CORRELATION OF MITOCHONDRIAL HAPLOTYPES WITH CUTICULAR HYDROCARBON PHENOTYPES OF SYMPATRIC RETICULITERMES SPECIES FROM THE SOUTHEASTERN UNITED STATES

TRACIE M. JENKINS,1,2 MICHAEL I. HAVERY,2
CHRISTOPHER J. RASTEN,3 LORI I. NELSON,2 MARION PAGE,2
and BRIAN T. FORSCHLIER4

1Department of Entomology, University of Georgia
Redding Building, Research Station
Griffin, Georgia 30223-1797
2Chemical Ecology of Forest Insects
Pacific Southwest Research Station, USDA Forest Service
P.O. Box 847, Berkeley, California 94701
3Department of Statistics, North Carolina State University
Raleigh, North Carolina 27695-8201
4Department of Entomology, University of Georgia
413 Biological Sciences Building
Athens, Georgia 30602-2603

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Abstract—Three sympatric Reticulitermes species have been identified in Georgia, USA, based on morphological characters from alates and soldiers: R. flavipes, R. virginicus, and R. hagani. However, species identification at individual collection sites is often difficult because alate production is seasonal and soldiers compose 1–3% of the colony. We therefore set up an experiment to determine if chemical phenotypes and mtDNA haplotypes can be used together to separate species of subterranean termites. Subterranean termites of the order Reticulitermes (Isoptera: Rhinotermitidae) were collected from 20 inspection points across four subsites in Georgia. Each collection was identified to species using dichotomous keys. Two collections, HH11 and BH25, however, could not be unequivocally keyed to species and were classified as unknown. The mitochondrial cytochrome oxidase II (COII) gene was sequenced from individual members of each collection, and the variation in cuticular hydrocarbon phenotypes from these same collections was characterized. The cuticular hydrocarbon and mtDNA phylogenetic analyses show agreement with both unknown collections falling out in a separate clade.

INTRODUCTION

Three species of Reticulitermes from the southeastern United States have been described: Reticulitermes flavipes (Kollar), R. virginicus (Banks), and R. hagani (Hartig, 1906; Weesner, 1965, 1970). These three species are sympatric throughout Georgia, the largest state in the region. Georgia covers 32.1 million hectares and provides a variety of subterranean termite habitats (Fig. 1): steeply sloped mountains in the Blue Ridge (BR) in the north, Southern Piedmont (SP) foothills underlain with metamorphic rock, gently sloping terrain covering marine sands, loam, and/or clay in the Southern Coastal Plain (SCP), and barrier islands in the Atlantic Coastal Flatswoods (ACF) along the Atlantic Coast. Although additional Reticulitermes species have been suggested in Georgia on the basis of soldier secretions (Clément et al., 1985, 1986), chemical analysis of cuticular hydrocarbons (Hartig et al., 1996b, 1999a), and phylogenetic analysis of mtDNA sequence data (Jenkins et al., 1999a), no new species have been confirmed or assigned.

Dichotomous keys have been published for identifying Reticulitermes species based on characters of alates and/or soldiers (Snyder, 1954; Weesner, 1965; Nutting, 1990; Scheffrahn and Sa, 1994), relying on synonymsies published in a catalog of the Isoptera (Snyder, 1949) and on material published nearly 80 years ago (Banks and Snyder, 1920). Difficulties arise in species determination at individual collection sites because the worker caste is the most abundant within a subterranean termite colony. Finding an imago (or alate) in a collection is seasonal and quite rare. Soldiers represent only 1–3% of Reticulitermes colonies and are morphologically variable; use of this caste alone often results in equivocal species determinations (Weesner, 1965). To make matters even more confusing, we have collected termites from a single piece of wood and keyed the soldiers to R. hagani and the alates to R. virginicus (Hartig et al., 1996b). Recent work using chemotaxonomic, genetic, and behavioral characters (Hartig and Nelson, 1997; Hartig et al., 1996b, 1999a,b; Polizzi and Forschler, 1998; Jenkins et al., 1999a) reiterate the need to revise the taxonomy of the genus Reticulitermes, as has been suggested frequently (Weesner, 1970; Nutting, 1990; Scheffrahn and Sa, 1994).
Hydrocarbon phenotypes and mitochondrial DNA (mtDNA) haplotypes have been independently examined to assess the validity of described species of *Reticulitermes* from Georgia. First, there is mounting evidence that hydrocarbon mixtures are species-specific in termites (Brown et al., 1990, 1996; Watson et al., 1989; Baguères et al., 1990; Haverty et al., 1988, 1990, 1991, 1992; Haverty and Nelson 1997; Kaib et al., 1991; Howard and Blomquist, 1982; Howard et al., 1988). Cuticular hydrocarbons were extracted from 91 different groups of workers sampled from colonies across four soil provinces in Georgia. Chemical analyses showed 15 unique hydrocarbon phenotypes among those *Reticulitermes* collections (Haverty et al., 1996a, 1999a). Based on keys to the alates and soldiers, three of the cuticular hydrocarbon phenotypes correspond with *R. flavipes* and one with *R. virginicus*. The remainder all key to *R. hageni*, based on soldiers, and either *R. virginicus* or *R. hageni* based on alates.

Second, assuming that a single pair of adults initiates a subterranean termite colony, each colony should have a unique and possibly species-specific mitochondrial DNA (mtDNA) genotype, because mtDNA is a haploid, maternally inherited molecule. We also recently extracted mtDNA from *R. flavipes* and *R. virginicus* individuals sampled from across four soil provinces in Georgia (Jenkins et al., 1999a). A 400-bp fragment from their 16S-rich region was sequenced and phylogenetically evaluated indicating population and species differences (Jenkins et al., 1999a). Subterranean termite colony associations are presently based on traditional field protocols that not only can provide ambiguous results (Forschler and Robinson, 1999), but cannot unequivocally delinete a colony, or its “movement and resource utilization patterns” (Jenkins et al., 1999b). We use the word population to refer to collections from inspection ports (Table 1), none of which are known to be connected by mark–release–recapture (MRR) (Forschler and Jenkins 2000). MRR is a technique in which termites are taken from an inspection port, marked with a dye, and released back into the inspection port from whence originally collected. After a specified time all the inspection
ports near where the marked termites were released are sampled. Inspection ports with marked termites are assumed to be connected and part of a colony unit.

Here we report the results of a study in which cuticular hydrocarbons (CH) and mtDNA sequence data were used to evaluate the accepted taxonomy of *Reticulitermes* in Georgia. Our primary goal was to determine if chemical phenotypes and mtDNA haplotypes could be used together to separate species. These data may then be used, along with morphological and soldier defense secretion characters, in future taxonomic and phylogenetic studies.

**METHODS AND MATERIALS**

**Specimens.** Termites were collected from established inspection ports located in four soil provinces in Georgia or by directed collections from infested wood (Forsyth and Townes 1995; and Jenkins et al., 1999a). Species were identified as *R. flavipes*, *R. virginicus*, or *R. hageni* using published dichotomous keys (Weest, 1965; Nutting, 1990), or as unknown based on lack of congruence with these published keys (Haverty et al., 1996a). Localities and specific details of each inspection port used in this study are presented in Figure 1 and Table 1. Sample collection methods and cuticular hydrocarbon analysis were detailed in Haverty et al. (1996a, 1999a). Voucher specimens for each collection were placed in 70% ethanol and are maintained by BTF at the University of Georgia. *Coptotermes formosanus* Shiraki, collected in New Orleans, was used as the outgroup to root both the mtDNA and CH cladograms. Each collection contained enough individuals for cuticular hydrocarbon characterization (200 workers), DNA sequencing (2–7 workers), and voucher specimens (25 soldiers, workers, and/or alates).

**Characterization of Cuticular Hydrocarbon Phenotypes.** Cuticular hydrocarbons were characterized and cuticular hydrocarbon phenotype separations were made according to procedures in Haverty et al. (1996a, 1999a), without prior knowledge of the species determinations. Cuticular hydrocarbons were identified by gas chromatography–mass spectrometry (GC-MS). The 142 cuticular hydrocarbons identified from *Reticulitermes* phenotypes GA-A, GA-AB, GA-C, GA-D, GA-I, and GA-L were used as phytophagic characters (Haverty et al., 1996a, 1999a). In the text and figures, we use shorthand nomenclature to identify hydrocarbons. The shorthand uses a descriptor for the location of methyl groups (X–m), the total number of carbons (CXX) in the molecule exclusive of methyl branches, and the number of double bonds follows a colon (CXX:Y). Thus 1,4-pentacosane becomes c–C25; 13-methylpentacosane becomes 13-mcC25; 11,15-dimethylpentadecanoic becomes 11,15-dimeC35; and pentacosane becomes C25:3. In no case were the locations of the double bonds determined. None of the individual *Reticulitermes* taxa or *C. formosanus* contained all 142 hydrocarbons.

**Phylogeny Analyses Based on Cuticular Hydrocarbon Phenotypes.** Parsimony analyses were performed using PAUP (Mac version 3.1.1 of Swofford (1993)) to compare degrees of relatedness among *Reticulitermes* based on chemical phenotypes and mtDNA analyses. Each discrete hydrocarbon character state was scored as a fraction of the total cuticular hydrocarbon. 0 = absent or not detected, 1 = 50.3%; 2 = 0–3.1%, 3 = 1.0–3.0%, 4 = 3.0–6.0%, 5 = 6.0–10.0%, 6 = 10.0–20.0, 5. and 7 ≥20.0. With the small data set of seven taxa and 142 hydrocarbon characters, the exhaustive search algorithm of PAUP was used to find the most parsimonious trees. Support for relationships among phenotypes was assessed by bootstrap analysis using 1000 replications.

**DNA Extraction, Amplification, Sequencing.** Genomic DNA was extracted from individual whole termites (Jenkins et al., 1999a) Primers Tn-M-N-193 (TGGGATGACAACTTACGTCG) and SR-1-14613 (TGAAGGCTAACTGCTGATGTG) were used to amplify the entire A+T-rich region. Primer AT-J-T-1 (CAGTAAGGTAATACCAATGTCG) was designed from *R. flavipes*, *R. virginicus*, and *R. hageni* original sequence data (Jenkins et al., 1999a) and was used as a reverse primer to amplify and sequence a 400-bp fragment.

Polymerase chain reaction (PCR) amplification was done according to Jenkins et al. (1999a) in a Perkin-Elmer Gene Amp PCR System 9600. Electrophoresis of amplification products and size standards, as well as fragment size estimation, was accomplished according to Jenkins et al. (1999a,b). Primers AT-J-T-1 and Tn-M-N-193 were then used to sequence complementary strands of a 400-bp fragment from 67 individual samples representing 20 collection sites or inspection ports (Jenkins et al., 1999a) in Georgia. (Table 1). Insect mtDNA was verified as in Jenkins et al. (1999a).

**Standardization.** Insect mtDNA has both a gene order and transcriptional orientation unique to its class. Therefore, we used the standard format for insect mtDNA primer names and strand identification (F for majority strand and N for minority strand) established by Simon et al. (1994). Also as per Simon et al. (1994), the primers that were designed from our unpublished sequence data do not have numerical locations. Sequence data have been submitted to GenBank. **Statistical Analysis.** Sequencer individual chromatograms were edited; contigs made, and alignments done to 312 segregating sites beginning at tRNA^Thr^ and ending 150 bp into the A+T-rich region. Consensus sequences were then assigned, multiple consensus sequences of all samples aligned with Malign (Hein, 1989), and aligned sequences reformatted for PHYLIP (Felsenstein, 1993); and bootstrapped 1000 times (Jenkins et al., 1999a). The same bootstrapped data were then used with DNAPARS and CONSENSE to produce the parsimony tree (F) (Felsenstein, 1993). The parsimony tree was drawn with TREEVIEW PPC (Page, 1999). A second neighbor-joining (NJ) tree was also constructed (not shown) as in Jenkins et al. 1999a,b.)
The Hudson, Boos, and Kaplan (HBD) (Hudson et al., 1992) statistic was used to test the null hypothesis of no genetic differentiation between populations as outlined in Jenkins et al. (1996).

The Kluge and Farris (1969) consistency index (CI) that measures the level of support for each tree and the Farris (1969) retention index (RI) that measures the congruency of characters to the cladogram and to each other were calculated for the PAUP parsimony analysis of cuticular hydrocarbon phenotypes (Figure 7 below). The CI will equal 1 when the data set explains the tree as well as possible, and the RI will equal 1 when the characters in a data set are totally congruent with each other and the tree.

RESULTS

All but two of the collections were positively identified using published morphometric keys. Specimens taken from inspection ports HH11 and BH25 could not be assigned to species unequivocally. The alates from HH11 keyed to R. virginicus and the soldiers keyed to R. hageni. We collected no alates from BH25, but soldiers collected keyed to R. hageni. Cuticular hydrocarbon phenotypes for these two equivocal samples were each unique and were identified as phenotype 2 (Figure 2) and phenotype 1 (Figure 3) for HH11 and BH25, respectively (Haverty et al., 1996a, 1999a). In addition, the cuticular hydrocarbon and mtDNA cladograms show HH11 (L) and BH25 (L) in separate clades (Figures 4 and 5), as do the neighbor-joining (NJ) phenograms for both (not shown).

Of the remaining collections, R. flavipes specimens were collected from all soil provinces, R. virginicus specimens from both the SP and ACP soil provinces. All of the R. hageni specimens, verified by both soldier and alate morphology, were collected from the SP soil province (Figure 1, Table 1). Specimens collected from BH2 and BH17 inspection ports were determined to be part of the same colony (Forschler, unpublished MRR data). Cuticular hydrocarbon phenotypes, like the mtDNA haplotypes, correspond well with the morphometric species determinations, with two exceptions. The chemical phenotypes are identified as A and AB for R. flavipes (Figure 6), C for R. virginicus (Figure 7), and D for R. hageni (Figure 8).

Phylogenetic analyses utilizing cuticular hydrocarbon characters indicate that phenotypes AB and C are closely related and different from the closely related phenotypes I and L. The phenotypes of the colonies studied here fall into two clades or lineages: phenotypes A, AB, and C belong to a lineage with a predominance of internally branched mono- and dimethyl alkanes, and phenotypes D, I, and L belong to a lineage with a predominance of unsaturated hydrocarbons (Figure 4). Phenotypes A and AB correlate with morphological determinations for R. flavipes: AB phenotype from R. flavipes collected from

Fig. 2. Total ion chromatogram of cuticular hydrocarbons from phenotype 1 specimens [Hill Hole (HH11)] from Plains, Sumter County, Georgia, and identified as Reticulitermes hageni from soldiers and Reticulitermes virginicus from alates.

Fig. 3. Total ion chromatogram of cuticular hydrocarbons from phenotype 1 specimens [Big House (BH12)] from Sapelo Island, McIntosh County, Georgia, and identified as Reticulitermes hageni from soldiers and Reticulitermes virginicus from alates.
inspection ports on Sapelo Island and A phenotype from *R. flavipes* collected from all other inspection ports. These phenotypes differ primarily by the large quantities of C25:3 in phenotype A and the near absence of C25:3 and C33 methylalkanes in phenotype AB (Haverty et al., 1996a). This difference is similar to the difference between *Reticulitermes* phenotypes CA-A and CA-A' from California: phenotypes CA-A and CA-A' likely represent variants of the same species (Haverty and Nelson, 1997). Phenotypes A and AB have apparent disjunct geographic distributions. Thus far, phenotype AB has only been collected from the coast of Georgia near Savannah and on nearby Sapelo Island. Phenotype C correlates with morphological determinations for *R. virginicus*. This is the only phenotype of *Reticulitermes* to have significant quantities (>3% of the total hydrocarbon component) of 11-meC31 and 11,15-dimeC31 (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). Phenotype C is consistent over a wide geographic range, from Louisiana to southern Florida, through Georgia to Maryland and Virginia (Haverty et al., unpublished observations). Phenotype D, I, and L all key to *R. hageni* based on soldier morphology: the width of the
Fig. 6. Total ion chromatogram of cuticular hydrocarbons from phenotype A specimens (Peach colony) from Blairsville, Union County, Georgia, and phenotype AB specimens (Big House (BH 2)) from Sapelo Island, McIntosh County, Georgia, and identified as *Reticulitermes flavipes* from soldiers and alates.

Fig. 7. Total ion chromatogram of cuticular hydrocarbons from phenotype C specimens (95) from Westbrook Farm, Spalding County, Georgia, and identified as *Reticulitermes virginicus* from soldiers and alates.

Fig. 8. Total ion chromatogram of cuticular hydrocarbons from phenotype D specimens (Dukes) from Barnesville, Lamar County, Georgia, and identified as *Reticulitermes hagenii* from soldiers and alates.
dienes (Havenly et al., 1996a, 1999a). These relationships are corroborated in the mtDNA analyses.

Cladistic analyses of both cuticular hydrocarbon and mtDNA data sets generally agree (Figures 4 and 5). Reticularitermes flavipes (hydrocarbon phenotypes A and AB) forms a very supported (99%) chemical clade (Figure 4) and a supported (533) mtDNA clade (Figure 5). Figure 4 shows a supported clade in which both R. virginicus (Nitra 4, 93, 94, 95), phenotype C and R. hagensi (Andy 1, Tyler 6, Duques, House 19-2), phenotype D belong. This major clade also includes collections from HH11 and BH25 (Figure 4). These individuals that could not be identified unequivocally using morphological keys have unique cuticular hydrocarbon phenotypes and mtDNA haplotypes. They also form a supported clade (682) separate from the three sympatric species. Although the mitochondrial COI gene marker has bootstrap support for several clades including R. flavipes, R. virginicus, R. hagensi, and others (Jenkins et al., 1999b) within Georgia, there is little nodal support within the R. flavipes using this A+T-rich mtDNA marker (Figure 4).

The HKB statistic was calculated to determine genetic partitioning among species groups and clades identified in the cladogram (Figure 4). Partitioning among R. flavipes, R. virginicus, and R. hagensi (Ks* = 2.2570, Zs = 3.1587–3.2891) was significant (P < 0.05), indicating genetic differentiation. The Ks* and Zs values for comparison at HH11/BH25 clade and R. hagensi, D clades (Ks* = 1.6690, Zs* = 1.0993) and for R. virginicus, C clade and HH11/BH25 clade (Ks* = 1.9033, Zs = 1.0965) were not significant (P > 0.05). HKB statistic for R. flavipes, A and AB clade and HH11/BH25 clade (Ks* = 2.4670, Zs* = 3.0672) was significant (P < 0.05). Thus, the HKB statistic genetically partitioned the HH11/BH25 clade from R. flavipes clades A and AB, but not with R. virginicus clade C and R. hagensi clade D.

**Discussion**

Mitochondrial DNA has been used to demonstrate unspecific geographical localization from which phylogeography has been deduced (Jenkins et al., 1996c) and colonization patterns elucidated (Jenkins et al., 1996a). Inspection of the rooted phylogenetic patterns in Figure 5 reveals low bootstrap values for R. flavipes that likely reflect a limited number of substitutions, a situation that we know improves when more sequence data are used (Jenkins et al., 1999b). The cladogram topology shows no geographic localization (Figures 1 and 5, Table 1). Gene flow, therefore, appears to be unrestricted, especially in the R. flavipes collections. Figure 5 demonstrates that the clade containing R. virginicus, R. hagensi, HH11 (phenotype I), and BH25 (phenotype L) is separated from the R. flavipes clade by supported nodes. The R. flavipes clade, however, demonstrates strikingly low levels of molecular divergence with unsupported nodes (Figure 5). Thus, it is difficult to resolve R. flavipes lineages using phylogenetic analyses with this mtDNA marker. Since individual internal nodes are not supported, they should be collapsed into a single node. This means that R. flavipes clades do not show geographical localization with this marker, whereas there is geographic separation, as mentioned previously, of the two hydrocarbon phenotypes of R. flavipes, A and AB. The phylogenetic analysis indicates that gene flow is unhindered by either geographic or physical barriers (Figure 5).

Cuticular hydrocarbon analysis was done on collection from inspection port BH2. MRR results (Forschler, unpublished data) indicated that BH2 and BH17 were connected. Thus, it was expected that mtDNA sequences from BH2 and BH17 would be the same. They were not (Figure 5). Although the bootstrap results were <50% (Figure 5), the mtDNA sequence between BH2 and BH17 was different (Figure 5). The molecular data for these two inspection ports, as in recent studies (Jenkins et al., 1999b), appear to differ from field data and expectations for colony structure. Furthermore, because the matrilinial line collected from BH2 was not the same as that collected from BH17, the cuticular hydrocarbon phenotype assigned to BH2 could not be designated, as expected, for BH17 (Figure 5).

Collections from inspection ports HH1 and BH25 both had soldiers that keyed to R. hagensi. The HKB statistics of the mtDNA sequence data revealed that, while the three sympatric species in Georgia were significantly different genetically (P < 0.05), neither R. virginicus nor R. hagensi was significantly different from the HH11/BH25 clade (P > 0.05). The R. flavipes A and AB clade, however, was significantly different from HH11/BH25 (P < 0.05). The statistical analyses appear to indicate that HH11 and BH25 are more closely related to either R. virginicus or R. hagensi than to R. flavipes; the hydrocarbon data suggest a much closer affinity to R. hagensi or phenotype D.

Although HH11 and BH25 were sampled from two disparate soil provinces, SP and ACF, respectively (Figure 1, Table 1) they are more related phylogenetically to each other than to either R. virginicus or R. hagensi (Figures 4 and 5). All external nodes for R. virginicus, R. hagensi, and HH11 and BH25 show that they form unique clades. The internal nodes in Figure 5 indicate the strength of support. HH11 and BH25 have the same ancestor as R. virginicus and R. hagensi, but have a supported node (683) that indicates that they are different from either R. virginicus or R. hagensi. Thus, the HH11 and BH25 inspection ports could represent a salvational speciation event (Mayr, 1964; Carson, 1968; Templeton, 1980), which could be explained by either molecular genetics or population genetics phenomena.

First, HH11 and BH25 could be the result of a molecular genetics phenomenon. A hybridization event could have caused a molecular recapitulation that resulted in changes in gene expression or regulation. This change in molec-
lar genes may have so influenced organismal evolution as to result in a rapid speciation event (Avise, 1994, p. 257). The genetic change, having an adaptive advantage, was established and perpetuated by the colony's social structure.

A population genetics argument for the genotypic and phenotypic uniqueness of HH11 and BH25 may have an explanation in a founder event (Mayr, 1954). If geographically isolated and relatively small, a founder event might have resulted in a "genetic revolution" (Mayr, 1954) that, coupled with a "founder-flush" or rapid expansion (Carson, 1968), produced novel recombinants that were adaptive and, therefore, perpetuated. This could then have caused a shift to a new adaptive peak (Whittaker, 1951) due to a rapid and temporary accumulation of inbreeding that did not deplete genetic variability (Templeton, 1980).

The agreement between the chemical phenotypes and the mtDNA haplotypes adds robustness to the phylogenetic analyses as it calls attention to the two variant taxa, HH11 and BH25. The use of multiple characters also provides more insight into the speciation process because the collective uniqueness of each assists in understanding organismal evolution generally. Collections from HH11 and BH25 inspection ports form a supported clade (682) (Figure 5) that differs from R. flavipes, R. virginicus, and R. hageni clades genetically, chemically, and morphologically. We suggest, therefore, that these two collections may represent at least one new taxon in Rhizotermeinae. The association of cuticular hydrocarbon phenotypes with mtDNA haplotypes not only provides further support for the species specific nature of cuticular hydrocarbon phenotypes first seriously proposed by Howard and Blomquist (1982), but for using multiple data sets to evaluate taxa. Furthermore, the phenotypic plasticity and genotypic similarity of colonies of R. flavipes provide some caution for overinterpreting slight differences in cuticular hydrocarbon mixtures.

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