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MITOCHONDRIAL DNA VARIATION AND DISTRIBUTION OF THE SUBTERRANEAN TERMITE GENUS *RETICULITERMES* (ISOPTERA: RHINOTERMITIDAE) IN ARKANSAS AND LOUISIANA

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ABSTRACT

Limited information exists on genetic variation and distribution of *Reticulitermes* from the south central United States. Focusing on molecular sequence data from the mitochondrial DNA 16S gene, this study records the distribution and genetic variation of *Reticulitermes* species in Arkansas and updates the current distribution in a neighboring State, Louisiana. Termite samples were collected from the field, subjected to DNA analysis with Polymerase Chain-Reaction (PCR), and sequenced. *Reticulitermes* sp. sequence data were aligned, genetic distances recorded, and their respective haplotypes were evaluated for possible geographic structure. From 35 Arkansas counties, 59 *R. flavipes*, 13 *R. hageni*, and seven *R. virginicus* were identified. In Arkansas, 11 mitochondrial haplotypes were observed in *R. flavipes*, three in *R. hageni* and three in *R. virginicus*. Among the 12 Louisiana parishes sampled, 13 *R. flavipes*, three *R. virginicus*, and one *R. tibialis* were identified with six, three, and one haplotypes for each species, respectively. Genetic variation among the *R. flavipes* haplotypes from both States ranged from 0.2 to 0.9%. *Reticulitermes flavipes* haplotype diversity observed in Arkansas and Louisiana was lower than observed in Texas and Oklahoma.

Key Words: 16S rRNA gene, DNA sequence, genetic variation, population genetics, *Reticulitermes*, termite.

RESUMEN

La información existente sobre la variación genética y la distribución de *Reticulitermes* en el sur central del los Estados Unidos es limitada. Enfocandose en los datos de las secuencias moleculares del gene 16S del ADN mitocondrial, este estudio registra la distribución y la variación genética de *Reticulitermes* spp. en Arkansas y pone al dia la distribución actual en el estado vecino, Louisiana. Las muestras de termitas recolectadas del campo, fueron sujetas al análisis de ADN por la reacción en cadena de la polímerasa (RCP), y secuenciadas. Los datos de secuenciación genética para *Reticulitermes* spp. fueron alineados, las distancias genéticas registradas, y sus haplotipos respectivos fueron evaluados para su posible estructura geográfica. De los 35 condados de Arkansas, 59 *R. flavipes*, 13 *R. hageni*, y siete *R. virginicus* fueron identificados. En Arkansas, 11 haplotipos de mitocondria fueron observados en *R. flavipes,* tres en *R. hageni* y tres en *R. virginicus.* Entre las 12 regiones de Louisiana muestreadas, 13 *R. flavipes*, tres *R. virginicus*, y una *R. tibialis* fueron identificados con seis, tres, y un haplotipos por cada especie, respectivamente. La variación genética entre los haplotipos de *R. flavipes* de ambos estados fue de 0.2 hasta 0.9%. La diversidad de los haplotipos de *Reticulitermes flavipes* observada en Arkansas y Louisiana fue menor de la que fue observada en Texas y Oklahoma.

In Arkansas and Louisiana, subterranean termites cause millions of dollars of damage annually. Damage caused by subterranean termite activity probably exceeds $2.5 billion annually in the United States (Anonymous 2003) and $22 billion globally (Su 2002). Several structurally important species in the genus *Reticulitermes* are common throughout the southeastern U.S. (Weesner 1965). Messenger et al. (2002) completed a comprehensive survey of termites in Louisiana, providing information for the likely occurrences of *Reticulitermes* spp. in Arkansas. Previously, Snyder (1954) listed four species in Arkansas: *Reticulitermes flavipes* (Kollar), *R. virginicus* Banks, *R. hageni* Banks, and *R. tibialis* Banks. All four species have been documented in Louisiana, including *R. tibialis*, which was recently discovered (Messenger 2003). Information on the distribution of *Reticulitermes* spp. in Arkansas has become available through a current national termite survey, which confirms that *Reticulitermes* spp. are common throughout the State (Messenger 2003). Moreover, surveys from the neighboring States of Texas and Oklahoma have included valuable information on *Reticulitermes* distribution and genetic composition (Austin et al. 2004a, b).

Previous genetic studies have primarily focused on phylogenetic relationships among *Reti-*

culitermes species from the eastern United States and Western Europe (Jenkins et al. 1998, 2001; Marini & Mantovani 2002; Uva et al. 2003; Ye et al. 2003). Recently, Austin et al. (2004a, b) have conducted the first comprehensive genetic surveys of *Reticulitermes* sp. for Texas and Oklahoma using DNA sequencing of a portion of the mitochondrial DNA (mtDNA) 16S rRNA gene. We investigated the extent of genetic variation within and among Arkansas and Louisiana *Reticulitermes* relative to Texas and Oklahoma (Austin et al. 2004[ab]), evaluated these genetic markers for identifying species, and updated the geographical distribution of these taxa.

Materials and Methods

Termites were collected in Arkansas and Louisiana and preserved in 100% ethanol (Table 1). We solicited the assistance of Pest Management Professionals (PMPs) throughout both States to determine which species are most frequently recovered from infested structures. We provided collection kits, and PMPs returned samples to our laboratory for analysis. From various geographic zones throughout Arkansas and Louisiana (Table 1), 96 samples were used for molecular analysis.

When alates or soldiers were available, *Reticulitermes* sp. were morphologically identified to species by applying the keys of Krishna & Weesner (1969); Scheffrahn & Su (1994); Hostettler et al. (1995); and Donovan et al. (2000). In addition, all samples were identified to species with mtDNA 16S sequences (Szalanski et al. 2003). All of the morphological species identifications agreed with the molecular species identifications. Three additional taxa (Table 1) were included as outgroup taxa to corroborate relationships within the genus for phylogenetic analysis. Voucher specimens preserved in 100% ethanol are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

DNA was extracted from alcohol-preserved specimens dried on filter paper according to Liu & Beckenbach (1992) and Jenkins et al. (1999) from individual worker termites with 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 with the primers LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati & Smith 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al., 1994). These PCR primers amplify an approximately 428-bp region of the mtDNA 16S rRNA gene. The PCR reactions were conducted with 1 µl of the extracted DNA (Szalanski et al. 2000), having 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 with minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega). Samples were sent to The University of Arkansas Medical School DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions. GenBank accession numbers were AY603499 to AY603509 for termite DNA sequence haplotypes new to this study. Consensus sequences for each sample were obtained with Bioedit 5.09 (Hall 1999), and sequences were aligned by CLUSTAL W (Thompson et al. 1994). Mitochondrial DNA haplotypes were aligned by MacClade v4 (Sinauer Associates, Sunderland, MA). Haplotype distribution between populations, number of haplotypes, number of unique haplotypes, haplotype diversity (h), and nucleotide diversity (pi) were calculated with DNAsp v3.51 (Rozas & Rozas 1999).

The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980). Mitochondrial 16S sequences from the desert subterranean termite *Heterotermes aureus* (Snyder) (GenBank AY380299) and Formosan subterranean termite *Coptotermes formosanus* Shiraki (GenBank AY558910) were added to the *Reticulitermes* DNA sequences as outgroup taxa. Maximum parsimony analysis on the alignments were conducted with PAUP* 4.0b10 (Swofford 2001). 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 and used the Branch and Bound algorithm of PAUP*. Because no previous accounts of the abundance of *Reticulitermes* in Arkansas have been published, we compiled all available data from existing sources and noted them on our distribution map (Fig. 1).

Results

The DNA sequencing of the 16S rDNA amplicon revealed an average size of 428 bp. The average base frequencies were A = 0.41, C = 0.23, G = 0.13, and T = 0.23. From 35 Arkansas counties 59 *R. flavipes*, 11 *R. hageni*, and seven *R. virginicus* were identified based on species diagnostic nucleotide sites from Szalanski et al. (2003) (Table 1). In Arkansas, 11 haplotypes were observed in *R. flavipes*, three in *R. hageni* and three in *R. virginicus* (Table 1, Fig. 1). Among the 12 Louisiana parishes sampled, 13 *R. flavipes*, three *R. virginicus*, and one *R. tibalis* were identified with six, three, and one haplotypes for each species, respectively (Fig. 2, Table 1).

Nine nucleotide sites were variable among the 11 *R. flavipes* haplotypes (Table 2), and Tajima-Nei distances (Tajima & Nei 1984) among the *R. flavipes* haplotypes ranged from 0.2 to 0.9% (Table 3). The most common haplotypes were F and G with 32 and 9 representatives, respectively.

TABLE 1. COLLECTION DATA, AND HAPLOTYPES FOR ARKANSAS AND LOUISIANA *Reticulitermes* AND OUTGROUP TAXA.

Species	City	County/Parish	State	Haplotype	n
R. flavipes	Amity	Clark	AR	F	1
	Brinkley	Monroe	AR	F	1
	Cave City	Sharp	AR	F	1
	Clifty	Madison	AR	F	1
		Polk	AR	F	1
	Cushman	Independence	AR	F	1
	Strickler	Washington	AR	F	1
	Fayetteville	Washington	AR	F	2
	El Dorado	Union	AR	F	1
	Eureka Springs	Carroll	AR	F	1
	Ft. Smith	Sebastian	AR	F	1
	Harrison	Boone	AR	F	1
	Jasper	Newton	AR	F	1
	Marvell	Phillips	AR	F	1
	McGehee	Desha	AR	F	1
	Mineral Springs	Howard	AR	F	1
	Nashville	Howard	AR	F	1
	Paragould	Greene	AR	F	1
	Stuttgart	Arkansas	AR	F	1
	Warm Springs	Randolph	AR	F	1
	Hardin	Jefferson	AR	F	1
	Hoxie	Lawrence	AR	F	1
	Walnut Ridge	Lawrence	AR	F	1
	Newport	Jackson	AR	F	1
	Hot Springs	Garland	AR	F	1
	Baton Rouge	E. Baton Rouge	LA	F	2
	Houma	Terrebonne	LA	F	1
	Cut Off	Lafourche	LA	F	1
	Delhi	Richland	LA	F	1
	Port Sulphur	Plaquemines	LA	F	1
	West Monroe	Ouachita	LA	J	1
	Ashdown	Little River	AR	M	1
	Ft. Smith	Sebastian	AR	M	1
	Warren	Bradley	AR	M	1
	Fayetteville	Washington	AR	M	1
	Pine Bluff	Jefferson	AR	M	1
	Shreveport	Caddo	LA	M	1
	Sulphur	Calcasieu	LA	M	1
	Camp Robinson	Pulaski	AR	G	1
	Glenwood	Pike	AR	G	1
	Jonesboro	Craighead	AR	G	1
	N. Little Rock	Pulaski	AR	G	1
	Newport	Jackson	AR	G	1
	Piggot	Clay	AR	G	1
	Morrilton	Conway	AR	G	1
	Shreveport	Caddo	LA	G	1
	Jonesboro	Jackson	LA	G	1
	Fayetteville	Washington	AR	Q	1
	Harrison	Boone	AR	Q	1
	Hoxie	Lawrence	AR	Q	1
	Newport	Jackson	AR	Q	1
	Walnut Ridge	Lawrence	AR	Q	1
	Fayetteville	Washington	AR	T	1
	Hoxie	Lawrence	AR	W	1
	Jonesboro	Craighead	AR	P	1
	Lake Charles	Calcasieu	LA	P	1
	Lake Dardanelle	Pope	AR	R	1

TABLE 1. (CONTINUED) COLLECTION DATA, AND HAPLOTYPES FOR ARKANSAS AND LOUISIANA *Reticulitermes* AND OUT-

Species	City	County/Parish	State	Haplotype	n
	Jonesboro	Craighead	AR	R	1
	Strickler	Washington	AR	R	1
	Fayetteville	Washington	AR	R	1
		Madison	AR	R	1
	Searcy	White	AR	R	1
		Faulkner	AR	S	1
	Chauvin	Terrebonne	LA	S	1
	Little Rock	Pulaski	AR	V	1
	Pocahontas	Randolph	AR	V	2
	Sheridan	Grant	AR	V	1
	Sherwood	Pulaski	AR	V	1
	Ft. Smith	Sebastian	AR	U	1
R. hageni	Eureka Springs	Carroll	AR	H1	1
	Fayetteville	Washington	AR	H1	1
	Weddington	Washington	AR	H1	3
	Pocahontas	Randolph	AR	H1	1
	Conway	Faulkner	AR	H1	1
		Polk	AR	H1	1
	Clifty	Madison	AR	H1	1
	Fayetteville	Washington	AR	H2	1
	Marmaduke	Greene	AR	H2	1
	Strickler	Washington	AR	H3	1
	Hamburg	Ashley	AR	H3	1
R. virginicus	Fayetteville	Washington	AR	V1	4
	Fayetteville	Washington	AR	V2	1
	Fayetteville	Washington	AR	V3	1
	Morgan Mtn.	Franklin	AR	V1	1
	Minden	Webster	LA	V1	1
	Raceland	Lafourche	LA	V4	1
	Delcambre	Vermilion	LA	V5	1
R. tibialis	Sulphur	Calcasieu	LA	T8	1
Coptotermes formosanus	Rockwall	Lamar	TX	outgroup	
Heterotermes aureus			AZ	outgroup	

Within *R. hageni*, one nucleotide site was variable between the two observed haplotypes. Haplotype diversity for *R. flavipes* from Arkansas was 0.759, and 0.782 for Louisiana (Table 4). Both States had high levels of genetic diversity. Tajima's test resulted in non-significant P values ($P < 0.05$) in both States leading to the acceptance of the null-hypothesis of neutrality for the mtDNA rRNA 16S gene (Table 4).

Bootstrap analysis of the aligned *Reticulitermes* species and the outgroup taxa resulted in a consensus tree with several distinct branches (Fig. 3). These distinct clades included *R. flavipes*, *R. tibialis*, *R. hageni*, and *R. virginicus*. No genetic relationship was observed among *R. flavipes* haplotypes.

DISCUSSION

This study represents the first attempt to update the current geographic distribution of *Reticulitermes* spp. and genetically categorize the genus *Reticulitermes* in Arkansas. At the same time, this is the first molecular description of *Reticulitermes* spp. from Louisiana, albeit on a limited scale.

Populations of nearly all species, social or otherwise, exhibit at least some degree of genetic differentiation among geographic locales (Ehrlich & Raven 1969). One of the purposes of the research presented herein was to estimate the baseline genetic variation which occurs both within and among *Reticulitermes* spp. in Arkansas and Louisiana. By combining haplotype observations in the present study with the studies of Austin et al. (2004a) from Texas and Austin et al. (2004b) from Oklahoma, we had hoped to observe some type of spatial continuity which may not have been revealed otherwise. As with animal populations, additional genetic structure normally is to be expected over increasing spatial scales, where populations can show additional differentiation due to spatial habitat structure, isolation by distance, or other factors (Avise 1994). There was no

Fig. 1. Species distribution of *Reticulitermes* haplotypes in Arkansas. Samples obtained from the National Termite Survey, but not subjected to genetic analysis are marked as "◆" *R. flavipes*, "●" *R. virginicus*, "■" *R. hageni*. Letters indicating haplotypes are given in Table 1.

Fig. 2. Species distribution of *Reticulitermes* haplotypes in Louisiana. Letters indicating haplotypes are given in Table 1.

apparent consistency of haplotype occurrence for *Reticulitermes* spp. in this study based on geography. However, we found genetic divergence values similar to those detected in our previous works (Austin et al. 2004a, b).

Of the four species of *Reticulitermes* presented in this study, *R. flavipes* was the most common, followed by *R. hageni* and *R. virginicus*. Haplotype F was the most common haplotype of *R. flavipes* observed in Arkansas, and represented 26 of the 79 (44%) samples from Arkansas. This haplotype is also present in both Texas and Oklahoma but in smaller distributions: 12 and 13% for Oklahoma and Texas, respectively. In Oklahoma and Texas, the most abundant *R. flavipes* haplotypes are L (23%) and G (28%), respectively (Austin et al. 2004a, b).

A haplotype or allele is defined by one unique form of the gene and differs from any other haplotype by at least one nucleotide. Haplotype diversity or gene diversity quantifies the number of haplotypes in relation to their relative frequency to each other, and is the probability that two sequences randomly selected from a population are different (Nei 1987). Haplotype diversity for *R. flavipes* from Arkansas and Louisiana was lower than Texas or Oklahoma (Austin et al. 2004a, b).

Tajima's D (Tajima 1989) is a standard test for the neutrality of a gene region. This is a useful measure because nucleotide diversity (Pi) calculations are dependant upon the infinite alleles model that assumes gene neutrality. Values of D indicate not only if natural selection is influencing gene frequencies but the type of selection pressure in operation. Negative values of D can indicate re-

TABLE 2. HAPLOTYPE VARIATION AT 9 NUCLEOTIDE SITES AMONG *Reticulitermes flavipes* FROM ARKANSAS AND LOUISIANA.

Haplotype	55	97	122	131	162	168	179	270	271
F	G	A	A	A	G	G	C	T	T
G	.	.	T	G	.	A	.	.	C
J	.	.	.	G	.	A	.	.	.
M	.	.	.	.	.	A	.	.	.
P	.	.	.	.	.	A	.	.	C
Q	.	.	.	.	.	.	T	.	.
R	.	.	.	.	.	A	.	.	.
S	.	.	.	.	A	A	.	.	.
T	.	.	.	.	.	A	.	C	C
U	.	.	.	.	.	.	.	C	.
V	.	.	.	.	A	.	.	.	.

TABLE 3. GENETIC DIVERGENCE AMONG RETICULITERMES FLAVIPES HAPLOTYPES (HAP) FROM ARKANSAS AND LOUISIANA.

Hap	G	T	P	U	V	F	Q	R	S	M	J
G	—										
T	0.009	—									
P	0.007	0.002	—								
U	0.009	0.005	0.007	—							
V	0.009	0.009	0.007	0.005	—						
F	0.007	0.007	0.005	0.002	0.002	—					
Q	0.009	0.009	0.007	0.005	0.005	0.002	—				
R	0.007	0.007	0.005	0.007	0.007	0.005	0.007	—			
S	0.007	0.009	0.007	0.009	0.005	0.007	0.009	0.002	—		
M	0.005	0.005	0.002	0.005	0.002	0.005	0.002	0.005	—		
J	0.002	0.007	0.005	0.007	0.007	0.005	0.007	0.005	0.007	0.002	—

cent expansions in population size while positive values indicate recent population bottlenecks. Tajima's D value was negative for both Arkansas and Louisiana, indicating that *R. flavipes* in these States may be expanding in population size. A similar result was observed from *R. flavipes* from Oklahoma, while Texas *R. flavipes* have a positive D value, indicating a recent population bottleneck. The null hypothesis of this test is that the gene region of interest is neutral. If the D value is significant ($P < 0.05$) then the null hypothesis of neutrality may be rejected. Tajima's test resulted in non-significant p values ($P > 0.05$) for Arkansas and Louisiana. This was also observed in Texas and Oklahoma (Austin et al. 2004a, b) leading to the acceptance of the null-hypothesis of neutrality for the mtDNA rRNA 16S gene.

The phylogenetic relationships of *Reticulitermes* evaluated in this study are consistent with those from neighboring states (Austin et al. 2004a, b) and in other areas (Austin et al. 2002; Jenkins 1998, 1999, 2001). Extensive collecting in Louisiana has produced only one sample of *R. tibialis* from Sulphur, Calcasieu Parish, which borders Texas. Although Snyder (1954) lists *R. tibialis* as occurring in Arkansas, we have not yet recovered this species to date.

We speculate that because *R. tibialis* was generally not recovered in our collecting efforts, an eastern transition zone (from east to west) for the distribution of this species may exist in the proximity of Calcasieu Parish, Louisiana**.** This species is known to prefer more arid climates which can be more readily found in Texas and Oklahoma where *R. tibialis* has been more frequently recovered. More intensive collecting efforts should be performed to validate this hypothesis.

The lack of a geographical haplotype continuity of *Reticulitermes* suggests that (1) we require samples from larger geographic zones, (2) we need to increase the number of samples sequenced, or (3) the observed lack of spatial continuity from *Reticulitermes* may be attributed to anthropogenic origins. To evaluate the latter element, evaluation of haplotype frequency from undisturbed habitats (e.g., protected forests) should be compared with our current data, which largely reflects samples obtained from urban landscapes. Selected sites in undisturbed locations are being evaluated for intensive collecting efforts in future studies where more comprehensive statistical measures can be applied, and a better overall understanding of population dynamics may be addressed than in the current study.

TABLE 4. GENETIC DIVERSITY PARAMETERS FOR EACH POPULATION OF *RETICULITERMES FLAVIPES* FROM TEXAS, OKLAHOMA, ARKANSAS, AND LOUISIANA.

Population	n	No. haplotypes	No. unique haplotypes	Haplotype diversity (h)	Nucleotide diversity (Pi)	Tajima's[a] D value (*P*-value)
TX[b]	69	13	3	0.864	0.0071	+0.6021 ns
OK[c]	41	10	1	0.811	0.0048	-0.6349 ns
AR	59	11	3	0.759	0.0044	-0.3488 ns
LA	13	6	0	0.782	0.0044	-0.0688 ns

[a]A non-significant value for this test indicates that the null hypothesis of neutrality in the 16S rRNA gene cannot be rejected. Non-significance of test when $P > 0.10$.

[b,c]Data from Austin et al. (2004a, b).

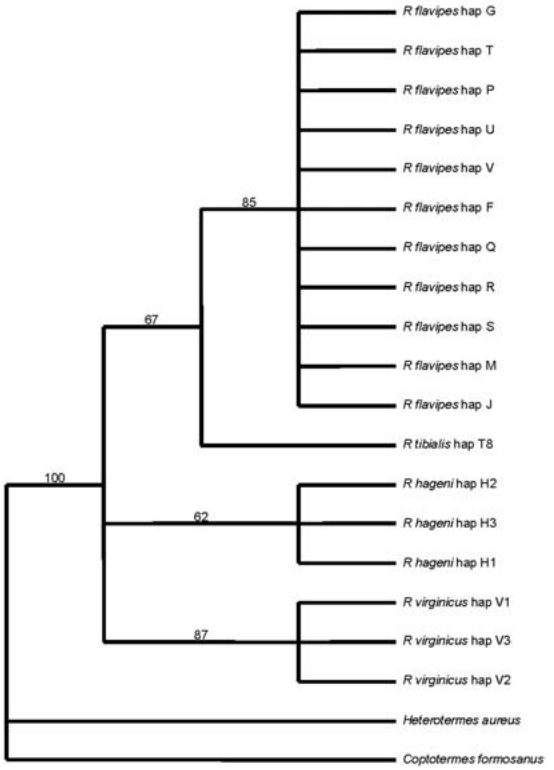

Fig. 3. Single most parsimonious tree based on the 16S rRNA gene during a branch and bound search with PAUP*. Bootstrap values for 1,000 replicates are listed above the branches supported at ≥50%.

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