

# Identification of *Reticulitermes* spp. (Isoptera: Reticulitermatidae) from South Central United States by PCR-RFLP

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**ABSTRACT** Because of morphological ambiguity, traditional identification of *Reticulitermes* Holmgren termites has always been difficult and unreliable. A molecular diagnostic method is presented for differentiating *Reticulitermes* species occurring in the south central United States, which are economically important urban pests. A 379-bp region of the mtDNA COII gene and a 415-bp region of the mtDNA 16S rRNA gene were amplified using polymerase chain reaction (PCR) and sequenced from *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes hageni* Banks, and *Reticulitermes tibialis* (Banks). Applying DNA sequence data, the PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of two restriction enzymes each for the COII amplicon and the 16S amplicon, were diagnostic for all of the *Reticulitermes* species analyzed. Based on putative mutation rates, >87% and 97% of the samples should be successfully identified to species with PCR-RFLP of COII and 16S, respectively. To verify the accuracy of our predictions, we examined unclassified *Reticulitermes* populations from Arkansas, Louisiana, Missouri, Oklahoma, Texas, and Virginia using PCR-RFLP. Applying PCR-RFLP, 97 samples were correctly classified to species. This technique allows the use of field-collected specimens preserved in alcohol and can identify termite specimens regardless of caste. PCR-RFLP, resolved with agarose or polyacrylamide gel electrophoresis, provided an efficient method for identification of *Reticulitermes* species from the south central United States for diagnostic purposes.

**KEY WORDS** PCR-restriction fragment-length polymorphism, molecular diagnostics, *Reticulitermes*, termites, mitochondrial DNA

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SPECIES OF THE GENERA *Reticulitermes* Holmgren are the major termite pests infesting wooden structures in the United States and in other countries around the world. Subterranean termite species, such as those found in the genera *Reticulitermes*, live in moist soil habitats, feeding on wood buried beneath or in contact with the soil. 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). More recent estimates by the National Pest Management Association suggest this figure to be closer to \$2.5 billion (NPMA 2003). A breakdown by costs caused by termite species, reveal that the five principal subterranean termite species in the United States are *Reticulitermes flavipes* (Kollar); *Reticulitermes virginicus* (Banks), the dark southern subterranean termite; *Reticulitermes hesperus* (Banks), the western subterranean termite; *Reticulitermes tibialis* (Banks), the arid subterranean termite; and *Coptotermes formosanus* (Shiraki). Ninety percent of the termite control business involves these five subterranean termite species (Forschler and Lewis 1997). Subterranean termites of the family Rhinotermitidae, genus *Reticulitermes*, cause the most structural damage in the United States, and are considered the most

economically important species (Su and Scheffrahn 1990). Similarly, in Europe and other parts of the world, *Reticulitermes* spp. are the most expensive and damaging pest species. The cost of treatment against termites in Europe is expected to top one billion euros by 2005 (UNEP and FAO 2000). In the south central region of the United States, four species of *Reticulitermes* occur: *R. flavipes*, *R. virginicus*, *R. tibialis*, and *R. hageni* (National Termite Survey 2002, Weesner 1965).

Correct identification is critical for pest insects, such as termites, that may require very different control methods depending on the target species. Identifying workers is nearly impossible and separating soldiers is especially difficult given that precise measurements are required and overlap may occur between species (Scheffrahn and Su 1994). Difficulties arise in species determination at individual collection

species determination. Molecular genetic methods are able to differentiate species regardless of the caste encountered or locality obtained.

Previous research on *Reticulitermes* genetics has focused on genetic variation within and among populations, or phylogenetic relationships within *Reticulitermes* and Isoptera but not molecular diagnostics (Austin et al. 2002; Miura et al. 1998; Jenkins et al. 1998, 2001; Clément et al. 2001). Research on genetic variation within *Reticulitermes*, using DNA sequencing of a portion of the mtDNA COII gene, has revealed sufficient variation to distinguish many species from North America, Europe, and Asia (Austin et al. 2002). Although developments in molecular biology have improved our ability to differentiate species, identification by DNA sequencing can be both costly and time consuming. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has become established as a reliable molecular technique for identifying economically important insects including *Rhagoletis* (Diptera: Tephritidae) species (Salazar et al. 2002), boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) populations (Roehrdanz 2001), and *Muscidifurax* wasps (Hymenoptera: Pteromalidae) (Taylor and Szalanski 1999). We chose PCR-RFLP for this study because the technique is simple and inexpensive, very reliable and repeatable, and provides discrete character states for quantitative comparisons of two or more species.

In this study, a PCR-RFLP technique was developed to study restriction site variation of two regions of the mtDNA genome. Digestion of PCR products with specific restriction enzymes yielded diagnostic patterns for the identification and differentiation of *Reticulitermes* termites, regardless of caste. This is of great importance because of the frequent lack of morphologically distinct caste members collected, namely soldiers or alates, which are essential for accurate deter-

mination of species from a given population of subterranean termites. This is the first application of a molecular genetics technique for the identification of *Reticulitermes* termite taxa from the south central United States.

## Materials and Methods

Termites were field collected continuously from 2000 to present at various locations in Canada, The Bahamas, Europe, and the United States, and preserved in 100% ethanol (Table 1 and see Table 5). Morphological identification of 32 *Reticulitermes* was performed applying the taxonomic keys of Krishna and Weesner (1969), Scheffrahn and Su (1994), and Hostettler et al. (1995) (Table 1). Voucher specimens, preserved in 100% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

Alcohol-preserved specimens were allowed to dry on filter paper, and DNA was extracted from individual worker, soldier, or alate heads using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50  $\mu$ l of Tris:EDTA and stored at  $-20^{\circ}\text{C}$ . PCR was conducted using two different mtDNA primer sets. The first primer set, *tercoII-idf* (5'-TCTTCTTCCACGAY-CAYACAYTAATAA-3') and primer *tercoII-idr* (5'-TTTATGGGTAGTACYATTCCGYTT-3') were used to amplify a 379-bp portion of the 5' end of the COII gene. The other primer set, 16S-r (5'-CGCCTGTT-TATCAAAAACAT-3')

**Table 2.** *Reticulitermes* genetic divergence (%), COII above, 16S below diagonal

Species	<i>R. flavipes</i>	<i>R. hageni</i>	<i>R. virginicus</i>	<i>R. tibialis</i>
<i>R. flavipes</i>	1.1/1.6	7.2	8.0	8.9
<i>R. hageni</i>	5.7	0.1/2.2	5.8	8.3
<i>R. virginicus</i>	5.5	2.6	0.2/1.0	7.4
<i>R. tibialis</i>	8.3	7.5	9.2	1.3/-

vidual 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, AR) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer (Foster City, CA). GenBank accession numbers for the termites subjected to DNA sequencing in this study and DNA sequences obtained from GenBank are presented in Table 1.

Restriction sites were predicted from the DNA sequence data using Web Cutter 2.0 (Heiman 1997). Amplified DNA was digested according to manufacturer's (Promega, Madison, WI) recommendations following Cherry et al. (1997) using the restriction enzymes *Hinf*I and *Taq*I for the COII gene and *Dra*I, and *Tsp*R I for the 16S rRNA gene. Fragments were separated by either 2% agarose or 8% polyacrylamide gel electrophoresis per Taylor et al. (1996). Gels were stained with ethidium bromide then photographed using a UVP BioDoc-it documentation system (Upland, CA).

**Results**

The mtDNA COII amplicon was 379 bp long for all of the *Reticulitermes* studied, and the 16S amplicon ranged from 425 to 429 bp. PCR was conducted on 2–8 individuals from each population and one individual from each population was sequenced (Table 1). Maximum DNA sequence variation within each species and average DNA sequence variation among the four *Reticulitermes* species is presented in Table 2. Analysis of the DNA sequencing data with Webcutter 2.0 software revealed that no single restriction enzyme was able to completely separate all of the *Reticulitermes* species. However, combinations of different enzyme digestion results could aid in species identification. Examining the COII marker first, the *Hinf*I and *Taq*I pair was able to differentiate all of the species studied (Table 3). No variation was observed within species for these two restriction enzymes. Viewing the 16S marker, two restriction enzymes, *Dra*I and *Tsp*RI, were required to differentiate all four species (Table 4).

We can estimate the possibility (*P*) of intraspecific polymorphism of PCR-RFLP analysis using the formula:  $P = (\gamma/N) \times [(N/4^n) \times n] = \gamma \times n/4^n$ , where  $\gamma$  is the average intraspecific diversity, *N* is the nucleotide length of the gene, and *n* is the number of recognition sequence sites of the restriction enzyme (Lin et al. 2002). The average intraspecific substitution of the 379-bp region of the COII gene is 6.0 nucleotides for *R. flavipes*, 8.3 nucleotides for *R. hag-*

**Table 3.** Restriction fragment length polymorphisms from the COII gene

Restriction enzyme	Species	Restriction site	Fragment(s)	Pattern
<i>Hinf</i> I (G/ANTC)	<i>R. flavipes</i>	97, 294	197, 97, 85	A
	<i>R. hageni</i>	184, 294	184, 110, 85	B
	<i>R. tibialis</i>	97, 294	197, 97, 85	A
	<i>R. virginicus</i>	294	294, 85	C
<i>Taq</i> I (T/CGA)	<i>R. flavipes</i>	292	292, 87	A
	<i>R. hageni</i>	–	379	B
	<i>R. tibialis</i>	–	379	B
	<i>R. virginicus</i>	–	379	B

*eni*, and 3.8 nucleotides for *R. virginicus*. *Hinf*I is a 5-bp recognition enzyme and *Taq*I is a 4-bp recognition enzyme. *P* for *Taq*I was thus calculated to be 0.094 for *R. flavipes*, 0.130 for *R. hageni*, and 0.059 for *R. virginicus* for the COII marker. Based on the 16S RNA sequences in this study, the average intraspecific substitution rate of this gene is 4.6 nucleotides for *R. flavipes*, 0.4 nucleotides for *R. hageni*, 0.8 nucleotides for *R. virginicus*, and 5.4 nucleotides for *R. tibialis*. For the 16S marker, *Dra*I is a 6-bp recognition enzyme, and *Tsp*R I has a 5-bp recognition site. Thus, the possibility of an intraspecific polymorphism, *P*, for *Tsp*R I is 0.022 for *R. flavipes*, 0.002 for *R. hageni*, 0.004 for *R. virginicus*, and 0.026 for *R. tibialis*. Applying these rates to our PCR-RFLP diagnostic technique, we were able to correctly classify 97 unknown samples collected from four south central states, Missouri, and Virginia to *R. flavipes*, *R. virginicus*, *R. tibialis*, or *R. hageni* (Table 5). The PCR amplicon from several of the unclassified populations occurring in Arkansas, Louisiana, Missouri, Oklahoma, and Texas were subjected to DNA sequencing, and confirmed the species identification achieved with PCR-RFLP.

**Discussion**

*Reticulitermes* species can be readily differentiated with *Hinf*I and *Taq*I digests of the 379-bp amplicon of the mtDNA COII gene, and *Dra*I, and *Tsp*RI digests of the 428-bp 16S amplicon. Although both markers require only two restriction enzymes to differentiate all four *Reticulitermes* species, the lower amount of intraspecific variation in the 16S region makes it a more reliable diagnostic marker relative to the COII region. Additionally, based on our evaluation of this tech-

**Table 4.** Restriction fragment length polymorphism from the 16S gene

Restriction enzyme	Species	Restriction site	Fragment(s)	Pattern
<i>Dra</i> I (TTTAAA)	<i>R. flavipes</i>	–	428	A
	<i>R. hageni</i>	–	425	A
	<i>R. tibialis</i>	–	425	A
	<i>R. virginicus</i>	254	254, 172	B
<i>Tsp</i> RI (CACTG)	<i>R. flavipes</i>	375	375, 53	A
	<i>R. hageni</i>	143	283, 143	B
	<i>R. tibialis</i>	141, 372	231, 141, 53	C
	<i>R. virginicus</i>	142	284, 142	B

**Table 5. Identification of unclassified *Reticulitermes* samples**

Identification	Collection site	N Colonies	Pattern COII <sup>a</sup>	Pattern 16S <sup>a</sup>
<i>R. flavipes</i>	Fayetteville, AR	3	AA	AA
	Chauvin, LA	1	AA	AA
	Houma, LA	1	AA	AA
	Beaver Co., OK	1	AA	AA
	Greer Co., OK	2	AA	AA
	McCurtain Co., OK	2	AA	AA
	Norman, OK	1	AA	AA
	Oklahoma City, OK	2	AA	AA
	Payne Co., OK	1	AA	AA
	Spiro, OK	1	AA	AA
	Stillwater, OK	2	AA	AA
	Wagoner Co., OK	1	AA	AA
	Addison, TX	1	AA	AA
	Arlington, TX	2	AA	AA
	Austin, TX	1	AA	AA
	Balch Springs, TX	1	AA	AA
	Beaumont, TX	1	AA	AA
	Beeville, TX	1	AA	AA
	Blanco, TX	1	AA	AA
	Bryan, TX	1	AA	AA
	Carrollton, TX	2	AA	AA
	College St., TX	1	AA	AA
	Combine, TX	2	AA	AA
	Corpus Christi, TX	2	AA	AA
	Dallas, TX	18	AA	AA
	DeSoto, TX	1	AA	AA
	El Paso, TX	2	AA	AA
	Ft. Worth, TX	3	AA	AA
	Hempstead, TX	1	AA	AA
	Irving, TX	2	AA	AA
	Mansfield, TX	2	AA	AA
	Milano, TX	2	AA	AA
	Odessa, TX	1	AA	AA
Post, TX	1	AA	AA	
Red Water, TX	1	AA	AA	
Rowlette, TX	2	AA	AA	
Stephenville, TX	2	AA	AA	
Taylor, TX	1	AA	AA	
The Woodlands, TX	2	AA	AA	
Suffolk, VA	2	AA	AA	
<i>R. virginicus</i>	Fayetteville, AR	8	CB	BB
	McGehee, AR	2	CB	BB
	Columbia, MO	1	CB	BB
	Oklahoma City, OK	1	CB	BB
	Bryan, TX	1	CB	BB
<i>R. hageni</i>	Roanoke, VA	2	CB	BB
	Conway, AR	1	BB	AB
<i>R. tibialis</i>	Fayetteville, AR	2	BB	AB
	Ft. Worth, TX	1	AB	AC
	El Paso, TX	1	AB	AC

<sup>a</sup> Restriction enzyme pattern in the order *Hinfl*, *Taq I* for COII and *Dra I*, *TspR I* for 16S (see Tables 2 and 3).

nique using unclassified field-collected *Reticulitermes*, the method is more reliable than predictions based on putative mutation rates. As a diagnostic tool, this process could eliminate much of the ambiguity involved in morphological identification of species within the genus *Reticulitermes* or with other taxa within the order Isoptera. The PCR-RFLP molecular diagnostic method presented herein can be used with workers, soldiers, or alate specimens. The ability to identify specimens to species regardless of caste is a great advantage over traditional morphological methods in which keys are designed primarily for soldiers and alates. Although the control tactic used for most *Reticulitermes* infestations is unlikely to vary between

species, correct identification of the species involved can assist professional pest managers with understanding of why their respective control efforts might have failed. Numerous tactics used by professional pest managers employ the biology and ecological demands of various termite species (e.g., baiting regimes for colony suppression or elimination), which might be jeopardized because of subtle differences that can occur between the various species and subspecies of *Reticulitermes*.

Molecular diagnostics will aid in the identification of future introductions of *Reticulitermes* species to the United States or in ongoing genetic studies that demand correct identification to ascertain what population the species in question may have originated from. Molecular diagnostics is also more cost effective than cuticular hydrocarbon examinations, which require more costly chromatographic techniques and materials, generally more termites per location, and may have chemical polymorphisms that vary among castes. Additionally, based on DNA sequence data (Austin et al. 2002), the 379-bp marker used in this study could be used to identify *Reticulitermes lucifugus* and *Reticulitermes grassei* from Europe and Asia. European introductions of *R. flavipes* from the United States have been documented to have occurred in Germany (Becker 1970) and Austria (Hrdy 1961). More recently, introductions of damaging exotic *Reticulitermes* spp. are known to occur in South America, including: *R. lucifugus* in Uruguay (Aber and Fontes 1993) and *R. flavipes* in Santiago and Valparaiso, Chile (Clément et al. 2001) where they have established themselves as serious structural pest of wooden timbers. In Europe, discoveries of *R. grassei* in southwestern England have prompted new studies to assist in understanding the risks associated with their occurrence and their associated threat to structural timbers in the United Kingdom. The anthropogenic introductions of hardy *Reticulitermes* species (existing beyond what would be considered their "normal" habitation range) are likely to continue. Clearly, problems with the classification of synonymous species are another area in which having the alternative viewpoint of this technique is not only valid, but necessary. This has been demonstrated when looking at the synonymy of the *R. lucifugus* complex in Europe and the Middle East (Austin et al. 2002), the *R. hesperus* complex in California and the southwest United States (Haverty and Nelson 1997, Haverty et al. 1999), and the various complexes of *R. tibialis* in the southwest United States (Myles 2000). Quick and accurate identification of these species will be a daunting task for inexperienced professional pest managers operating in areas in which previously undescribed species are now occurring. Molecular diagnostics is just one way that we might gain insight as to the origins of newly introduced species so that intervention may be directed in the most economically effective manner (whether identifying the source of introduction, discriminating species for the application of corrective treatment measures, or for cataloging species with the intent to correctly classify species that will likely be discovered

later). It also supports the possibility of classifying samples currently in museums that have not yet been resolved. For example, within the insect collection at Texas A&M University, College Station, TX (<http://entowww.tamu.edu/new/research/systematics/collection.html>), there are presently 227 *Reticulitermes* samples, of which only 96 have been classified to species (85% *R. flavipes*, 7% *R. virginicus*, 7% *R. hageni*, and 1% *R. tibialis*). Fifty-eight percent, representing 131 vials, have not yet been classified to species.

The PCR-RFLP method presented herein was developed to provide researchers and professional pest managers with a rapid and simple molecular method for accurately differentiating four species of *Reticulitermes* encountered in the south central United States, and could be used to identify *Reticulitermes* species that may be introduced to the United States. Field-collected samples can be preserved in ethanol at the collection site and stored for several years until identification is required. The PCR amplification is robust, and the use of a small amplicon, <450 bp, facilitates the use of specimens that have not been optimally preserved (Taylor et al. 1996). The restriction enzymes used in this study are economical, costing less than \$12.50 per 1,000 U, and yield fragments that can be readily visualized under less-than-optimal PCR and electrophoretic conditions. The entire set of procedures, from DNA extraction to final identification, can be completed within a single working day.

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