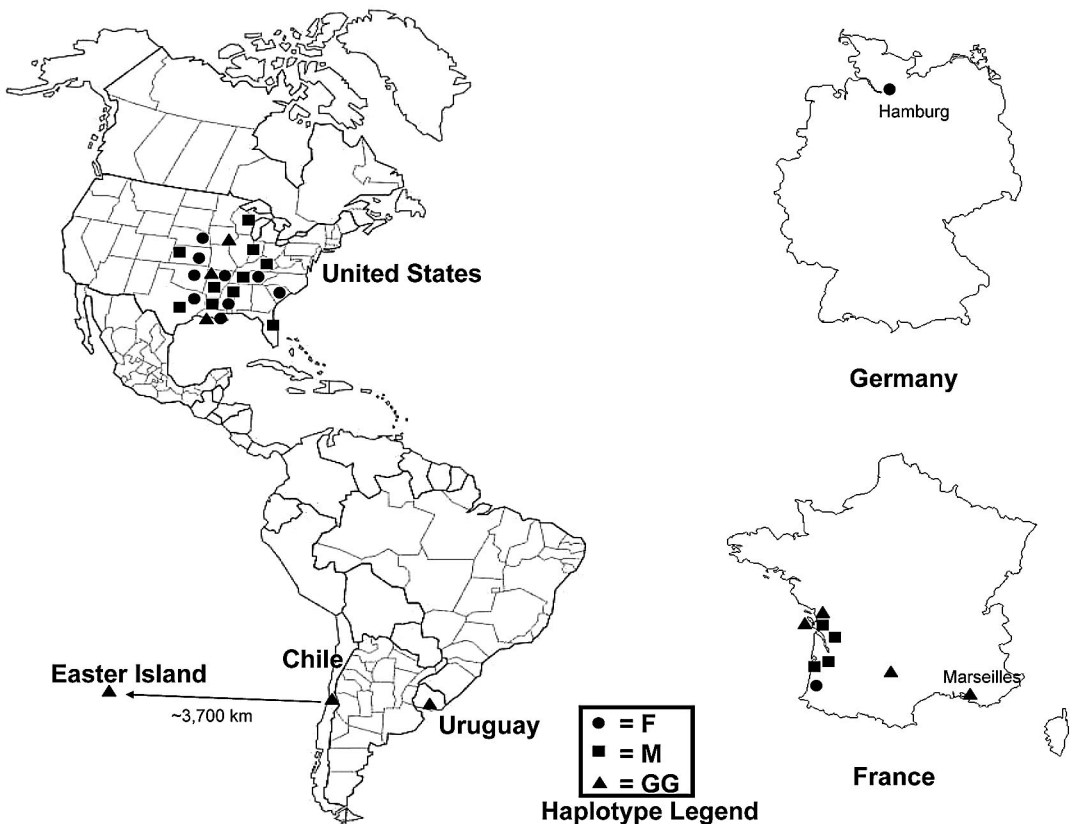


Fig. 1. Distribution of three *R. flavipes* haplotypes from United States, France, Germany, Easter Island, Chile, and Uruguay.

chondrial), both in Europe and North America, have confirmed the relationship between *R. flavipes* and *R. santonensis*, and there is already an open discussion concerning the synonymy of these two species (Jenkins et al. 2001; Austin et al. 2002; Marini and Mantovani 2002; Uva et al. (2004a), J.W.A. et al., unpublished data). Our genetic analyses confirm that *R. flavipes* occupies the largest established geographic range of any termite within *Reticulitermes*, making it arguably the most damaging pest species within the genus and among other pestiferous *Heterotermitinae*, but *R. flavipes* still needs some phylogeographic clarifications because of the complexity of the species. This is the first evidence, for any genetic marker, which specifically identifies exact matching haplotypes between North American *R. flavipes* and European *R. santonensis*. This provides the framework for

Table 2. Nucleotide variation among three *R. flavipes* haplotypes from United States, France, Germany, Easter Island, Chile, and Uruguay

Haplotype	131	133	158	168	170
F	A	C	G	G	—
M	G	A	A	A	—
GG	—	—	—	—	G

synonymizing these two species, particularly when considering the number of occurrences each haplotype was found in the United States. We have documented 47 distinct mtDNA 16S *R. flavipes* haplotypes (Austin et al. 2004a, b, c; J.W.A. et al., unpublished data), representing the majority of its currently known distribution (globally), and all haplotype GG samples in this study were identical to *R. santonensis* samples from undisturbed (nonurban) habitats from the western forests of France. A concurrent study using microsatellite markers and another mitochondrial gene (COII) will likely corroborate these data and should provide additional data on the phylogeny between the two species (S.D. et al., unpublished data). Perhaps additional haplotypes from urban zones (e.g., Paris and other infested districts in France) will reveal additional haplotypes that are commonly found across North America. Future studies of *R. flavipes* from these areas may be important for potential improvement of various control regimes that may vary due to subtle differences between various haplotypes of this pest and other *Reticulitermes*.

Because nonendemic establishments of *R. flavipes* probably originated from single reproductive pairs and endemic distribution of this species is broad, it should not be difficult to imagine that termite taxon-

omists would observe significant variation in this species. Clinal variations (in size and shape) are already documented for *Reticulitermes* termites (Weidner 1960, 1970) and many other insects. Similarly, chemotaxonomic studies (of *R. flavipes/santonensis*) applying cuticular hydrocarbons (Haverty et al. 1999) have not been as resolute in clarifying this relationship as the 16S rRNA gene, or other molecular markers for that matter. Since 1925, *R. santonensis* has repeatedly changed its taxonomic status, contributing to a great deal of confusion for this morphologically and chemotaxonomically challenging species. Intraspecific variation of *R. flavipes* seems to be more pervasive than found in other *Reticulitermes* (J.W.A., unpublished data), promoting adaptive phenotypic plasticity (DeWitt et al. 1998) and rendering nongenetic-based identification methods less certain. Last, a more comprehensive review of established populations of *R. flavipes* in South America needs to be evaluated. Because there are no records of *R. flavipes* in Montevideo, Uruguay, and previous taxonomic uncertainty has been established, the occurrences of *Reticulitermes* in South America should be evaluated. Constantino (2002) suggests that invasion of *Reticulitermes* to neighboring Argentina, Paraguay, and Southern Brazil are likely.

Variation among populations "makes more sense" when compared and interpreted through a phylogenetic framework (Avisé 1994) and ideally would be assessed for individuals from all possible populations, but practicality necessitates selective sampling (Foster et al. 2004) as presented herein. Additionally, there are no specific rules that govern how many populations or individuals should be evaluated, but specimens should be sampled from populations evenly distributed within the species range (Mayr and Ashlock 1991). To date, our study represents the most comprehensive (in terms of samples evaluated) genetic evaluation of *R. flavipes* known. This has been a limiting factor for most studies that have attempted to elucidate the relationships between these two species, either relying on significantly fewer samples or from fewer geographic locations. One of our objectives is to simplify the chaotic and oftentimes outdated phylogeny of *Reticulitermes* worldwide. Although a revision of the genus is definitely needed, few would endeavor to attempt this given the significant time and financial constraints that it would likely entail. This study is a small, but important, step toward that goal. Furthermore, we provide compelling genetic evidence that these species are in fact one—*R. flavipes*—an American exotic pest introduced to many of America's global trading partners. The most parsimonious conclusion, given the broad geographic occurrence of haplotypes in the United States, which are identical to haplotypes in Europe and elsewhere, suggests that synonymizing these two species is justified.

We do not suggest that genetic markers (or any other nonmorphological identification scheme) are necessarily superior taxonomic tools or that they should be exclusively applied to describing termite species in general. However, when dealing with dif-

ferent taxonomic groups such as the genus *Reticulitermes*, which has populations separated by large geographic distances, they are especially important. Genetic markers such as the 16S rRNA gene may be one of the few ways to offer clarity for this problematic genus.

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