DNA-Based Identification of the Eastern Subterranean Termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae)

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ABSTRACT A DNA-based system for the identification of *Reticulitermes flavipes* (Koller) was established based on the following criteria: adequate molecular variation, comprehensive specimen sampling, explicit morphological species identification, and cladistic analysis. Termite soldiers were identified by labral shape, and these specimens and associated pseudergates were used in subsequent molecular analyses. Mitochondrial DNA from the AT-rich region was sequenced for 173 *R. flavipes*, *Reticulitermes hageni* Banks and *Reticulitermes virginicus* (Banks) soldiers and pseudergates collected from several widely dispersed Texan localities. Cladistic analysis was performed after exploration of an optimal sequence alignment inclusive of DNA sequences from Texas and published sequences from Georgia and Canada. Among the Texan individuals, 29, 5, and 1 mitochondrial DNA haplotypes were identified respectively with *R. flavipes*, *R. hageni*, and *R. virginicus*. One haplotype from El Paso likely represented a different *Reticulitermes* species. Species were monophyletic; however, individual relationships were unresolved in a strict consensus of 700 most parsimonious trees. Texas haplotypes were isolated by distance, and a correlation between genetic and geographic distance was observed among the Texas, Georgia, and Canada haplotypes. These results suggest that the methods outlined in this study will allow for the identification of *R. flavipes* from localities between Texas and Canada.

KEY WORDS molecular, taxonomy, phylogenetics, insect

SUBTERRANEAN TERMITES, SUCH AS *Reticulitermes flavipes* (Koller), cause millions of dollars in damage to wooden structures throughout the United States per year (Cornelius et al. 1995). Research concerning their control has received much attention; however, the dynamic and cryptic nature of termites and the lack of diagnostic morphological characters for the pseudergate caste of termite species have hampered progression toward control methods (Su and Scheffrahn 1991, Getty et al. 2000). Integration of molecular, morphological, and chemical characters augments traditional field and laboratory techniques and increases the understanding of termite biology and relationships (Forschler and Jenkins 1999).

Prospects for DNA-based identification of *Reticulitermes* species is encouraging (Forschler and Jenkins 1999, Jenkins et al. 2000). Particularly, mitochondrial nucleotide variation of protein coding genes and the AT-rich control region facilitate species diagnoses, phylogenetic reconstruction, and verification of introduced exotic species (Jenkins et al. 1999, 2000, 2001; Austin et al. 2002). However, the above-mentioned studies of *Reticulitermes* molecular systematics were not designed to establish a DNA-based identification system. Like morphologically based identification, accurate species and population DNA-based diagnostic systems are dependent on delimination of character variation for a specific locus within the species. Four criteria should be addressed to assure an accurate and "universally" applicable DNA-based identification system (Mallet and Willmott 2003, Tautz et al. 2003). First, the genetic locus must exhibit enough variation to potentially diagnose the species or population. Nucleotide diversity for mitochondrial DNA (mtDNA) cytochrome oxidase I and II (COI and COII) loci seems too conserved for population diagnosis because phylogenetic analysis did not resolve relationships among *R. flavipes* populations (Jenkins et al. 2001, Austin et al. 2002). A more variable locus may provide added phylogenetic characters and elucidate additional systematic diversity. Length variation of the AT-rich region among *R. flavipes* populations suggested that nucleotide variation sufficiently differentiated *Reticultermis* species and populations (Jenkins et al. 1998). Second, and ideally, genetic variation would be assessed for individuals from all possible populations, but practicality necessitates selective sampling. Although there is no regulated number of populations or individuals that should be assessed, specimens should be sampled from populations evenly distributed within the species range (Mayr and Ashlock 1991). Currently, AT-rich mtDNA haplotype

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variation for 78 specimens from only seven populations within Georgia and one Canadian specimen have been characterized (Jenkins et al. 1998). Third, molecular diagnostic characters should be associated with other specific diagnostic characters, including biological, chemical, and morphological data (Jenkins et al. 2000, Brunner et al. 2002). Association of specific genetic markers with soldier and alates of *R. flavipes*, and the sympatric termite species *Reticulitermes hageni* Banks and *Reticulitermes virginicus* (Banks) is necessary. In addition, the retention of voucher specimens should be mandatory (Jenkins et al. 2000, Austin et al. 2002). Fourth, phylogenetic reconstruction provides an objective method for summarization of haplotype variation and their relationships. A monophyletic group of species haplotypes indicates that all haplotypes were derived from a common ancestor (Hennig 1966). Observation of rampant polyphyly among haplotypes may necessitate taxonomic revision or indicate recent speciation or hybridization (Sperling 1990). Phylogenetic reconstruction of *Reticulitermes* spp. by using mtDNA COI and COII DNA sequence suggests the synonymy of *Reticulitermes santonensis* De Feytaud with *R. flavipes* (Jenkins et al. 2001, Austin et al. 2002). Given the above-mentioned criteria, the system for DNA-based identification of *R. flavipes* can be improved.

In this study, we included samples of *R. flavipes* from multiple populations and individuals to assess haplotype variation in a phylogenetic context, which improved the DNA-based identification of this species. We sequenced a portion of the AT-rich mtDNA region for 28 *R. flavipes* soldiers from 28 widely dispersed (between 55 and 1,190 km) localities within Texas, and for 145 pseudergates of *R. flavipes*, *R. hageni*, and *R. virginicus* individuals from one Texas locality. These DNA data are compared using phylogenetic and genetic isolation by distance analyses with published data for 14 Georgian and one Canadian *R. flavipes*, and five Georgian *R. virginicus* haplotypes (Jenkins et al. 1998, Jenkins et al. 2000). Following the above-mentioned criteria, we established a DNA-based identification system for *R. flavipes.*

Materials and Methods

Collection and Morphological Identification of Termite Samples. Alcohol preserved termite alates and soldiers were provided by licensed pest control professionals from 28 dispersed Texan localities in spring 2001. *Reticulitermes* spp. soldiers were identified to species by using keys based upon labral shape (Hostettler et al. 1995). Labra from these specimens were slide mounted in Hoyers or PVA media and then viewed at 100× magnification. Labra were photographed for taxonomic comparisons (Fig. 1). DNA was extracted and sequenced from these identified soldiers.

Termite pseudergates and soldiers were also collected at monitoring stations (Houseman et al. 2001) in an undeveloped park in College Station, TX, between July and December 2000 and 2001. Specimens were preserved in 95% ethanol and stored at −20°C. Soldiers collected with the pseudergates were identified to species based on labral shape. These identifications were initially applied to the associated pseudergates; 18, 20, and 10 specimens of *R. flavipes*, *R. hageni*, and *R. virginicus*, respectively, were included. An additional 97 temporally sampled (biweekly between July 2000 and August 2002), unidentified pseudergate specimens were included in the molecular analysis to increase the probability of sampling genetic diversity. Mounted labra, specimen remnants, and DNA are vouchered in the Texas A&M University insect collection.

DNA Extraction, Amplification, and Sequencing. DNA was extracted from the thorax of each specimen by using a DNeasy tissue kit manufactured by QIAGEN (Valencia, CA). Two microliters of extracted DNA (>50 ng/μl) from each sample was prepared for polymerase chain reaction (PCR) by adding to it 35 μl of PCR grade H_2O, 5 μl of 10× $MgCl_2$-free Promega buffer (Promega, Madison, WI), 4 μl of 25 mM Promega $MgCl_2$, 1 μl of 40 mM dNTPs, 0.2 μl of 100 U of Promega *Taq* polymerase, and 2 μl of 5 mM solution of each PCR primer. PCR primers TM-N-193 (TGGGGTATGAACCAGTAGC) (Taylor et al. 1993) and AT-J-T1 (CACTAAGGATAATCAATTATACGTC) (Jenkins et al. 1998) were used to amplify 324–337 base pairs of the mtDNA AT-rich region. PCR was carried out in a Peltier thermal cycler (PTC-200) under the following conditions: an initial 194°C for 120 s; 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s for a total of 36 cycles, followed by 72°C for 5 min. PCR products were then electrophoresed in a 1× Tris borate-EDTA buffer at 100 V for 30 min on 1.5% agarose gels stained with ethidium bromide (10 mg/ml solution), and then visualized in UV light. PCR products were cleaned of unincorporated dNTPs and primers by using a QIAGEN PCR cleanup kit following the manufacturer's protocol. Cycle sequencing followed and was performed with fluorescently dyed terminator nucleotides (Big Dye kit, Applied Biosystems, Foster City, CA) in a cocktail of 8 μl of PCR grade H_2O, 2 μl of Big Dye, 3 μl of 5× sequencing buffer, 3 μl of primers, and 2 μl of cleaned PCR product. Both strands of DNA for each PCR product were sequenced. Cycle sequencing products were cleaned with Sephadex and then visualized on an ABI 377 automated sequencer (Applied Biosystems) in the Gene Technologies Laboratory (Department of Biology, Texas A&M University). The chromatographs of complementary DNA were edited into consensus sequences with Sequencer 3.11 (Gene Codes Corporation, Ann Arbor, MI). Unique sequences were submitted to GenBank (AY351593-AY351620).

DNA Sequence Analysis. The variable length AT-rich sequences generated for the above-mentioned specimens and those published *R. flavipes* and *R. virginicus* sequences from Georgia (Jenkins et al. 1999, 2000) were aligned with Clustal X (Thompson et al. 1997). Alignment of nucleotide positions has been debated because different values for multiple sequence analysis can result in different alignments and subsequently different phylogenies (Morrison

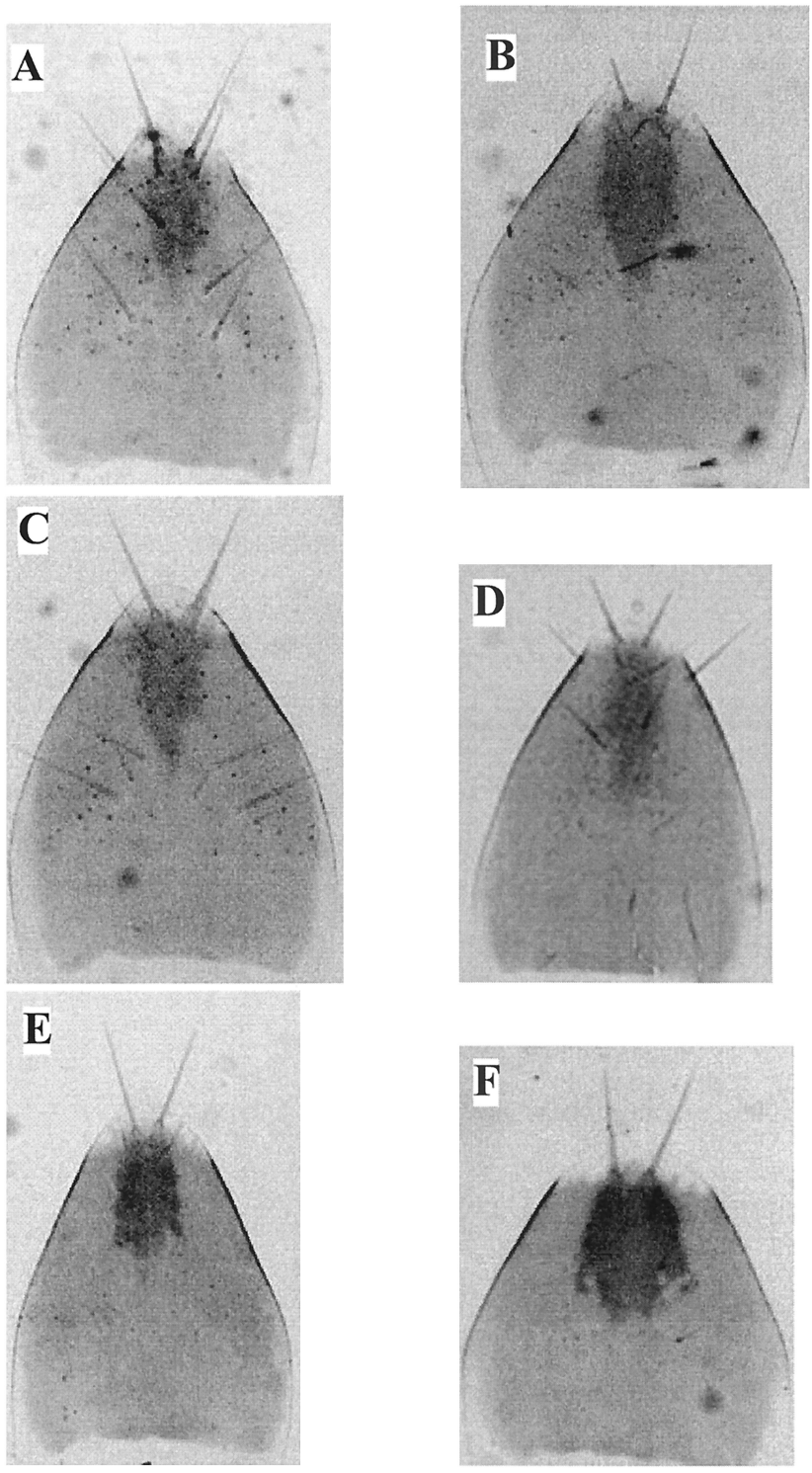

Fig. 1. Variation of labral shape of *Reticulitermes* collected in Texas (100× magnification). Labral width is diagnostic for species, see Hostettler et al. (1995) for details. (A) *R. flavipes*-Amarillo. (B) *R. flavipes*-Austin. (C) *R. flavipes*-College Station. (D) *R. flavipes*-El Paso. (E) *R. hageni*College Station. (F) *R. virginicus*-College Station.

and Ellis 1997). Therefore, it is necessary to explore alignment with different values, most importantly the gap to nucleotide change ratio. Further alignment subjectivity is removed by an explicit criterion for selection of the alignment results. This criterion maximizes character congruence among multiple data sets as measured with incongruence length difference (Phillips et al. 2000). However, this criterion cannot be applied to studies that use only one locus. For this study, sequence alignment was chosen based on a maximum phylogenetic resolution criterion where the alignment that resulted in the most resolved phylogeny was considered optimal. Alignments were made with gap penalties of 5, 10, 15, and 20; all other Clustal X settings were left as defaults. Phylogenetic resolution was lost with a gap penalty of 20 and no difference was observed among the other values. We chose the sequence alignment created with the 10-gap penalty value. This alignment in a NEXUS file format can be obtained from http://hisl.tamu.edu/research.

Phylogenetic analysis under parsimony optimality criterion was followed using PAUP* (Swofford 2002). Tree reconstruction was conducted using a heuristic search with 100 stepwise-addition replicates. At most, 100 most parsimonious trees were kept at each replicate. Gaps were treated as missing data and fifth character states. Although the latter provided an additional seven phylogenetic informative characters, the resulting tree topologies did not greatly vary. Gap positions can change with additional data and revised analyses. To increase the stability of DNA-based identification, gaps were treated as missing in final analysis because diagnostic nucleotides are regulated to conserved regions. All other PAUP* settings were left as defaults. In PAUP, bootstrap values were determined from 10,000 psuedoreplicates by using the "fast" stepwise addition search.

Geographical and Genetic Distance Comparison. Pairwise genetic and geographical distances were calculated for mtDNA haplotypes from the Texas and Georgia locations. Genetic distances were calculated under a Jukes–Cantor nucleotide evolution model in PAUP (Jukes and Cantor 1969). The geographical distances (kilometers) between cities were determined with an online distance calculator (http://www.indo.com/cgi-bin/dist). This program corrected the distance for global circumference. Genetic and geographic distances were associated with Mantel's approximate *t*-test by using the R Package computer program (Casgrain and Legendre 2001).

Results

Soldier labral shape was diagnostic for all three species as described by Hostettler et al. (1995), although some variation was observed among *R. flavipes* individuals (Fig. 1). This variation was within established limits for the species (Hostetter et al. 1995). A total of 28 haplotypes was identified from the 173 termite individuals. Nineteen mtDNA AT-rich haplotypes were found among 28 *R. flavipes* individuals from the Texas collection. Eleven haplotypes were identified from the 145 College Station specimens. Of these, three *R. flavipes* haplotypes were unique to College Station, whereas two haplotypes were identical to haplotypes found in other Texan locations. In addition, five and one haplotypes were identified with *R. hageni* and *R. virginicus*, respectively. Among the 48 pseudergates identified by their association with soldiers, three *R. flavipes* and four *R. hageni* soldier-associated identifications were alternatively identified by their DNA as *R. hageni* and *R. flavipes*, respectively. Two soldier-associated *R. virginicus* identifications were alternatively identified by their DNA as *R. flavipes* and *R. hageni.*

Nucleotide diversity (π) among the 22 Texan *R. flavipes* haplotypes was moderate (0.03 ± 0.016) and corresponded to 0.029 ± 0.035 (0.018 ± 0.007, excluding El Paso individual because of ambiguous species status; see Discussion) mean pairwise nucleotide difference among individuals. The Jukes–Cantor corrected sequence difference among haplotypes ranged from 14.9 to 0.3% (3.3 to 0.3%, excluding the El Paso individual). Comparison among all *R. flavipes* haplotypes (including Georgian and Canadian haplotypes) revealed little difference; $\pi = 0.10 \pm 0.044$, $s = 0.037 \pm 0.034$, JC distance range = 19–0%. Interspecific (*R. flavipes*– *R. virginicus* and *R. flavipes*–*R. hageni*) mean pair-wise nucleotide difference (0.17 ± 0.05 and 0.16 ± 0.04, respectively) was 5 times greater than *R. flavipes* intraspecific comparisons. Comparison among *R. hageni* and *R. virginicus* haplotypes revealed a mean nucleotide difference of 0.09 ± 0.05. Gap positions comprised 4% of the data.

Cladistic analysis of *R. flavipes*, *R.* hageni, and *R. virginicus* haplotypes resulted in 700 most parsimonious trees based on 95 parsimony informative nucleotides (Fig. 2). Resolution among haplotypes for each species was low and can be explained in part by homoplasy (rescaled consistency index = 0.69) (Kitching et al. 1998) and a lack of informative characters. However, the data contained phylogenetic information and the three species were reciprocally monophyletic. Each species clade had high bootstrap values and 15, 6, and 7 synapomorphies were observed for *R. flavipes* (8, excluding El Paso), *R. hageni*, and *R. virginicus*, respectively. These results show that the El Paso haplotype is basal to the other *R. flavipes* haplotypes and suggest that *R. flavipes* and *R. hageni* are sister species.

Texan *R. flavipes* haplotypes are isolated by distance (Mantel $t = 3.34$, $P = 0.0004$), and the El Paso haplotype is genetically and geographically the most distance to the other haplotypes. A significant isolation by distance (Mantel $t = 3.62$, $P = 0.0001$) exists among the Texas, Georgia, and Canadian haplotypes.

Discussion

Following the criteria stated in the Introduction, we provide a DNA-based identification system for *R. flavipes.* The AT-rich region provided nucleotide diversity that contained species-level diagnostic characters. It also identified unique haplotypes for several

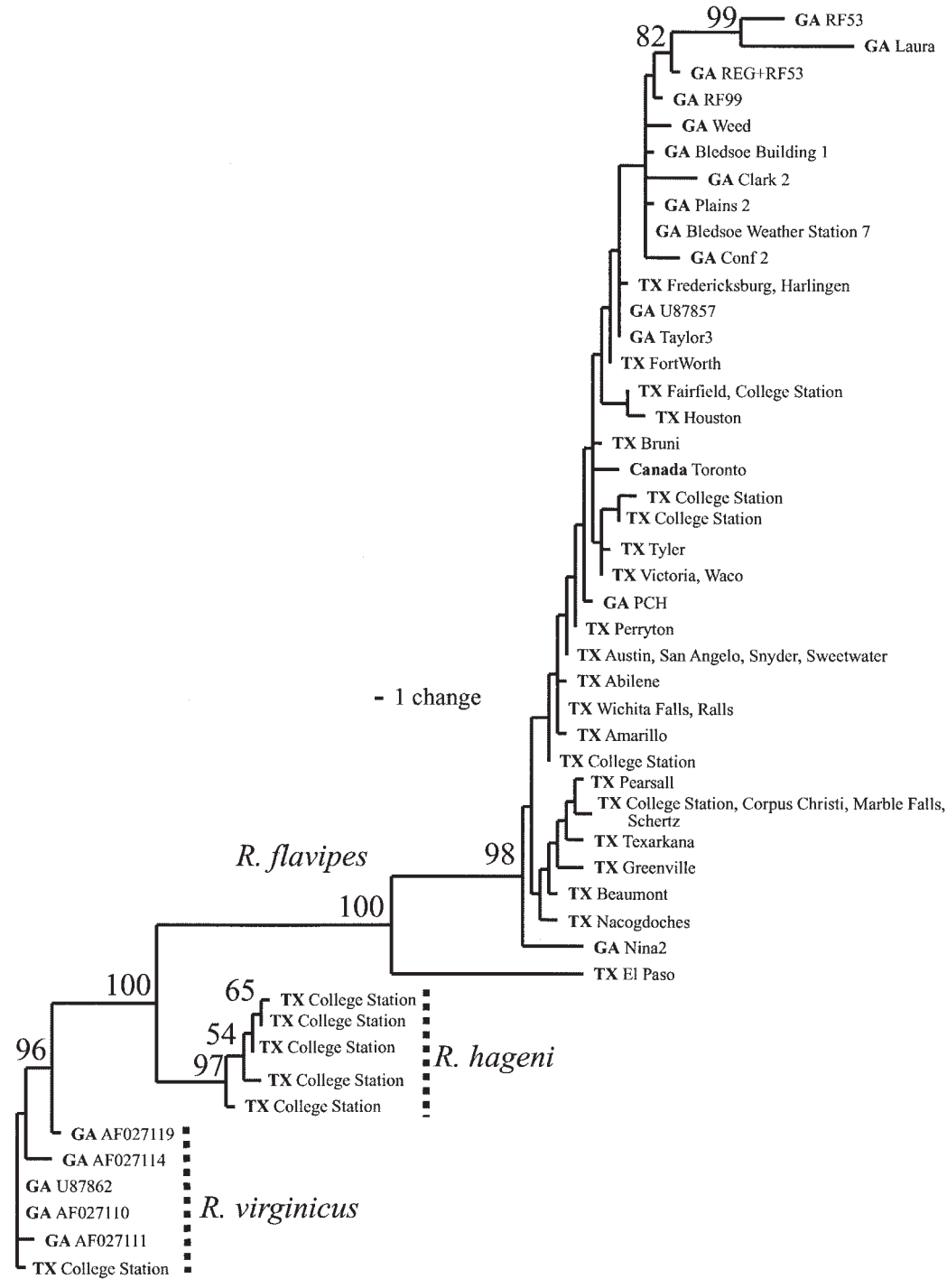

Fig. 2. Phylogram of 37 *R. flavipes*, five *R. hageni*, and six *R. virginicus* mtDNA AT-rich region haplotypes. Clades with bootstrap values (numbers above branches) were resolved in a strict consensus of 700 most parsimonious trees. See text for analysis details.

Texas, Georgia, and Canadian populations (Fig. 2). However, resolution among the populations was poor, likely due to a lack of phylogenetically informative characters. Inclusion of other mitochondrial and nuclear gene loci (especially introns) will likely provide phylogenetic resolution among the populations, as ob-

served in other studies (Scheffer and Lewis 2001). This lack of resolution does not preclude the use of the AT-rich region haplotypes to track termite infestations (Forschler and Jenkins 1999). Origins of an infestation could be identified given thorough assessment of local population haplotype variation, including queens and that the infestation is identified by a rare halpotype. Other molecular markers such as microsatellites and allozymes may provide higher resolution phenetic diagnostic characters for termite populations (Vargo 2000, Bulmer et al. 2001).

Specimen sampling seemed adequate for the *R. flavipes* populations. Texas populations were genetically isolated by distance. Potentially new haplotypes will be described with analysis of additional *R. flavipes* populations from more northern areas. Despite significant isolation by distance, phylogenetic analysis finds *R. flavipes* monophyletic.

Mitochondrial haplotypes were associated with soldier morphology. All *R. flavipes* soldiers identified based on labral shape were associated with *R. flavipes* haplotypes (Figs. 1 and 2); however, not all pseudergate species identifications based on soldier association were confirmed with molecular diagnostic characters. Eighteen percent were misidentified mostly between *R. flavipes* and *R. hageni* determinations. A similar misidentification rate (20%) occurs with the use of soldier pronotal width and labral shape (Hostettler et al. 1995). This suggests either an imperfect association of labral shape with genetic affinities or the occurrence of multiple species at bait stations. Molecular or chemical characters provide a better means for pseudergate species identification (Haverty et al. 1996).

Cladistic analysis of mtDNA haplotypes will allow for the identification of *R. flavipes* and will likely diagnose other *Reticulitermes* species. Hence, a revised cladistic analysis by using the protocol outlined (in the Materials and Methods) and DNA sequence from the AT-rich region from unknown specimens would be performed. The unknown identities would be resolved by their placement within the *R. flavipes* clade. Given that the widely disjunct Canadian population fell within the *R. flavipes* clade, it is likely that this method will correctly identify other *R. flavipes* individuals from populations between Texas and Canada. It is also possible to develop PCR-restriction fragment length polymorphism species diagnostics (as in Sperling and Hickey 1994). However, we refrain because DNA sequence data provides more phylogenetic information, which will yield a better understanding of *R. flavipes* haplotype diversity and taxonomy.

The individual from El Paso most likely represents a different species. Relative to the other species this individual has an average pairwise JC nucleotide difference (16.7%) that is similar to the other interspecific comparisons. Also, the El Paso haplotype is basal to the other *R. flavipes* haplotypes and has 14 unique nucleotides, which is double the most derived *Reticulitermes* haplotype (**GA** Laura) (Fig. 2). Similar nucleotide and phylogenetic patterns have been observed for specimens with uncertain species determinations (Jenkins et al. 2000, Austin et al. 2002). Potentially, the El Paso specimen is a *Reticulitermes tibialis* (Banks) soldier given the species distribution in Mexico and southwestern United States (Snyder 1949). However, the shape of its labrum was similar to *R. flavipes*, which leaves morphological identification ambiguous (Fig. 1). In a more inclusive cladistic analysis of *Reticulitermes* species based on mtDNA COI and COII sequence, the relationship of *R. flavipes*, *R. hageni*, *R. virginic*us, and *R. tibialis* is unresolved (Austin et al. 2002). A well-founded DNA-based identification system for all *Reticulitermes* species awaits a more thorough sampling of individuals and the use of the analyses outlined in this study.

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