

Molecular Phylogeography of *Reticulitermes* (Isoptera: Rhinotermitidae) Termites from Florida

by

Allen L. Szalanski¹, James W. Austin², Jackie A. McKern¹, Rudolf H. Scheffrahn³,
Carrie B. Owens⁴ & Matthew T. Messenger⁵

ABSTRACT

Limited information exists on the extent of *Reticulitermes* genetic variation in Florida. We conducted a molecular genetics study involving DNA sequencing of a portion of the mitochondrial DNA 16S gene to determine the extent of genetic variation within *Reticulitermes* from urban and rural areas in Florida. Forty-six samples from rural areas and 134 samples from urban areas were analyzed. From these samples, 142 (81%) were *R. flavipes*, 37 (18%) were *R. virginicus*, and 1 (1%) were *R. hageni*. A total of 47 haplotypes were observed in *R. flavipes*, with 36 haplotypes (72% of the *R. flavipes* samples) unique to Florida. Twenty six of the *R. flavipes* haplotypes occurred only once and three haplotypes occurred more than three times. There did not appear to be a difference in the occurrence of *Reticulitermes* haplotypes between urban and rural areas. A major finding of this study is the high degree of *R. flavipes* and *R. virginicus* haplotype diversity in Florida. A possible explanation for this is the geological history of Florida.

Keywords: 16S, DNA sequencing, genetic variation, population genetics, termite.

INTRODUCTION

In Florida, subterranean termites cause hundreds of millions of dollars in damage annually. Damage caused by subterranean termite activity probably

¹Department of Entomology, University of Arkansas, Fayetteville, AR USA 72701.

²Center for Urban and Structural Entomology, Department of Entomology, Texas A&M University, College Station, TX USA 77843-2143.

³Fort Lauderdale Research and Education Center, University of Florida, Institute of Food and Agricultural Sciences, 3205 College Ave., Fort Lauderdale, FL UYSA 33314.

⁴City of New Orleans Mosquito and Termite Control Board, 6601 Stars and Stripes Blvd., New Orleans, LA USA 70126.

⁵Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN, USA 46268

*Corresponding author. email: aszalan@uark.edu

exceeds \$2.5 billion annually in the United States (Anonymous 2003), and subterranean termite control accounts for an 80% share, with the majority of this probably due to *R. flavipes* and *R. virginicus*, while infestations of *R. hageni* are less common (Su *et al.* 2001). These three structurally important species in the genus *Reticulitermes* are common throughout Florida (Weesner 1965). Scheffrahn *et al.* (1988) found that 59% of sampled *Reticulitermes* in Florida were *R. flavipes*, 39% were *R. virginicus*, and 1% were *R. hageni*. The only previous genetic study of Florida *Reticulitermes*, was a study of *R. flavipes* mtDNA 16S genetic variation across the United States (493 samples from 39 States) and found 6 haplotypes from 8 Florida samples (Austin *et al.* 2005a). Because morphological variability has been observed in *Reticulitermes* from Florida (Hostettler *et al.* 1995), and molecular methods of identification have proven more reliable for accurate species identification (Szalanski *et al.* 2003), it would be plausible to assume moderate genetic variability among *Reticulitermes* in Florida. The nature of this variability remains unknown.

To evaluate the degree of variability among *Reticulitermes* from Florida, a phylogeographic analysis of Florida *Reticulitermes* applying the 16S mtDNA gene was conducted using samples from both urban and rural areas. Isolation of populations recovered from rural areas is presumed to contribute to greater genetic variability of observed haplotypes.

MATERIALS AND METHODS

Termites were obtained from both urban and rural areas of Florida from the 2002 National Termite Survey and from Dr. Rudolf H. Scheffrahn (Fig. 1, Table 1). When either alates or soldiers were available, *Reticulitermes* spp. were morphologically identified to species by applying the keys of Krishna & Weesner (1969), Scheffrahn & Su (1994), and Hostettler *et al.* (1995). In addition, all samples were identified to species with mtDNA 16S sequences (Szalanski *et al.* 2003). In general, there were no discrepancies between morphological species identifications of alates with molecular species identifications in this study. Morphological identification of soldiers was more difficult and validates the molecular approaches applied (Szalanski *et al.* 2003). 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.

From alcohol-preserved specimens dried on filter paper, the DNA was extracted 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 μ l of Tris:EDTA and stored at -20°C . Polymerase chain reaction (PCR) was conducted with the primers LR-J-13007 (5'-TTACGCTGTTATC-CCTAA-3') (Kambhampati & Smith 1995) and LR-N-13398 (5'-CGC-CTGTTTATCAAAAACAT-3') (Simon *et al.* 1994). These PCR primers amplify an approximately 428 bp region of the mtDNA 16S rRNA gene. The

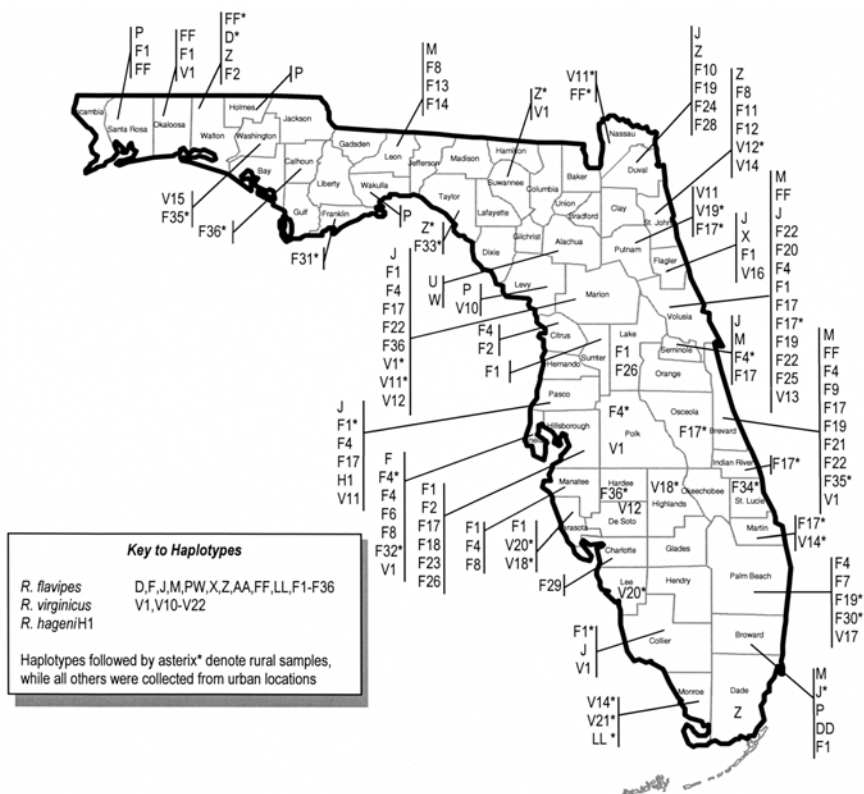


Fig. 1. Species distribution of *Reticulitermes* haplotypes in Florida. Haplotypes D-Z, AA-SS, F1-F36 are *R. flavipes*; V1, V10-V22 are *R. virginicus*; and H1 is *R. hageni*. Samples collected from rural areas are underlined.

PCR reactions were conducted with 1 μ l 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. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999), and mitochondrial DNA haplotypes were determined using MacClade v4 (Sinauer Associates, Sunderland, MA). GenBank accession numbers were EU259734 to EU259782 for termite DNA sequence haplotypes new to this study. The genealogical relationship of *R. flavipes* haplotypes was constructed using TCS (Clement *et al.* 2000). 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).

Reticulitermes haplotypes found in Florida that have been previously described elsewhere (Szalanski *et al.* 2003, Austin *et al.* 2005a) and the *Reticulitermes* haplotypes new to this study were used for the phylogenetic analysis. The *Reticulitermes* sequences were then aligned using Clustal W (Thompson *et al.* 1994) using sequences from the desert subterranean termite *Heterotermes aureus* (Snyder) (GenBank AY380299) and *Coptotermes formosanus* Shiraki (GenBank AY558910) as outgroup taxa. The best-fitting nucleotide substitution model was chosen according to the general time reversible + gamma (GTR+G) model among 64 different models using the ModelTest v 3.7 (Posada and Crandall 1998) and PAUP* 4.0b10 (Swofford 2001) programs. Maximum parsimony (MP) analysis was conducted with the best-fitting evolutionary model as implemented in PAUP*. Bootstrapping was performed using neighbor joining or MP (1000 replicates) to determine the reliability of the obtained topologies. Phylogenetic trees were also obtained using Bayesian inference with the GTR+G model using Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.4.2 software (Drummond & Rambaut 2003). For Bayesian inference, four Markov chains run for 10^6 generations with a burn-in of 2×10^4 were used to reconstruct the consensus tree.

RESULTS AND DISCUSSION

A total of 180 individual termites were analyzed, of these 142 (81%) were *R. flavipes*, 37 (18%) *R. virginicus*, and 1(1%) were *R. hageni*. Forty-eight haplotypes were observed in *R. flavipes*, with 27 occurring only once and 34 of the haplotypes were unique to Florida (Tables 1-2, Fig. 1). The most common *R. flavipes* haplotypes were F4(16), F17(15), J(15), and F1(12). *Reticulitermes flavipes* haplotype M was the common ancestor to all of the observed haplotypes (Fig. 2), and it is a haplotype that is common in the United States (Austin *et al.* 2005a). The most divergent haplotype, F16, is

Table 1. Florida *Reticulitermes* and outgroup taxa collection locations, haplotypes and haplotype frequencies (n).

County (n)	Haplotype(s) (n)	County (n)	Haplotype(s) (n)
Alachula (1)	F27(1)	Martin (2)	F17(1); V14(1)
Brevard (12)	M(1), FF(1), F4(1), F9(1), F17(1), F19(1), F20(1), F21(1), F22(1), F35(1); V1(2)	Miami-Dade (1)	Z(1)
Broward (2)	J(1), M(1)	Monroe (3)	LL(1); V14(1), V21(1)
Charlotte (1)	F29(1)	Nassau (2)	FF(1); V11(1)
Citrus (2)	F4(1), F21(1)	Okaloosa (6)	FF(4), F16(2)
Collier (3)	J(1), F1(1); V1(1)	Orange (7)	J(3), AA(1), F2(1), F4(1), F31(1)
Columbia (3)	V18(2), V19(1)	Osceola (3)	F17(1), F20(1); V1(1)
De Soto (1)	V12(1)	Palm Beach (6)	F4(2), F7(1), F19(1), F30(1); V17(1)
Duval (7)	J(2), Z(1), F10(1), F19(1), F24(1), F28(1)	Pasco (7)	J(1), W(1), F4(1), F17(1); H1(1); V11(1)
Flager (4)	J(1), X(1), F1(1); V16(1)	Pinellas (7)	F4(1), F6(1), F8(2), F32(1); V1(1)
Hardec (1)	F36(1)	Polk (2)	F4(1); V1(1)
Highlands (1)	V18(1)	Putnam (3)	F17(1); V11(1), V19(1)
Hillsborough (10)	F1(2), F2(1), F17(1), F18(1), F23(2), F26(1), V11(2)	Santa Rosa (4)	P(1), FF(1), F16(1), SS(1)
Holmes (1)	P(1)	Sarasota (3)	F1(1); V18(1), V20(1)
Indian River (1)	F17(1)	Seminole (3)	J(1), M(1), F4(1)
Jackson (1)	F36(1)	St. Johns (7)	Z(1), F8(1), F11(1), F12(1), F20(1); V12(1), V14(1)
Lake (2)	F1(1), F26(1)	St. Lucie (1)	F34(1)
Lec (1)	V20(1)	Suwanee (4)	Z(1), V1(1), V15(2)
Leon (7)	P(1), M(2), F8(2), F13(1), F14(1)	Taylor (2)	Z(1), F33(1)
Levy (2)	P(1); V10(1)	Volusia (25)	J(4), M(3), FF(1), F1(4), F3(1), F4(2), F17(1), F19(1), F20(1), F22(1), F25(1); V13(1), V22(1)
Manatee (5)	F1(1), F4(3), F8(1)	Wakulla (2)	P(1), F31(1)
Marion (6)	J(1), F4(1), F17(1), F22(1), F36(1); V12(1)	Walton (4)	D(1), Z(1), FF(1), F2(1)
Sumpter (1)	F17(1)	Washington (2)	F35(1); V15(1)

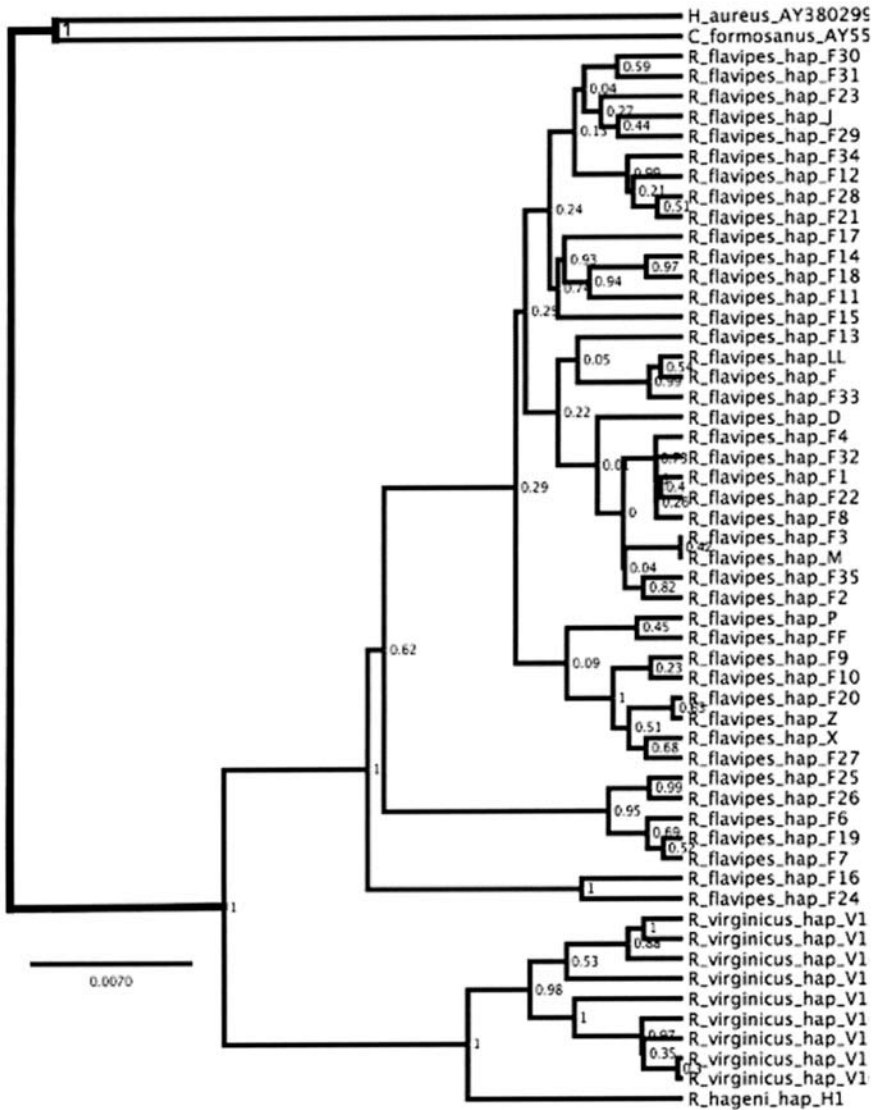


Fig. 2. Genealogical relationships among 48 *R. flavipes* haplotypes estimated by TCS (Clement et al. 2000). A unit branch represents one mutation, a small oval (see arrow) indicates haplotypes that were not observed. Shaded ovals indicate common haplotypes observed among US populations, while open ovals indicate haplotypes unique to Florida.

Table 2. Frequency of *Reticulitermes* haplotypes (Hap) from Florida.

Species	Hap	n	Species	Hap	n	Species	Hap	n
<i>R. flavipes</i>	D	1	"	F11	1	"	F32	1
"	J	15	"	F12	1	"	F33	1
"	M	8	"	F13	1	"	F34	1
"	P	5	"	F14	1	"	F35	2
"	W	1	"	F16	3	"	F36	3
"	X	1	"	F17	13	<i>R. virginicus</i>	V1	6
"	Z	6	"	F18	1	"	V10	1
"	AA	1	"	F19	4	"	V11	5
"	FF	1	"	F20	4	"	V12	3
"	LL	1	"	F21	2	"	V13	1
"	SS	1	"	F22	3	"	V14	3
"	F1	11	"	F23	2	"	V15	3
"	F2	3	"	F24	1	"	V16	1
"	F3	1	"	F25	1	"	V17	1
"	F4	16	"	F26	2	"	V18	4
"	F6	1	"	F27	1	"	V19	2
"	F7	1	"	F28	1	"	V20	2
"	F8	5	"	F29	1	"	V21	1
"	F9	1	"	F30	1	"	V22	1
"	F10	1	"	F31	2	<i>R. hageni</i>	H1	1

found only in the Florida panhandle. In *R. virginicus*, 14 haplotypes were observed; of these, seven haplotypes occurred only once and 12 of the haplotypes were unique to Florida. The most common *R. virginicus* haplotypes were V1(8), V11(6), and V18(4). The only *R. hageni* haplotype observed, H1, is a common haplotype found throughout the species range. The phylogenetic relationship between all three *Reticulitermes* species was identical for both the maximum parsimony and Bayesian analysis (Fig. 3). There did not appear to be a difference in the occurrence, distribution or phylogenetic relationship of urban and rural samples (Table 1, Fig. 3). There were, however, two distinct *R. flavipes* clades or lineages based on Bayesian and TCS analysis (Figs. 2-3). One, consisting of haplotypes F16 and F24 occurs only in northern Florida, the other one, consisting of haplotypes F6, F19, F25, F26, and F27 was only found in central Florida.

A major finding of this study is the high degree of *R. flavipes* and *R. virginicus* haplotype diversity in Florida. A possible explanation for this can be attributed to the geological history of Florida. During the Pleistocene Epoch (1.8 million to 10,000 years ago) seas alternately flooded and retreated from Florida, resulting in isolated 'island' land formations (Lane 1994). This his-

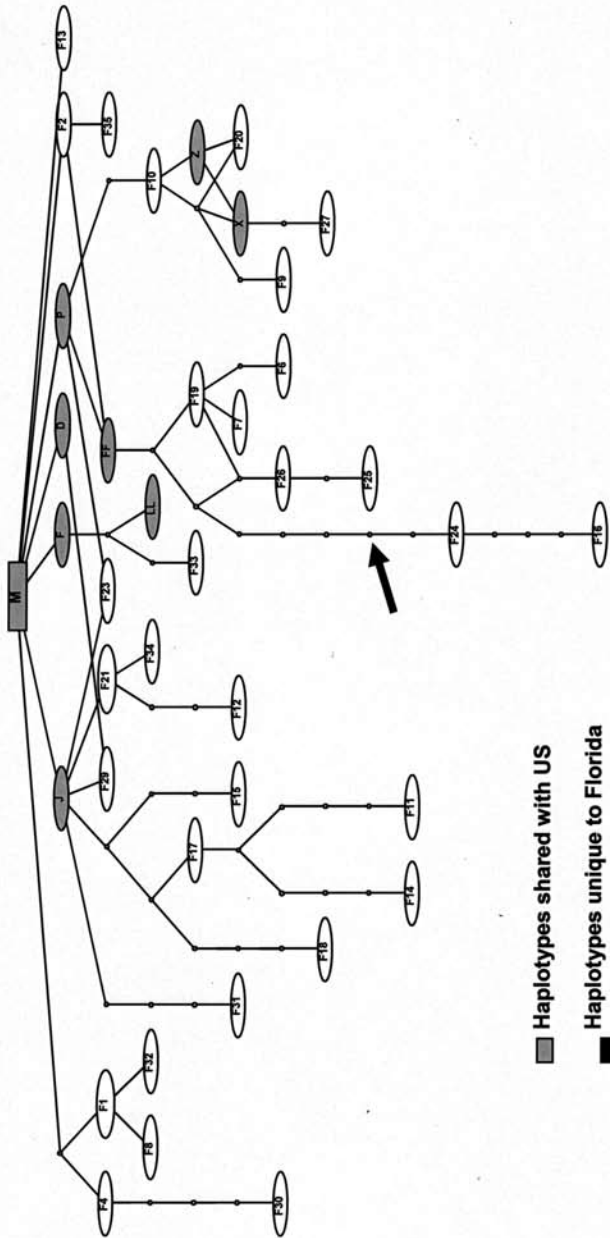


Fig. 3. Bayesian tree for the mtDNA 16S dataset. Posterior bootstrap values are provided for each node.

tory of flooding is well documented (Faught & Carter 1998, Froede 2002, Cunningham *et al.* 2003, Portell *et al.* 2003), and a general pattern of animals expanding their range from their refuges with the end of the last glaciation period is likely. The historical isolation of different populations of identical species could have created distinct strains (Ribera & Vogler 2004) with their own history and genetic compositions. No apparent relationship exists between *R. flavipes* and *R. virginicus* haplotypes and geographical distribution on the Florida peninsula, but there does appear to be a relationship in the Florida panhandle based on known inundation patterns exhibited by other terrestrial organisms (Alt & Brooks 1965, Flint 1971, Caine 1974). Multiple haplotypes may be more related to adaptations to coastal rise versus interior habitats, competition, and human transportation.

This suggests that a lack of gene flow between termite populations isolated for thousands of years could result in a large amount of haplotype diversity, without necessarily constituting speciation events. Why these haplotypes are not found outside of Florida remains unknown, as comprehensive genetic evaluations across the entire known range for *R. flavipes* have been conducted (Austin *et al.* 2005a) and identification of introductions to foreign locations has been well documented (Austin *et al.* 2005b, McKern *et al.* 2006, Su *et al.* 2006). It may simply be preferential behavior for the climatic zones that Florida possesses. Glacial refugia theories concerning speciation of *Reticulitermes* from the Palearctic (Uva *et al.* 2003, Luchetti *et al.* 2004, 2005 Austin *et al.* 2006a) and the Nearctic have been postulated before (Austin *et al.* 2006b, McKern *et al.* 2007), and these theories might provide some indication of the types of geographic isolation events that have formed the basis for the genetic variability observed among these populations. It may be the adaptive capacity of these surviving populations, evolving with extreme alternating climatic events, that fostered such haplotypic heterogeneity in *R. flavipes* in Florida, the likely (apparent) point of origination for the subsequent radiation of this species throughout the Nearctic. In turn, this environmentally adaptive heterogeneity may partially explain why *R. flavipes* appears to have an enhanced capacity for establishment in foreign regions around the world when compared to all other members of the genus. For this reason, future studies which attempt to identify establishment capabilities of exotic termite species to or from the United States should first identify focal points of

maximal heterogeneity which contribute to radiation events versus artificial introduction events. This is not to suggest that all natural radiation points may be revealed. This was the dilemma with low heterogeneity observed in *Coptotermes formosanus* Shiraki (Austin *et al.* 2006c) from their points of origin in southern China and Taiwan; however, until comparative studies of other termite species have been performed, this phylogeographic perspective offers the greatest insight to exotic invasion scenarios.

These biogeographic examples outlined herein are well supported as demographic responses to climate change and resulting range changes usually result in genetic manifestation, making them genetically traceable with adaptively neutral genetic markers (Hewitt 2000, Lessa *et al.* 2003). Other possibilities involve anthropogenic introduction of termites (e.g., the movement of infested wood materials), but this would likely exclude the same rationale for populations of *R. hageni* that are more homogeneous in terms of genetic diversity. Although only one haplotype was uncovered for this species in Florida (in the present study) ongoing investigations have revealed at least 5 distinct genetic haplotypes across its known range (JWA and ALS, unpublished). Rather than geological explanations, *Reticulitermes hageni* may have reduced genetic variability due to its inability to disperse great distances (Vargo & Carlson 2006) and a predilection for wood which is typically smaller in diameter (JWA, personal observations), as has been observed with other *Reticulitermes* species (Waller 2007). This may suggest some type of seasonal or occupational partitioning for available resources (Houseman *et al.* 2001, Green *et al.* 2006) in this scenario. It may be due to reproductive differences, whereby outbred primary reproductive pairing is less common in *R. hageni* as observed in other *Reticulitermes* groups (Vargo *et al.* 2006).

Regardless, it appears that strong isolation mechanisms are enforcing the scope and degree of genetic variability observed among these species in Florida, as has been observed in other studies (Austin *et al.* 2002). The origins of species such as *R. flavipes* may owe their historical radiation to events that were fostered in Florida, whether through some vicariant activity or from the natural dispersal and establishment accompanied by time throughout the Nearctic region. Conversely, the isolation of *R. virginicus* may be a function of limited resource availability at distances (Suárez & Thorne 2000) which retard greater foraging and dispersal. Although Su *et al.* (2001) suggest that

R. virginicus is just as commonly observed as a nuisance pest of structures as *R. flavipes*, we did not recover *R. virginicus* at the same frequency, nor did we see as much genetic diversity within this species from Florida. This study demonstrates that morphological variability among *Reticulitermes* in Florida (Hostettler *et al.* 1995) may be tied to their genetic variability and environmental plasticity. While genes ultimately control phenotypes, they are susceptible to environmental isolating mechanisms which may demonstrate preferences that translate into where the respective species of Florida may be found.

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