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# *Reticulitermes malletei* (Isoptera: Rhinotermitidae): a valid Nearctic subterranean termite from Eastern North America

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## Abstract

The taxonomic status of *Reticulitermes* Holmgren in North America has been in need of revision for years, but because of morphological ambiguity, traditional morphological identification of *Reticulitermes* species has always been difficult and unreliable. Analysis of termites, applying non-morphological genetic comparisons of mitochondrial DNA from numerous populations across North America, has implicated new species which are presently under investigation. Applying the 16S rRNA gene with biometric, cuticular hydrocarbons, and ethological data, a cryptic species of Nearctic *Reticulitermes* has been identified from Delaware, Georgia, Maryland, North Carolina, and South Carolina and determined to constitute a valid species with an apparently Atlantic distribution. Epicuticular hydrocarbon analysis showing the presence of rare triunsaturated alkenes, and a total absence of methyl branched alkanes also support this cryptic group as a distinct species. The name for this species is *Reticulitermes malletei*, previously described, but not generally accepted by termite experts in the United States. Comparisons from a 403 bp region of the mtDNA 16S rRNA gene was used to discriminate known *Reticulitermes* species from North America: The eastern subterranean termite *R. flavipes* (Kollar), dark southern subterranean termite *R. virginicus* (Banks), light southern subterranean termite *R. hageni* Banks, arid subterranean termite *R. tibialis* Banks, and western subterranean termite *R. hesperus* Banks. When compared to this new species, both maximum parsimony and maximum likelihood support their genetic isolation from sympatric populations of known species and eliminate either exotic Palearctic introductions or western Nearctic involvement.

**Key words:** *Reticulitermes*, Rhinotermitidae, Nearctic, mtDNA, cuticular hydrocarbon

## Introduction

In North America there are 6 species of termites presently known in the genus *Reticulitermes* Holmgren: *R. hesperus* Banks is a western species not found east of the Rocky Mountains; *R. tibialis* Banks occurs in the West, Southwest, Central plains states, and is known to occur as far east as Indiana and through northern Mexico; *R. flavipes* (Kollar) has the most significant distribution occurring from Canada down through Mexico and throughout the eastern United States (Banks and Snyder 1920, Snyder 1954, Weesner 1965, Messenger 2003), and established populations have recently been confirmed in the western states of New Mexico, Ari-

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zona, Nevada, California, (Austin *et al*. 2005a), and Oregon (McKern 2006, J.W.A., unpublished) with multiple introductions around the world (Scheffrahn *et al*. 1999, Austin *et al*. 2005b, Su *et al*. 2006); *R. hageni* Banks, is found as far west as Texas, but is principally in the southeastern US; and *R. virginicus* (Banks) is similarly found in sympatry with *R. hageni*, but may have a broader northern distribution than *R. hageni* with more recent discoveries of western establishment (Austin *et al*. 2006a). Recent evaluation of the sand-dwelling subterranean termite *R. arenicola* Goellner, which is noted to be distributed around the sand dunes of eastern Michigan and Indiana (Goellner 1931), appears to be yet another variant of *R. flavipes* (JWA, unpublished data) and is supported by recent phylogenetic evidence (Austin *et al*. 2004e, 2005a, Ye *et al*. 2004) therefore, we consider its current status *nomen dubium*. In fact, it shares identical 16S rRNA haplotypes to *R. flavipes* haplotype "M" which has been observed throughout the Central United States (Austin *et al*. 2004a, b, c, Austin *et al*. 2005a, b). There are multiple genetic (Austin *et al*. 2005) and morphological variants (Bagnères *et al*. 1990, Clément *et al*. 2001) of this species which have contributed significantly to taxonomic confusion for both Nearctic and Palearctic descriptions of *Reticulitermes* (Austin *et al*. 2005b) and in locations where it has been accidentally introduced (Clément *et al*. 2001, Dronnet *et al*. 2004, 2005, Su *et al*. 2006).

Morphological identification methods have been successful in identifying these species, but are less capable of discriminating variants which may be attributed to clinal morphological variations over large geographic zones within the genus, as was observed in Palearctic *Reticulitermes* (Weidner 1960, 1970). This is especially true when attempting species identification without diagnostic castes. Identifying workers is nearly impossible and separating soldiers is especially difficult given that precise measurements are required and overlap may occur between species (Sheffrahn and Su 1994, Vieau, 1993, 1999). For this reason, more robust multivariate analyses have been applied to both morphological and non-morphological data which offer an increased ability to discriminate *Reticulitermes* groups (Austin *et al*. 2002, Bagnères *et al*. 1990, Clément 1977a, b, 1978, 1979, 1982a, b, 1986a, b, Clément *et al*. 2001, Delphia *et al*. 2003, Uva *et al*. 2004a, b). One example of non-morphological identification is the use of cuticular hydrocarbons (HCS).

Cuticular hydrocarbons have been applied successfully to discriminate numerous *Reticulitermes* species in Europe (Bagnères *et al*. 1988, 1991, Clément *et al*. 2001) and North America (Bagnères *et al*. 1990, Clément *et al*. 1985, Haverty and Nelson 1997, Haverty *et al*. 1996, 1999a, b). They offer an important non-morphological identification method, but they can also be problematic and should be evaluated carefully. For taxonomic purposes, cuticular hydrocarbons should represent fixed patterns within taxa. While they have an important role in the ecology and behavior of arthropods (Howard, 1993; Howard and Blomquist 2005), variations in hydrocarbons that occur between colonies within supposed species can lead to false conclusions about the number of species in a given area (Kaib *et al*. 2002), even though they are among the prime candidates for nestmate recognition in social insects (Kaib *et al*. 2004). Often, HCS analyses coincide with phylogeographic data (Clément *et al*. 2001, Kutnik *et al*. 2004, Copren *et al*. 2005, and Bagnères, unpublished). For example, Austin *et al*. (2002) identified *Reticulitermes* from Turkey as *R. lucifugus*, applying cuticular hydrocarbons from both workers and soldiers. This identification was also supported with genetic data: the cuticular hydrocarbon and the defensive substance analysis showed the close relatedness between Turkish samples with *R. clypeatus*, *R. balkanensis*, and *R. urbis sp. nov.*, which coincide with phylogeographic data (Austin *et al*. 2002, 2006b, Bagnères *et al*. 2003, Quintana *et al*. 2003, Uva *et al*. 2004a). Haverty *et al*. (1999) suggests that 26 different hydrocarbon phenotypes occur in North America, representing distinct taxa, when conventional taxonomy only recognizes six species. Our own efforts at evaluating this genus suggest that there are indeed more species of *Reticulitermes* in North America, but the number proposed by Haverty *et al*. (1999) would appear inflated compared to our most current evaluations of this genus (J.W.A., unpublished). For this reason the use of different genetic markers (e.g., mtDNA and nuclear genes), non-morphological tests such as reproductive isolation (Clément 1979b), biochemical (Clément 1981, 1984), and behavioral (Clément 1986a, b, Grace 1996, and Getty *et al*. 2000) characters are often applied. Most recently, genetic data have been used quite successfully to discriminate numerous termite groups. Austin *et al*. (2002, 2004a, b, c, d, e,), Clément *et*

*al*. 2001; Jenkins *et al*. 2001; Luchetti *et al*. 2004; Marini *et al*. 2002; Szalanski *et al*. 2003, 2004; Kutnik *et al*. 2004; Uva *et al*. 2004a, b; Ye *et al*. 2004; have demonstrated the objective power of genetically based identifications of *Reticulitermes*, and a new clarity for species determinations that have been somewhat lacking with previous endeavors.

In 1986, a new species of *Reticulitermes* was found in southeast United States. Conducting chemical olfactometry experiments (sternal extracts of reproductive females in hexane), agonistic bioassays (inter- and intraspecific aggression between disjunct termite groups), and comparisons of alate flight dates, Clément *et al*. (1986a) were able to discriminate this new *Reticulitermes* species from sympatric populations of *R. flavipes* and *R. virginicus*. Although not originally evaluated, *R. hageni* Banks also occurs in sympatry in this region. Subsequent genetic evaluation has confirmed its relationship to other congeners from this region (J.W.A., unpublished). This newly identified species' somewhat diminutive size (Hostettler *et al*. 1995, Sheffrahn and Su 1994, Whitney-King *et al*. 2007) makes it more discernable than either *R. flavipes* or *R. virginicus* (Banks and Snyder 1920), and its asynchronous flight dates with either *R. flavipes* or *R. virginicus* suggest temporal isolation (Krishna and Weesner 1970). Clément *et al*. (1986a) cited three mechanisms of isolation for this new species: sexual alate swarm dates, sexual attraction by pheromones, and aggression. On the basis of these species isolation mechanisms, a new species named *R. malletei* was proposed. Further, they collected similar samples from numerous locations across the United States. Clément *et al*. (1986a) suggest that *R. malletei* are abundant in Georgia, Tennessee, and in South Carolina. However, because no further investigations of this new species were conducted, nor was a proper description of the species published in accordance with the international code of zoological nomenclature (ICZN), termite researchers in the United States basically ignored this species, and it was not generally accepted—a *nomen nudum* (Scheffrahn *et al*. 2001).

While conducting more current investigations of *Reticulitermes* across the United States, we have found genetic evidence for unknown species (based on mtDNA 16S rRNA gene sequence data) which could not be identified with documented species from Nearctic, Palearctic, Neotropical, or Indomalayan regions (J.W.A., unpublished data). Samples from Delaware, Maryland, South Carolina, and Georgia were genetically identical, and were excluded as possible sibling/subspecies (from North American biotypes) based on their unique gene sequence data. In most instances, this new species had been misidentified as *R. virginicus*, based on specimens sent to our laboratory for investigation. Clément *et al*. (1986a) found that reproductive alates of this new species, *R. malletei*, swarmed at approximately the same time as *R. virginicus*, but could be easily distinguished from morphological data. Unfortunately, the work of Clément *et al*. (1986a) has not been widely circulated in the United States. Termite researchers have had to rely on antiquated dichotomous keys which, due to morphological overlap and geographic variations in size within the genus *Reticulitermes*, are notoriously poor; there appears to be some geographic restrictions to the broad application of morphologically-based identification keys for the genus (Heintschel *et al*. 2006). Additionally, the failure to evaluate *R. hagen*i, which is found in sympatry from the same region, casted doubt on the validity of this newly proposed species. Undoubtedly, this has led to some of the taxonomic confusion of this species, particularly for collections that are not made at the time of sexual flights (i.e., not all diagnostic castes were compared to other congeners).

Applying morphometric measurements to soldiers labra (Scheffrahn and Su 1994, Hostettler *et al*. 1995), samples collected from Delaware appeared somewhat smaller, and their size was more similar to *R. hageni* (Whitney-King *et al*. 2007). However, comparisons of reproductive alate size appeared more similar *to R. virginicus*, although wing color is generally different. Applying the 16S rRNA gene to HOLOTYPE specimens collected from Clément *et al*. (1986a) and Bagnères *et al*. (1990), we identified identical 16S rRNA gene sequence data from samples that had been tentatively identified as *R. malletei* (A-G. Bagnères, unpublished data) and which were identical to unknown species from the eastern United States collected previously during a national survey of termites (Messenger 2003). Additionally, epicuticular hydrocarbon analysis and morphometric evidence has been performed which support our findings.

Herein, we validate the description of *R. malletei* based on chemical, behavioral, morphometric, and genetic evidence. When combined with previous data by Clément *et al*. (1986a), Bagnères (1989) and Bagnères *et al*. (1990), who demonstrated ethological, chemical (sexual pheromones of alates and soldier defensive excretions), and temporal isolation of this species, we propose that the validated species, *R. malletei*, should be recognized as a native, cryptic species which should be investigated further. While the distribution of this species (from the present study) appears to occur in the Eastern Atlantic states, additional surveys and comparison with existing material may clarify its occurrences in North America and its relationship to other *Reticulitermes* within the genus.

## Materials and Methods

**Collection and DNA preparation.** Termites that were subjected to DNA sequencing in this study were collected from several Palearctic and Nearctic locations and preserved in 100% ethanol (Table 1). Voucher specimens preserved in 100% ethanol are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR and the Center for Urban and Structural Entomology, Department of Entomology, Texas A&M University, College Station, Texas. Collections of *R. malletei* from Clément occurred in 1983, and from Bagnères in November 1987 and June 1988 in Athens, Georgia (UGA). Attempts were made to collect in some of the identical areas where *R. malletei* was originally found, and in some new areas.

From alcohol-preserved specimens dried on filter paper, the DNA was extracted according to Liu and 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 –20ºC. Polymerase chain reaction (PCR) was conducted with the primers LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati and 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 94ºC for 45 s, 46ºC for 45 s and 72ºC 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 for *R. malletei* 16S rRNA haplotypes RM1 and RM2 were DQ422137 and DQ422138, respectively. Additional sequences not previously submitted to GenBank are listed in Table 1 and will be made available through GenBank. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999), and sequences were aligned using CLUSTAL W (Thompson *et al*. 1994). Mitochondrial DNA haplotypes were aligned by MacClade v4 (Sinauer Associates, Sunderland, MA). 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). For maximum likelihood analysis, the default likelihood parameter settings were used (HKY85 6-parameter model of nucleotide substitution, empirical base frequencies, and transition / transversion ratio set to 2:1). These parameters were used to carry out a heuristic search using PAUP*, using either the single most parsimonious tree as the starting tree or step-wise addition. Mitochondrial DNA 16S sequences of *R. grassei* from Bilbao, Spain (GenBank Accession AF292027), and *R. lucifugus* Grossetto, Italy (GenBank Accession AF292010), were also compared to other *Reticulitermes* haplotypes from North America and various other locations around the world (Table 1)(Austin *et al*. 2004a, b, c, d, Szalanski *et al*. 2003, Austin unpublished data).

**Cuticular hydrocarbon and Principal Component Analysis (PCA).** Analysis of two western Palearctic *Reticulitermes* species, *R. grassei* Clément and *R. lucifugus* Rossi, were conducted to exclude the possibility

that *R. malletei* is simply an exotic Palearctic introduction to the eastern United States. Six discrete populations of *R. malletei* collected in 1987 and 1988 (Bagnères 1989) were evaluated (3 from each year), from which pools of 100 workers (6) and 20 soldiers (5) have been extracted in pentane. Mean weights were calculated before extraction for both workers and soldiers. The total extract was performed in 2ml of the apolar solvent which was evaporated and resuspended in 1 ml of pentane with an internal standard (n-C21). 100µl was used for quantification by GC analysis. The remaining volume was used for GC-MS analyses. Extracts were applied to SepPak separation to remove the non-HC components. Gas chromatography was performed with a HP GLC 5890a connected to a HP 5880a integrator. SE54 capillary columns (25 m) were used (ID 0.32 mm x 30 m; 0.1µm of SE54) with an initial injection temperature of 150 ºC for 5 min. and a ramping temperature of 5 ºC/min to 320 ºC. The same worker and soldiers analyses have been performed on different species of *Reticulitermes*, i.e., *R. flavipes* from GA, USA*; R. grassei* from France, and *R. lucifugus* from France and Italy in Europe (see Bagnères *et al*. 1990 and 1991, Clément *et al*. 2001) to have the relative proportions of different species calculated in Excel software and transferred to Statgraphics software (Statgraphic v. 4.0 and Uniwin Plus v. 3.0) for the PCA analysis. We calculated the weight of worker and soldier *R. malletei* and quantified the total amount of HCs per mg of insect on several dozen individuals from different colonies. We also performed this quantification for different species from the USA (*R. flavipes*) and Europe (*R. grassei*).

**Behavioral Tests (Intra- and interspecific aggression behavior)**. Sufficient quantities of all species to conduct behavioral tests was not possible, but aggression behavior could be performed for the 2 most commonly found termites from the Athens, GA area: *R. flavipes*, *R. malletei* (Bagnères, 1989), and to a lesser extent *R. virginicus* (see Clément *et al*. 1986a). Tests were performed in Athens (GA) in 1987 following Clément (1986a) with the aggression index [Ag = 2.5(M+m/2) where M = mean number of dead, and m = mean number of mutilated]. Eleven tests were performed with 7 colonies of *R. malletei*. 23 tests were performed with 12 *R. flavipes* colonies (Table 3). In this case the tests were performed with one or the other of the species colored with the cresyl blue (a vital colorant) to differentiate which species was exerting the aggression during termite pairings.

**Biometric Study.** Biometric measurements were taken on scanning electron microscope (SEM) photographs (Figure 3) per Bagnères (1989), and Bagnères *et al*. (1990). Three measurements were obtained for each individual from groups of approximately 12 or more for each species from SEM photographs (Fig. 3). The measurements L, AB, and BC, were taken after verification that the tangent T appeared horizontal on the screen. All measurements were taken in millimeters, and the mean ± SD was calculated for *N* samples. The resulting numeric index was calculated with the formula: (distance AB + distance BC) x length L. Tangent *T* served as a reference point for vertical measurements, *T*' for measurement L, and *T*" as a reference from the location of bristles x and x', which were always present (Fig. 3).

In addition to these measurements, soldier labra were evaluated in comparison to all species which are known to coexist in sympatry with *R. malletei* according to Hostettler *et al*. (1995). Images were recorded and viewed in SigmaScan® Pro 5 (SPSS Inc. 1999) so that labra lengths and widths could be measured (Fig. 5). A hierarchical cluster analysis, applying Ward's method (See Ward 1963), was performed on four different combinations of measurement means described in Heintschel *et al*. (2006), and a morphological dendogram was constructed to demonstrate the relationship of *R. malletei* to its congeners (Fig.7). Soldier pronotum widths and alate coloration were compared with existing taxonomic keys (Scheffrahn and Su 1994) and local observations of material collected throughout the eastern Nearctic (Whitney-King *et al*. 2007, Austin *et al*. 2005a).

Additional measurements were recorded for head capsule (with and without mandibles) width, length and ratio; Gula width 'w' (w = widest point), width 'n' (n = narrowest point), length, ratio 'w'(ratio w = length/ width w), and ratio 'n' (ratio n = length / width n); Mandible length for both right and left mandibles measured from the madibular apex to the hinge on a diagonal); Pronotum width 'w' (w = widest point), width 'n' (n = narrowest point), length, ratio 'w' (ratio w = length / width w), and ratio 'n' (ratio n = length / width n); Labral length width and ratio.

**TABLE 1.** Collection data, and haplotypes for *Reticulitermes* from USA and World.

| **Species** | Location | Haplotype | N | Source |
|---|---|---|---|---|
| *R. balkanensis* | Dionissos-GRE | - | 1 | Austin *et al*. (2006b) |
| *R. flavipes* | TX-USA | A | 1 | Austin *et al*. (2004a) |
| | TX-USA | B | 1 | Austin *et al*. (2004a) |
| | TX-USA | E | 1 | Austin *et al*. (2004a) |
| | TX-USA | F | 1 | Austin *et al*. (2004a) |
| | TX-USA | M | 1 | Austin *et al*. (2004a) |
| *R. flavipes* (=*santonensis*) | AR-USA | JJ | 1 | Austin *et al*. (2005b) |
| *R. guangzhouensis* | Hong Kong-CHN | | 1 | This study |
| *R. hageni* | TX-USA | H1 | 1 | Austin *et al*. (2004a) |
| | AR-USA | H2 | 1 | Austin *et al*. (2004c) |
| | AR-USA | H3 | 1 | Austin *et al*. (2004c) |
| *R. hesperus* | CA-USA | HE1 | 1 | Szalanski *et al*. (2006) |
| | CA-USA | HE2 | 1 | Szalanski *et al*. (2006) |
| | OR-USA | HE3 | 1 | McKern *et al*. (2006) |
| | OR-USA | HE4 | 1 | McKern *et al*. (2006) |
| *R. urbis* (sp. nov.) | Domène-FRA | | 1 | Austin *et al*. (2006b) |
| *R. lucifugus* | Grosseto-ITA | | 1 | Luchetti *et al*. (2004) |
| | Antalya-TUR | | 1 | Austin *et al*. (2006b) |
| *R. grassei* | Devon-ENG | | 1 | This study |
| | Ile d'Oleron FRA | | 1 | This study |
| | Bilbao-SPN | | 1 | Marini & Mantovani (2002) |
| *R. labralis* | Hong Kong-CHN | | 1 | This study |
| *R. malletei* | DE-USA | RM1, RM2 | 9 | Whitney-King *et al*. (2007) |
| | GA-USA | RM1 | 4 | This study |
| | MD-USA | RM1, RM2 | 2 | This study |
| | NC-USA | RM1, RM2 | 5 | This study |
| | SC-USA | RM2 | 2 | This study |
| *R. tibialis* | TX-USA | T1 | 1 | Austin *et al*. (2004a) |
| | TX-USA | T2 | 1 | Austin *et al*. (2004a) |
| | TX-USA | T3 | 1 | Austin *et al*. (2004a) |
| | TX-USA | T4 | 1 | Austin *et al*. (2004a) |
| | TX-USA | T5 | 1 | Austin *et al*. (2004a) |
| | TX-USA | T6 | 1 | Austin *et al*. (2004a) |
| | OK-USA | T7 | 1 | Austin *et al*. (2004b) |
| | OK-USA | T8 | 1 | Austin *et al*. (2004b) |
| | UT-USA | T9 | 1 | This study |
| | UT-USA | T10 | 1 | This study |
| *R. virginicus* | TX-USA | V1 | 1 | Austin *et al*. (2004a) |
| | OK-USA | V2 | 1 | Austin *et al*. (2004b) |
| | AR-USA | V3 | 1 | Austin *et al*. (2004c) |
| | LA-USA | V4 | 1 | Austin *et al*. (2004c) |
| | AR-USA | V5 | 1 | Austin *et al*. (2004c) |
| | GA-USA | V6 | 1 | This study |
| | MO-USA | V7 | 1 | This study |
| | MS-USA | V8 | 1 | This study |

**Photographs.** Soldier and alate NEOTYPE specimens, collected from Georgia in 1987 (A.-G. Bagnères) were taken using Auto-Montage digital 3-D Syncroscopic software (Synoptics Ltd., UK) attached to an Olympus YS2-T compound microscope (Tokyo, Japan). Additional photos were taken with an Olympus SZX12 microscope equipped with a Olympus DP12 digital camera system (Olympus Optical, Tokyo, Japan).

## Results

**Genetic Analysis.** DNA sequencing of the 16S rRNA amplicon revealed that it averaged 428 bp in size. The average base frequencies were A = 0.39, C = 0.23, G = 0.14, and T =0.24. To facilitate comparisons with other samples from GenBank, 25 bp from the 3' end of the 16S rRNA gene were removed and samples were compiled into our existing database for analysis. *Reticulitermes malletei* sequence data (represented by either haplotype RM1 or RM2) from Delaware, Georgia, Maryland, North Carolina, and South Carolina, were distinct from all other taxa, forming a sister clade to *R. flavipes*. This distinction was well supported by bootstrap analysis (Fig. 9), and the proposed relationship was consistent with previous data from Clément *et al*. (1986a). Pairwise Tajima-Nei distances (Tajima and Nei 1984) among *Reticulitermes* from North American taxa ranged from 1.8% between *R. malletei* and *R. hageni* (haplotype H2 from Arkansas), to 3.5% between *R. malletei and R. tibialis* (Utah haplotype T10). There was little genetic variation among *R. malletei*, even from disjunct groups in the four states presented in this study with only two distinct haplotypes RM1 and RM2. Comparisons to taxa from outside North America resulted with larger distances, ranging from 3.1% to 4.0% with *R. malletei* and *R. sp. nov.* (= *R. urbis* Bagnères), France, and *R. malletei* and *R. labralis* from Hong Kong, respectively.

The aligned DNA data matrix, including the outgroup taxa resulted in a total of 403 characters. Of these characters, 86 (21%) were variable and 63 (16%) were phylogenetically informative. Bootstrap analysis of the aligned *Reticulitermes* species and the outgroup taxa resulted in a consensus tree (Fig. 9), (length = 200, CI = 0.570, RI = 0.778), as documented using the Branch and Bound search algorithm of PAUP*. Phylogenetic relationships among disjunct *Reticulitermes* outside North America were poorly resolved (Figs. 9 and 10). However, *Reticulitermes* from North America formed distinct clades and no polytomy was observed: *R. flavipes* shared a sister clade to *R. malletei*, but both were clearly distinct with strong bootstrap support (Fig. 10); both *R. hageni* and *R. virginicus* were more clearly resolved with maximum likelihood trees, where they formed a sister clade (Fig. 10). Additionally, *R. hesperus* and *R. tibialis* shared a sister clade, implicating their more western distributions in the United States. With the exception of *R. malletei*, relationships among these *Reticulitermes* groups are consistent with our previous studies (Austin *et al*. 2002, 2004a, b, c, Szalanski *et al*. 2003).

Regardless of whether the starting tree was the most parsimonious tree or was obtained via step-wise addition, the maximum-likelihood search found only one tree (Fig. 10). The maximum-likelihood tree did not vary much from the maximum-parsimony tree (Fig. 9), but *R. malletei* is clearly delimited from all other known North American *Reticulitermes* groups, suggesting genetic isolation from other sympatric species. Likewise a clear delimitation between eastern and western nearctic species (Fig. 10) supports geographic isolation of the genus in North America via glacial refugia routes (Austin *et al*. 2006b).

**Cuticular hydrocarbon analysis.** Workers of *R. malletei* weigh on average 1.8mg, whereas the average weight for *R. flavipes* and *R. grassei* was 2.8 and 2.4 mg, respectively. Although not evaluated in the cuticular hydrocarbon analysis, the workers of *R. hageni* weigh on average 1.7 mg. Quantification of total worker hydrocarbons gave 45 ng /mg per termite for *R. malletei*, 50 ng for *R. flavipes*, and 80 ng/mg per termite for *R. grassei*. Hydrocarbons from soldiers were shown to posses 150 ng/mg per termite for *R. malletei*, 300 ng/mg for *R. flavipes*, and 700 ng/mg per termite for *R. grassei*. There is no apparent relationship between HCs and the weight of individuals. It was interesting to note that there are qualitatively more common HCs between *R.*

*malletei* with the other US species (20% HCs common with *R. flavipes*) than with European species such as *R. grassei* (8%). *R. malletei* is the only *Reticulitermes* species to have no chemical polymorphism between workers and soldiers (Fig 4). The relative proportions of different chemical families being identical (i.e. 9 to 11% of monoenes (C23:1 to C27:1), 24 to 25 % of dienes (C25:2 to C29:2), 25 to 26 % of trienes (two C29:3) for unsaturated; 17 to 19% of mono-methyl branched alkanes and 22 to 23% of n-alkanes both ranking from C23 to C27) (Figure 4, Table 2).

**Aggression index analysis.** *Intra-specific aggression index*. The average between the 11 tests is very low (Ag=10.90, SD= 25.53). The high standard deviation is due to one test with an index of 85.6, and a second with 21.25, the others with range between 0 to 4.5. We might assume that this species has open colonies (i.e. non aggressive). We had also a low aggression index for the other American species *R. flavipes* (N=25, Ag=7.49 in Winter; N=26, Ag= 3.57 in Summer (from Clément and Bagnères, 1998). This changes with the data from European species having generally higher index values (Bagnères 1989, Clément and Bagnères 1998, Clément *et al.* 2001). It is interesting to note that in June 1988 we found sexuals in different colonies (in November we had colonies with several nymphs), but it did not change the agonistic behavior.

*Inter-specific aggression index*. The average between the 23 tests *R. malletei* versus *R. flavipes* was relatively high (Ag= 60.93, SD= 26.33) with 20 positive tests).

**Biometric analysis.** From two distinct 16S rRNA haplotypes (RM1 and RM2): mean soldier head capsule width and length were 0.906 and 1.553 mm, respectively with a ratio of 1.714; Mandible lengths were 0.896 and 0.914 mm for left and right mandibles, respectively; Head width and length with mandibles were 0.908 and 2.351 mm, respectively, with a ratio of 2.590; Gular measurements were 2.897, 6.995 and 0.896 mm for width ‘w’, width ‘n’, and length, respectively with ratios ‘w’ and ‘n’ of 0.914 and 0.494; Pronotal measurements were 1.612, 1.780, and 0.397 mm for width ‘w’, width ‘n’, and length, with ratios 0.397 and 1.254 for ratios ‘w’ and ‘n’, respectively; labral measurement were 0.908 and 2.351 mm for width and length, respectively with a ratio of 2.590. Comparisons of soldier labral morphology appeared most capable of distinguishing *R. malletei* from all other congeners (Figure 6). However, distinct morphological differences in size could be observed in the soldier pronota (Figure 4) and Head capsules (Figure 5). Worker specimen measurements were more equivocal, but clear differences among some taxa were observed (Figure 3).

**Types:** HOLOTYPE Colony. Athens, GA-USA. Samples from 1984, 1987, and 1989 are maintained by l’Institut de Recherche sur la Biologie de l'Insecte (C.N.R.S., Université de Tours), France. PARATYPE Colony Lewes, DE-USA, Elkton, MD-USA, Duke Forest, NC-USA, Clemson, SC-USA collected from 1997-2005 are maintained at the University of Arkansas, Department of Entomology Museum. NEOTYPE specimens from Lewes, DE-USA will be submitted to the American Museum of Natural History (AMNH), New York, New York, and to the Smithsonian Institution, Invertebrate Zoology Section, National Museum of Natural History (NMNH) and assigned catalog accession transaction number 2043708).

**Etymology.** The origin of this species name is attributed to E. J. Mallette, a USDA forestry researcher working for the Forestry Sciences Laboratory, Southern Forests Experiment Station in Gulfport, Mississippi along with R. W. Howard. Dr. Howard alerted J. L. Clément about the possibility of an unknown cryptic species from the area when the original HOLOTYPE specimens were procured and subsequently evaluated. Both Drs. Howard and Clément thought it fitting to name the termite in honor of E. J. Mallette. Although the original publication of Clément *et al.* (1986a) did not provide a complete comparative description of the species and was subsequently relegated by Scheffrahn *et al.* (2001) to *nomen nudem* status via Article 13 of the International Code of Zoological Nomenclature (ICZN), this application was likely based on incomplete data. By definition, *R. malletei* meets the criterion of “description or definition” requirement that is contained in the first subsection of Art. 13 of the ICZN (Art. 13(a)(i) of the 3rd Edition, 1985; Art. 13.1.1 of the 4th Edition, 1999), and thus, *R. malletei* can not be considered unavailable on the basis of failing that subsection.

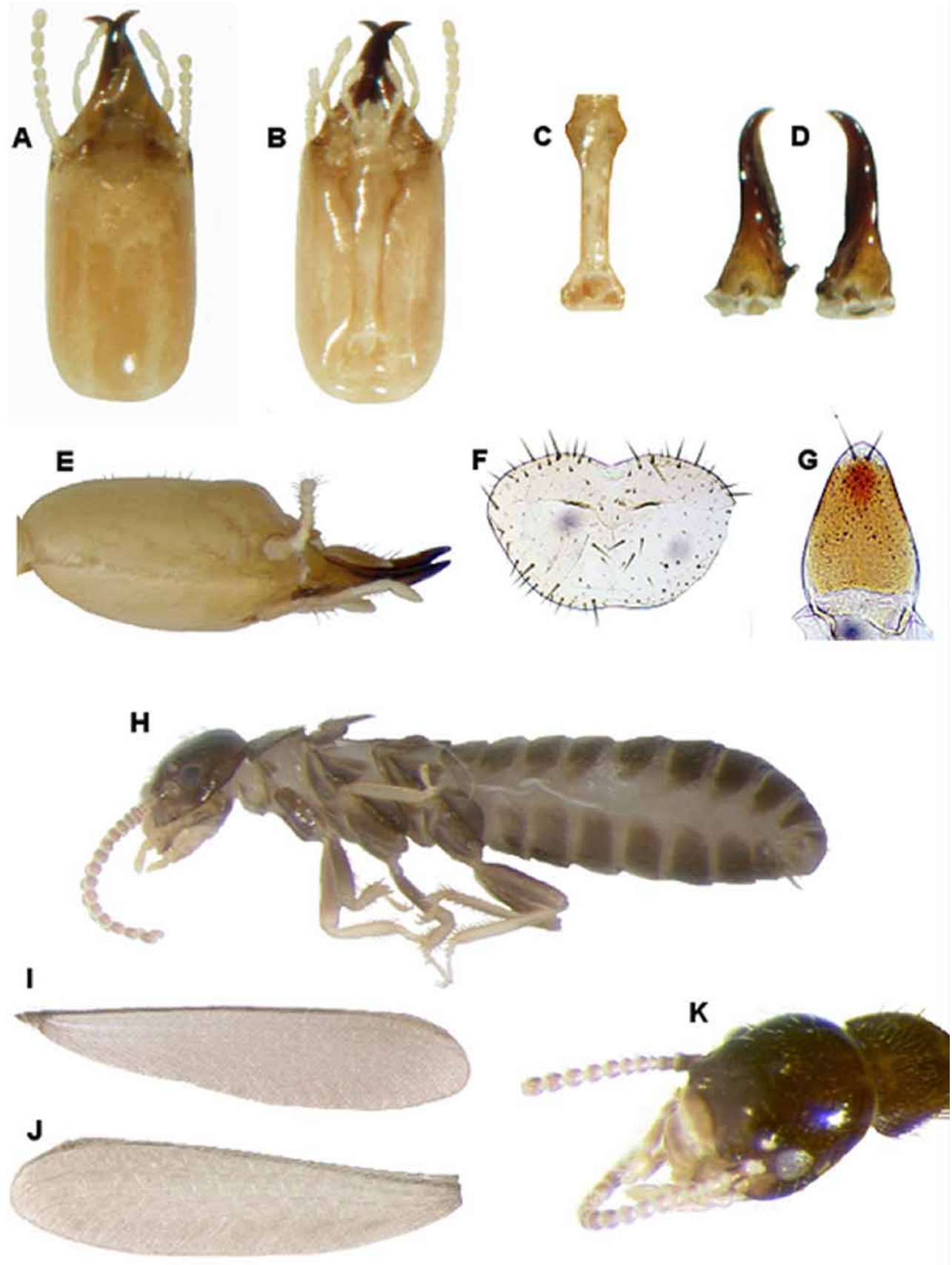


**FIGURE 1.** Automontage and slide mounted photographs of *R. malletei* from Georgia, collected in 1987: A) dorsal view of soldier head capsule with mandibles, B) ventral view of soldier head capsule with mandibles, C) soldier postmentum, D) soldier mandibles, E) pleural view of soldier head capsule with mandibles, F) soldier pronotum, G) soldier labrum, H) alate pleural view, I) alate forewing, J) alate hindwing, and K) alate head capsule.

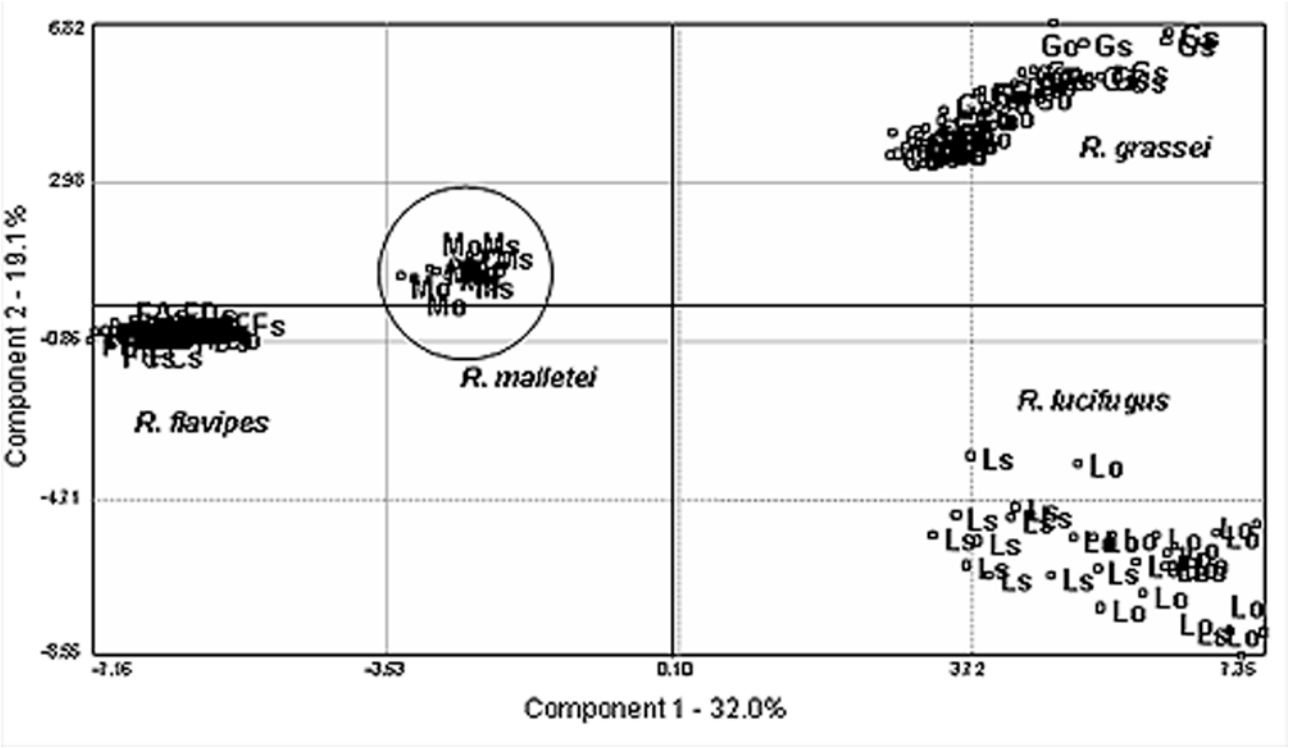


**FIGURE 2.** Principal component analysis of epicuticular hydrocarbons from *R. malletei*, *R. flavipes from Georgia (USA), R. lucifugus* from France and Italy, and *R. grassei* from France and Spain.

While it is clear that Clément *et al*. (1986a) did not provide an extensive, traditional, morphological description of the new species, they do appear to have met the "definition" criterion. For nomenclatural purposes, a "definition" is defined in the ICZN glossary as: "A statement in words that purports to give those characters which, in combination, uniquely distinguish a taxon" [4th Edition]; and "A statement in words that purports to give characters differentiating a taxon" [3rd Edition]. For clarification of findings from Clément *et al*. (1986a), it is important to note some important criteria for our justification in validating this taxonomic group on the basis of 'availability.' The ICZN Code does not require that a publication have in it a "diagnosis section"; there is no provision that requires that an author must discuss (although this would be helpful for subsequent scientists) any or all other apparently closely related or possibly identical species in order to establish the "availability" of a new name (e.g., Clément *et al*. omitted the congener *R. hageni* from their study); The Code contains very few, and very restricted, provisions under which the use of some "improper" names in a publication might affect the proper establishment of other new names contained in the same publication ((e.g., Clément *et al*. (1986a) used '*flavipes II*' to describe another taxa)). While there were no TYPE specimens provided in the original paper by Clément *et al*. (1986a), the requirement that a primary TYPE be fixed in the original publication containing the establishment of a new name was not incorporated into the Code until the 4th Edition, with effective date 1 January 2000 (see Art. 16.4). Clearly, Clément *et al*. did not fix a TYPE for "*malletei*" in the original publication. However, because the specific name "*malletei*" was proposed in 1986, Art. 16.4 does not apply to it. Consequently, whether or not its type was or was not fixed in its original publication is irrelevant to whether or not the name "*malletei*" is available. If the "recognition" of what Clément *et al*. (1986a) meant by the name "*malletei*" is an issue, the Code contains ample provisions for fixing this identity through the application of subsequent type designation procedures (e.g., lectotype or neotype; see Art. 74 & 75).

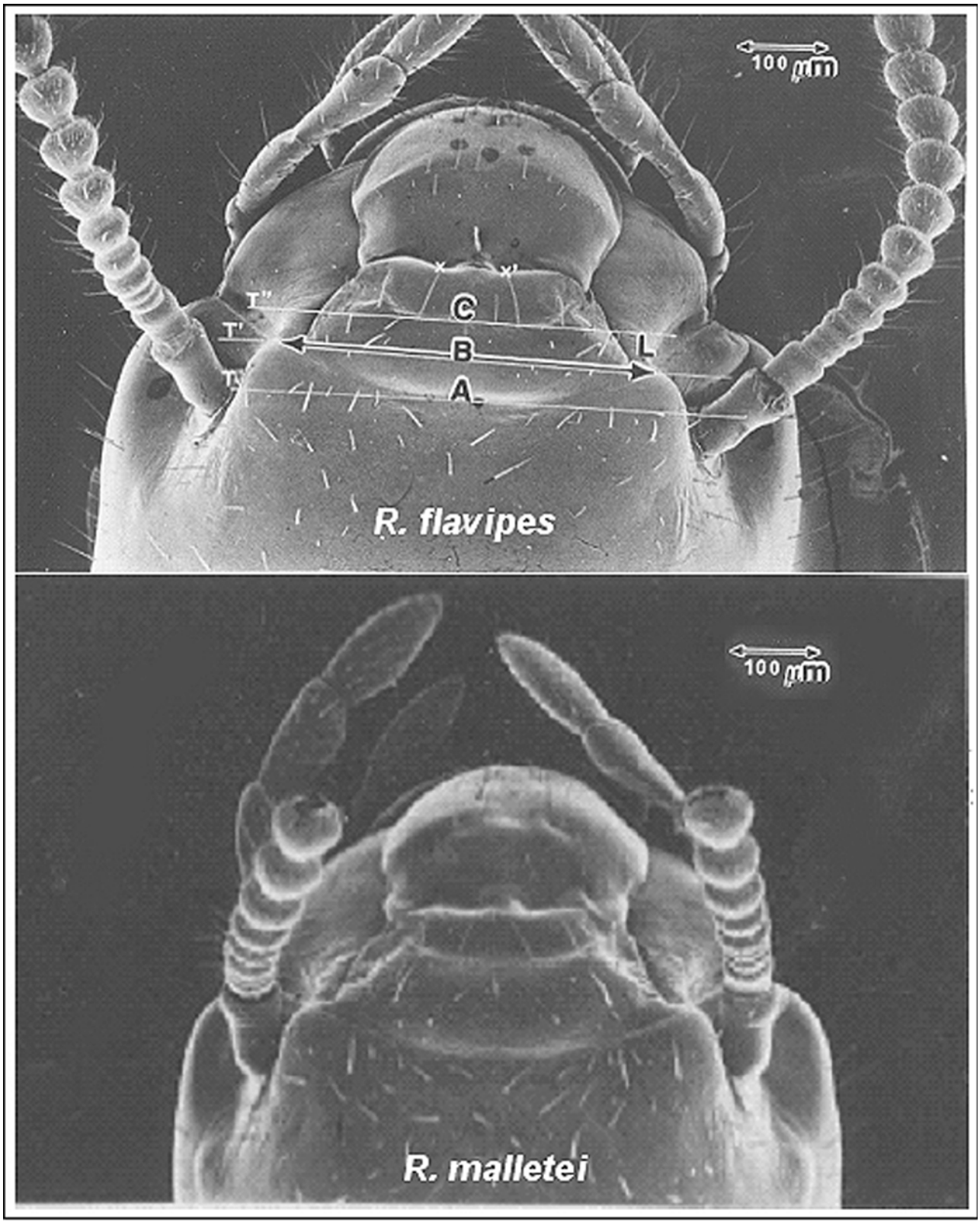


**FIGURE 3.** Scanning electron microscope (SEM) photography of: *Reticulitermes flavipes* worker (top) and *Reticulitermes malletei* worker, (bottom). Biometric measurements were taken to calculate our index: (distance AB + BC) + length L as described in Bagnéres *et al.* (1990). This procedure was repeated for both western Palearctic and eastern Nearctic *Reticulitermes* species.

**TABLE 2.** Hydrocarbons identified by GC-MS EI and CI for *R. malletei*.

| Code Peak[a] | Compound | CN[b] | ECL[c] | Diagnostic | EI-MS | CI-MS |
|---|---|---|---|---|---|---|
| T | *n*-C21 | 21 | 21.00 | | 296 | 295 |
| T | *n*-C22 | 22 | 22.00 | | 310 | 309 |
| e1 | 9-C23: 1 | 23 | 22.65 | | 322 | 323 |
| a2 | *n*-C23 | 23 | 23.00 | | 324 | 323 |
| m3 | 11Me-C23 | 24 | 23.36 | 168/169,196/197 | 338 | 337 |
| m4 | 4/2Me-C23 | 24 | 23.61 | 294/295 | 338 | 337 |
| m5 | 3Me-C23 | 24 | 23.72 | 308/309 | 338 | 337 |
| a6 | *n*-C24 | 24 | 24.00 | | 338 | 337 |
| T | 12+11Me-C24 | 25 | 24.38 | 182/183,196/197 | 352 | 351 |
| | | | | 168/168,210/211 | 352 | 351 |
| m7 | 4/2Me-C24 | 25 | 24.64 | 308/309 | 352 | 351 |
| T | 3Me-C24 | 25 | 24.70 | 322/323 | 352 | 351 |
| T | *x*-C25: 1 | 25 | 24.72 | | 350 | 351 |
| | + *x*, *y*-C25: 2 | 25 | 24.72 | | 348 | 349 |
| a8 | *n*-C25 | 25 | 25.00 | | 352 | 351 |
| m9 | 13+11Me-C25 | 26 | 25.37 | 196/197 | 366 | 365 |
| | | | | 168/169,224/225 | 366 | 365 |
| m10 | 4/2Me-C25 | 26 | 25.66 | 322/323 | 366 | 365 |
| m11 | 3Me-C25 | 26 | 25.75 | 336/337 | 366 | 365 |
| a12 | *n*-C26 | 26 | 26.00 | | 378 | 379 |
| me13 | 11Me-C26 | 27 | 26.35 | 168/169, 238/239 | 380 | 379 |
| | + *x*-C27: 1 | 27 | 26.35 | | 378 | 379 |
| m14 | 4/2Me-C26 | 27 | 26.63 | 336/337 | 380 | 379 |
| e15 | 9-C27: 1 | 27 | 26.71 | | 378 | 379 |
| a16 | *n*-C27 | 27 | 27.00 | | 380 | 379 |
| n17 | *x*, *y*-C27: 2 | 27 | 27.08 | | 376 | 377 |
| n18 | *z*, *w*-C27: 2 | 27 | 27.13 | | 376 | 377 |
| n19 | *u*, *v*-C27: 2 | 27 | 27.33 | | 376 | 377 |
| n20 | 11Me-C27 | 28 | 27.35 | 168/169,252/253 | 394 | 393 |
| e21 | *x*-C28: 1 | 28 | 27.38 | | 392 | 393 |
| T | 8+9-C28: 1 | 28 | 27.75 | | 394 | 393 |
| T | *n*-C28 | 28 | 28.00 | | 404 | 405 |
| n22 | *x*, *y*-C29: 2 | 29 | 28.30 | | 404 | 405 |
| n23 | *x*, *w*-C29: 2 | 29 | 28.38 | | 406 | 407 |
| T | 8-C29: 1 | 29 | 28.70 | | 408 | 407 |
| T | *n*-C29 | 29 | 29.00 | | 402 | 403 |
| t24 | *x*, *y*, *z*-C29: 3 | 29 | 29.03 | | 402 | 403 |
| t25 | *u*, *v*, *w*-C29: 3 | 29 | 29.12 | | 402 | 403 |
| n26 | *x*, *y*-C-30: 2 | 30 | 29.32 | | 418 | 419 |
| n27 | *u*, *v*-C29: 2 | 29 | 29.41 | | 404 | 405 |
| T | *x*-C30: 1 | 30 | 29.77 | | 420 | 421 |
| T | *n*-C30 | 30 | 30.00 | | 422 | 435 |
| T | *x*-C31: 1 | 31 | 30.79 | | 434 | 435 |
| T | *n*-C31 | 31 | 31.00 | | 436 | 435 |
| T | *n*-C32 | 32 | 32.00 | | 450 | 451 |
| T | *n*-C33 | 33 | 33.00 | | 464 | 463 |
| m28 | 13Me-C33 | 34 | 33.30 | 196/197,308/309 | 478 | 477 |
| T | *n*-C34 | 34 | 34.00 | | 478 | 479 |

[a]a = alkane, e = monosene, n = diene, m = monomethyl branched, d =dimethyl branched, and x = unknown.

[b]CN = carbon number

[c]ECL = equivalent chain length

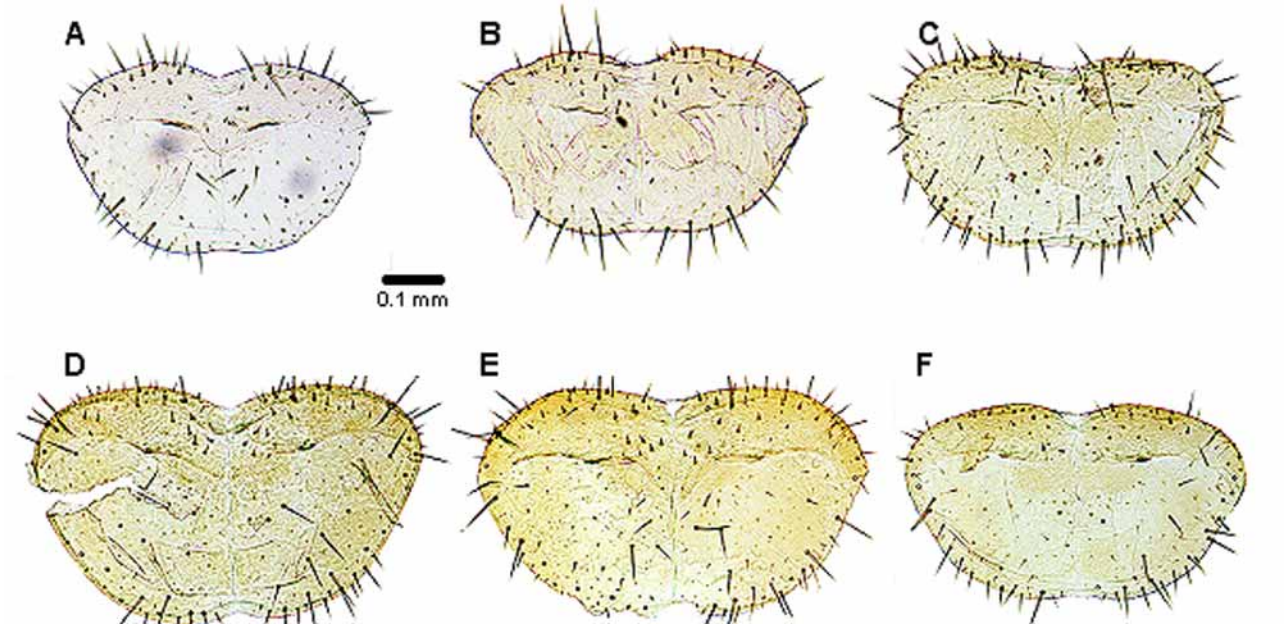


**FIGURE 4.** Slide mounted comparisons of Soldier Pronota from (A) *Reticulitermes malletei* (B) *R. virginicus* (C) *R. hageni* (D) *R. flavipes* (E) *R. tibialis* (F) *R. hesperus.*

Therefore, it is our opinion that Clément *et al*. (1986a) clearly provided, in text, several characters whose combination was purported to distinguish or differentiate the new species, thus meeting the "definition" criterion; and thus meeting Article 13.1. As the remaining subsections of Art. 13 do not apply to this case, *R. malletei* does not appear to fail Art. 13. Thus, since there are no other articles (from ICZN) applied to the application of *nomen nudem* for this species on which to base the unavailability of *R. malletei* that can be demonstrated, we propose that this name is nomenclaturally available, and thus can be used as the valid name for a species.

## Discussion

Clément *et al*. (1986a) provided evidence of three strong species isolation mechanisms for *R. malletei*. Reproductive male alates were evaluated in olfactometer studies by exposing them to hexanic extracts of the sternal glands of females to test for response. When exposed to *R. flavipes* extracts, *R. malletei* males responded positively 19% of the time to *R. flavipes* versus 81% to *R. malletei.* This was repeated with an additional, but different population of *R. flavipes* (referred to as "*R. flavipes II"* in Clément *et al*. 1986a), which responded positively only 25% of the time to *R. malletei* versus 75% by another *R. malletei* population (n = 33 and n = 36, respectively). When applying the same strategy to *R. virginicus*, which swarms at the same time, *R. malletei* males responded positively only 28% of the time, whereas *R. virginicus* did not respond at all 0%, (n = 50 and n=30, respectively). This simple test, provides significant evidence that chemical pheromones play a significant role in species isolation of *R. malletei* from sympatric populations of *R. flavipes* and *R. virginicus* (Clément *et al*. 1986a).

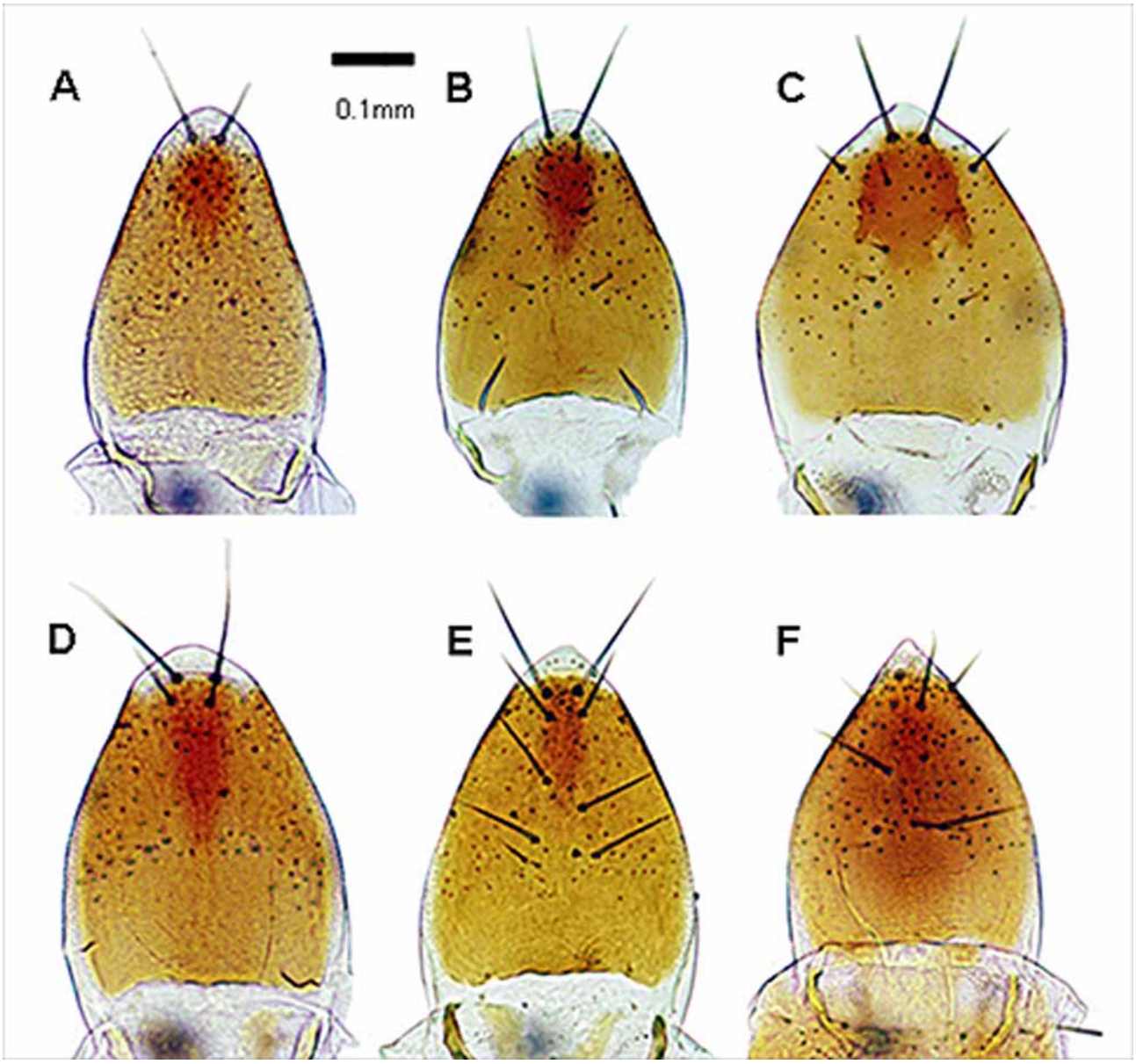


**FIGURE 5.** Slide mounted comparisons of Soldier labra from (A) *R. malletei* (B) *R. hageni* (C) *R. virginicus* (D) *R. flavipes* (E) *R. tibialis* (F) *R. hesperus*

In their preliminary study on this species, Clément *et al*. (1986a) suggests that alate swarming flights occur between May 30th and the 6th of June in the forests near the University of Georgia. Subsequent collecting endeavors at the type locality from where the original HOLOTYPE specimens were collected a few years later support the swarming dates proposed by Clément (Bagnères, unpublished). Furthermore, collections of alates from the northern identified range of this species from 1998, 2001, 2005, and 2006 in Delaware also support these dates (Whitney-King, personal communication). Asynchrony of alate dispersal flights from another congener, *R. hageni*, appears to exclude this group as a possible synonym for *R. malletei*. *Reticulitermes hageni* alate flights from Florida usually begin in early December and last until early February (Scheffrahn *et al*. 1988) and do not appear to overlap with *R. malletei*. Also, *R. hageni* frequently swarm at night while *R. malletei* have only been observed to swarm during the day. A comparison of HOLOTYPE samples from 1987 were genetically evaluated with other unknown *Reticulitermes* sp. sequenced during a survey of Nearctic *Reticulitermes* (Austin, unpublished data). Isolation between *R. flavipes* and *R. malletei* can be observed because of the high level of agonism, and can be reinforced by sexual attraction pheromones and

contact pheromones (i.e. HCs). Clément *et al*. (1986a) show that 75% males of *R. malletei* always prefer their own species over *R. virginicus*. Clément *et al*. (1986a) suggest that the reproductive alates of *R. malletei* are easily distinguished from *R. virginicus* by the black color of their wings. *Reticulitermes virginicus* alate wings are described as clear (Krishna and Weesner 1970). Alates of *R. hageni* can not be mistaken for either *R. virginicus* or *R. malletei* due to their smaller size and pale brown coloration. Similarly, alates of *R. flavipes* are generally much larger and darker than any other eastern Nearctic species. Although nearly all alates observed in Georgia during the 1987 collections and subsequent collections in 1989 were small with black wings, we suggest that wing coloration can be variable, and should be applied to species descriptions carefully. Clément also discussed the application of aggression indexes to discern these sympatric species. The aggression index for *R. malletei* also suggests isolation from other sympatric species.

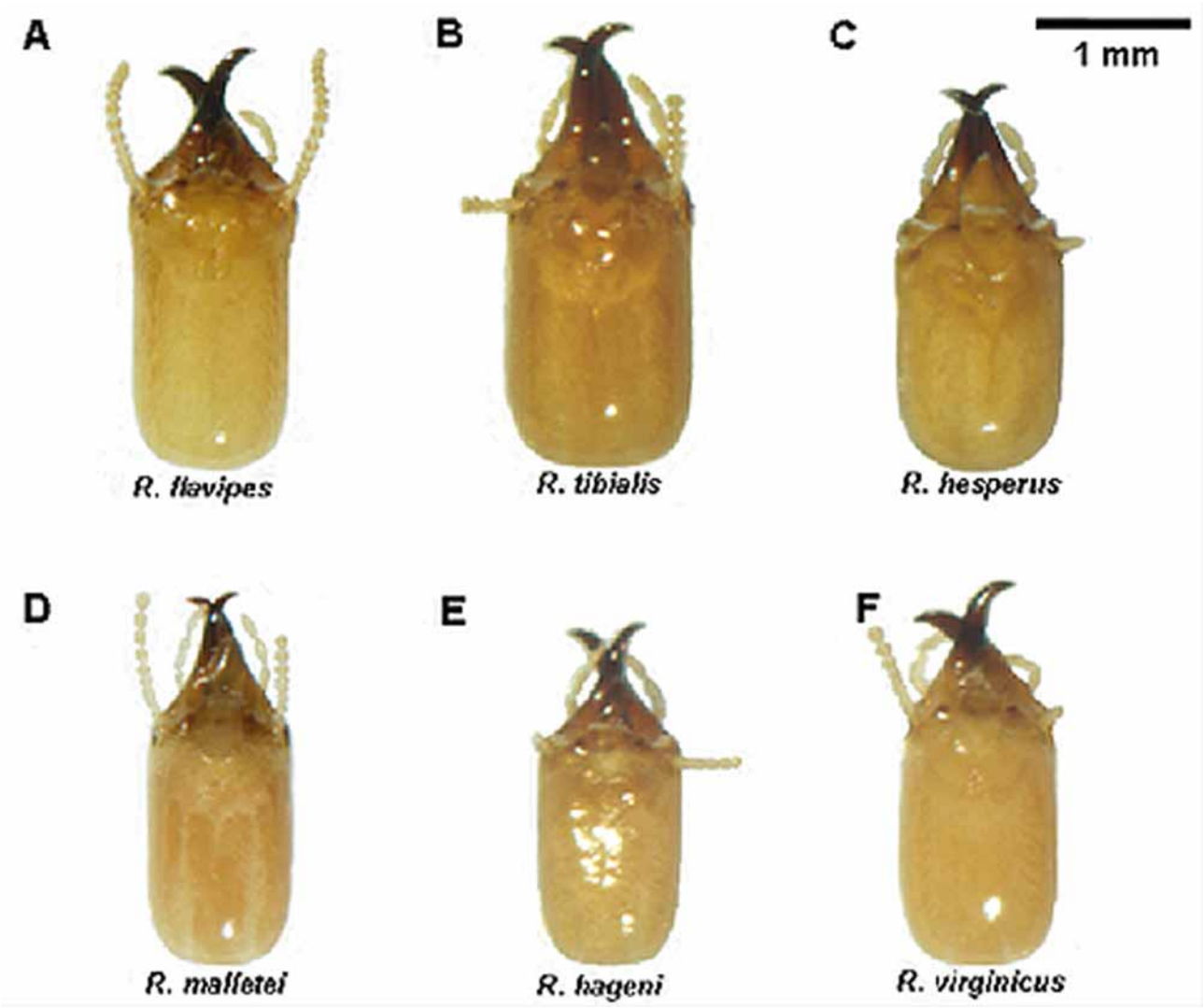


**FIGURE 6.** Comparison of Soldier head capsules from (A) *Reticulitermes flavipes* (B) *R. tibialis* (C) *R. hesperus* (D) *R. malletei* (E) *R. hageni* (F) *R. Virginicus.*

**TABLE 3.** Mean inter- and intraspecific Agression (Ag) index ( SE for $P > .95$). Ag index between two colonies is (M + (m/2)) x 2.5, where M is the mean numer of dead workers, m of injured workers (without antennae or one leg) in each assay. A confrontation between two colonies is composed of five assays of a mixture of 40 workers (20 from each colony) in a 80 mm petri dish during 15 hrs. A positive (+) aggressive response and negative ( - ) response to each confrontation is indicated between parenthesis (from Clément *et al*. (1986)).

| Colonies[1] | | (Ag)[2] | Result |
|---|---|---|---|
| *R. flavipes* / *R. malletei* | *R. malletei* / *R. malletei* | | |
| 1-1 / 1-2 | | 69.50 | (+) |
| 1-1 / 3-3 | | 24.25 | (+) |
| 1-3 / 1-2 | | 76.50 | (+) |
| 1-3 / 1-4 | | 45.75 | (+) |
| 1-3 / 2-2 | | 76.25 | (+) |
| 1-3 / 4-1 | | 69.50 | (+) |
| 1-3 / 4.3.1 | | 67.25 | (+) |
| 2-1 / 2-2 | | 69.75 | (+) |
| 2-1 / 3-3 | | 13.25 | (-) |
| 3-4 / 1-4 | | 86.50 | (+) |
| 3-4 / 2-2 | | 26.25 | (-) |
| 4.3.2 / 1-4 | | 63.50 | (+) |
| 4.3.2 / 4-1 | | 83.25 | (+) |
| 4.3.2 / 4.3.1 | | 76.50 | (+) |
| 5-1 / 1-4 | | 62.00 | (+) |
| 5-1 / 5-2a | | 81.25 | (+) |
| 5-2b / 2-2 | | 50.75 | (+) |
| 6-1 /1-4 | | 95.25 | (+) |
| 6-2 / 1-4 | | 79.25 | (+) |
| 7 / 1-4 | | 99.75 | (+) |
| 8 / 1-4 | | 30.50 | (+) |
| 8 / 5-2a | | 53.25 | (+) |
| 9 / 1-4 | | 1.50 | (+) |
| n = 23 independent tests Mean Aggression = 60.93 ± 26.33 | | | |
| | 1-2 / 1-4 | 4.50 | (-) |
| | 1-2 / 3-3 | 2.00 | (-) |
| | 1-4 / 2-2 | 3.00 | (-) |
| | 1-4 / 3-3 | 0.00 | (-) |
| | 1-4 / 4-1 | 0.00 | (-) |
| | 1-4 / 4.3.1 | 0.00 | (-) |
| | 2-2 / 3-3 | 21.25 | (-) |
| | 2-2 / 4-1 | 85.60 | (+) |
| | 2-2 / 4.3.1 | 0.00 | (-) |
| | 3-2 / 3-3 | 0.00 | (-) |
| | 3-3 / 4-1 | 3.50 | (-) |
| N = 11 independent tests Mean Aggression = 10.90 ± 25.53 | | | |

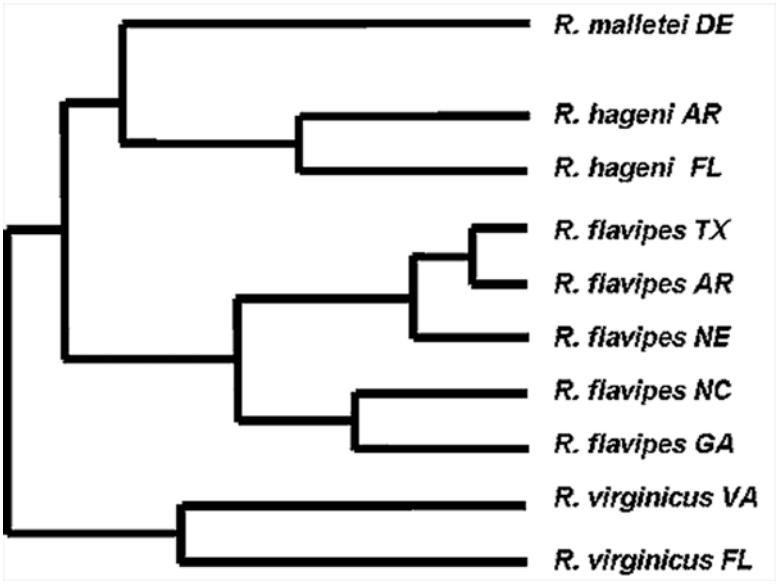


**FIGURE 7.** Dendogram from a cluster analysis of Nearctic *Reticulitermes* soldier labra applying Ward's method to the Labra ratio and labra width as described in Heintschel *et al*. (2006).

In agonistic bioassays performed in 1983, exposure of 40 workers, 20 from each pairing with 5 replicates (n =100 workers), demonstrated lower aggression within *R. malletei* (Ag = 19), than was recorded among *R. flavipes* (Ag = 79) or *R. virginicus* (Ag = 86) (Clément *et al*.1986a). This result is clearly supported from our genetic evaluation of these groups. Tests conducted between *R. malletei* and *R. flavipes* 4 years later consistently produced a very high aggression index (Ag = 65). This confirms the permanent aggressive pattern between the 2 species, inhibiting introgression in Georgia. We could not confirm the result with *R. virginicus* obtained in 1983, because we were not able to find this species at the same time as *R. malletei* the following years. It is also unclear from recent collections of *R. malletei* from Delaware if these patterns are consistent in more northern distributions of the species. As was observed in Georgia, there were synchronous alate dispersal flights of both *R. virginicus* and *R. malletei* (Whitney-King *et al*. 2007), and these dates seem to be corroborated by flight records (of *R. virginicus*) in Delaware occurring from May 18th through June 21st and in Maryland from May 19th to June 7th for *R. virginicus* (Krishna and Weesner 1970). Dispersal flights of *R. hageni* generally occur later in the year for this region, from July through August, with some forms slightly darker than "typical" *R. hageni* (Weesner 1965). These seemingly different forms and the lack of robust morphological comparisons from various Nearctic regions have made the identification of this cryptic species difficult.

In a recent study by Whitney-King *et al*. (2007), DNA sequences from *Reticulitermes* collected on the Delmarva Peninsula, Delaware and Maryland, originally thought to be *R. virginicus*, were in fact *R. malletei*. This study evaluated the pronotal width for 1,447 soldiers taken from 33 colonies, and pronotal width range for *R. virginicus* soldiers in Lewes, DE (0.63-0.83 mm.) overlaps with that of *R. hageni* in Florida (Hostettler *et al*. 1995), a species not presently found in Delaware. In fact, it was the incongruence with published measurements that alerted the senior author to the possibility that these termites could actually be *R. malletei*. Still further investigations into the abundance of *R. malletei* in Delaware suggests a strong reproductive advantage in the formation of 3rd form replacement reproductives when compared to its principle congener *R. flavipes* (Whitney-King, S., personal communication). This is an important biological difference when considering the succession of competing species for available food resources.

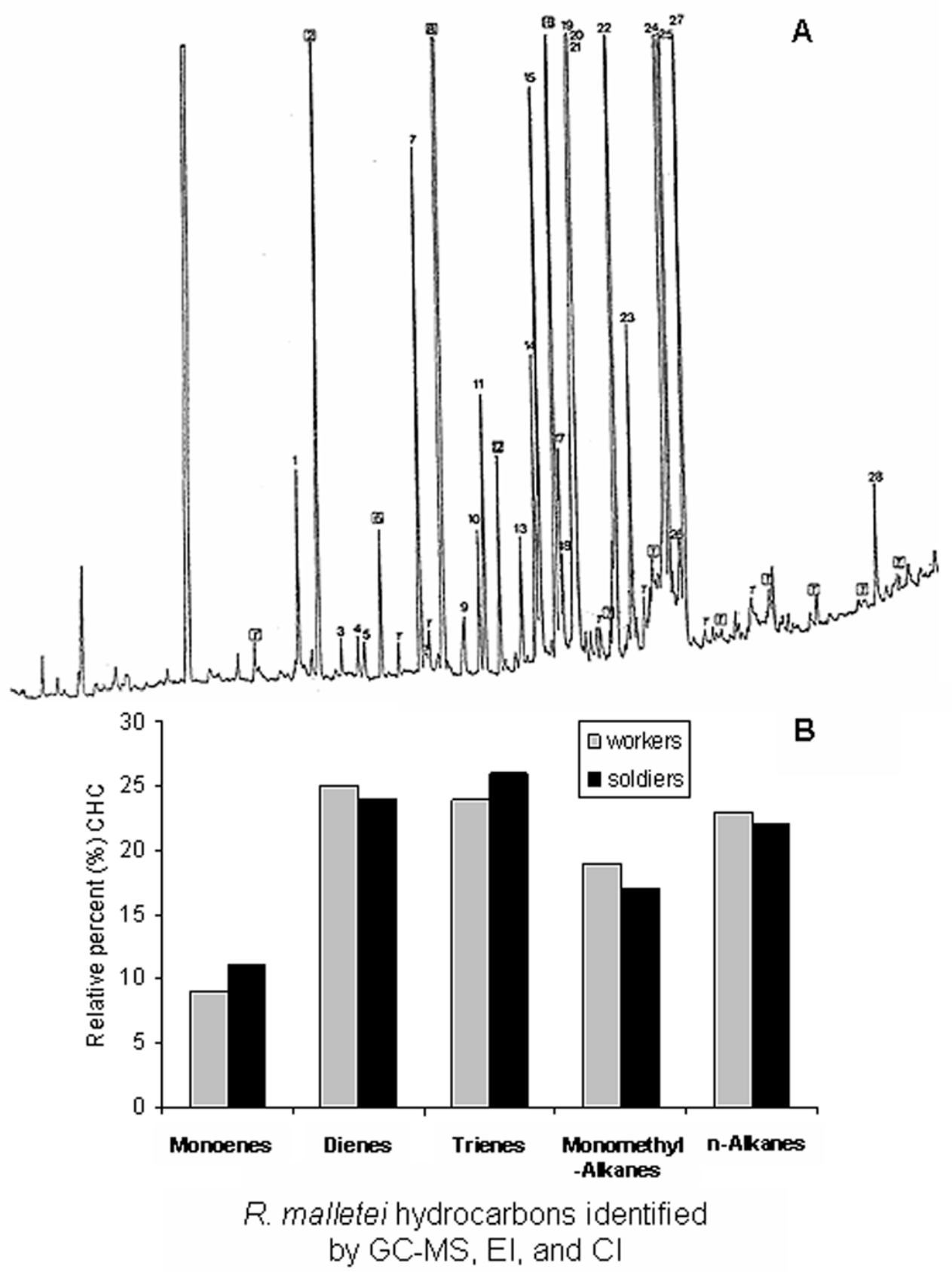


**FIGURE 8.** (A) Gas chromatogram of cuticular hydrocarbons (HCs) of both soldiers and workers of *R. malletei*. Peaks and associated HCs are listed and described in Table 2. (B) Hydrocarbon proportions for workers and soldiers of *R. malletei* were not significantly different at $\alpha = .05$ level when evaluated by ANOVA.

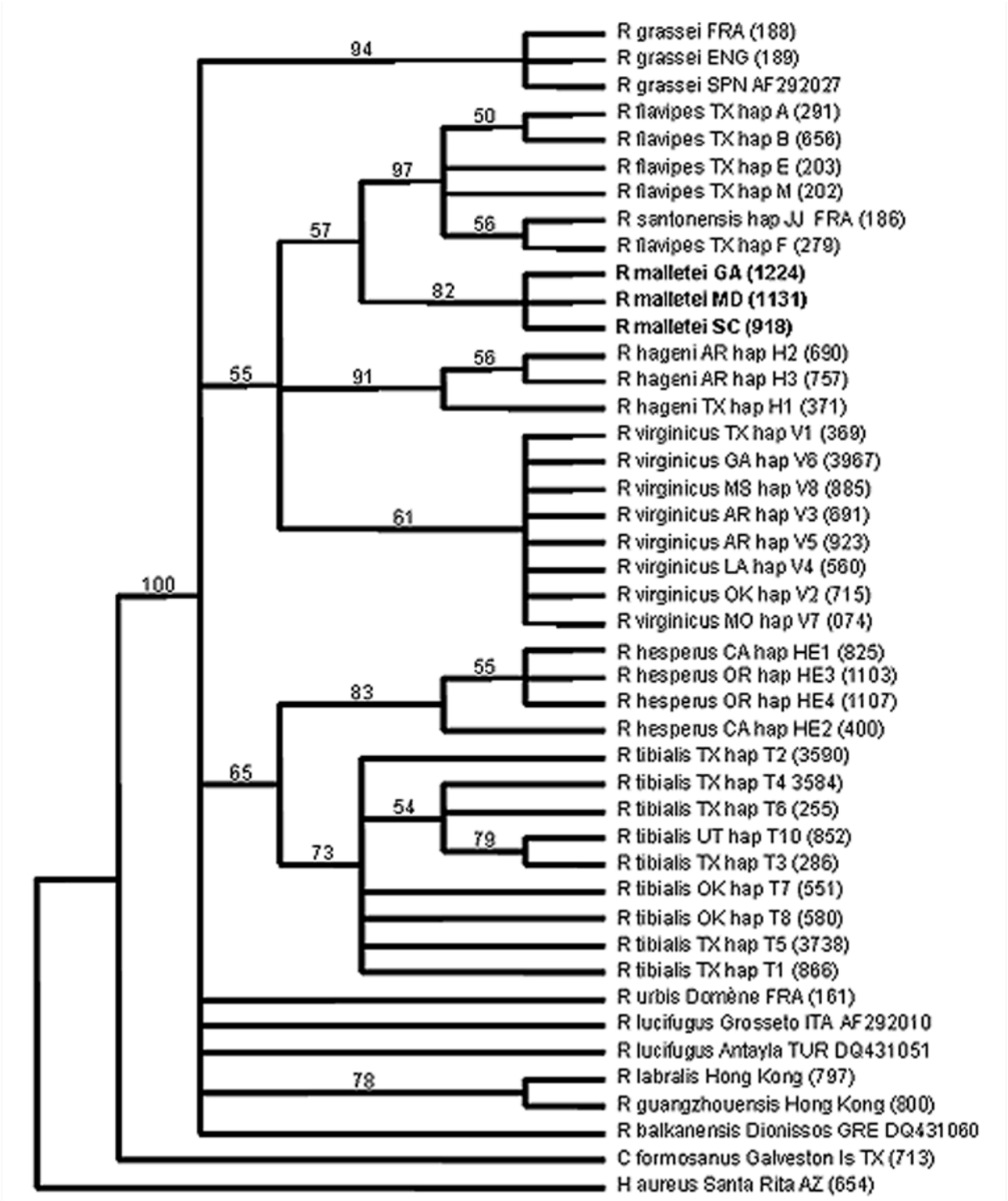


**FIGURE 9.** Single most parsimonious tree during a branch and bound search using PAUP* (Swofford 2001). Bootstrap values for 1,000 replicates are listed above the branches supported at =50%.

When evaluating *Reticulitermes* from both Southern Piedmont and Atlantic coastal Flatwoods in Georgia, Jenkins *et al*. (2000) identified two populations of *Reticulitermes* that could not be identified with available taxonomic keys (specimens HH11 and BH25). These specimens were evaluated and alates keyed to *R. virginicus* while the soldiers keyed to *R. hageni* (B. T. Forschler, personal communication). Likewise, their cuticular hydrocarbon phenotypes for these two equivocal samples were each unique, with both chemotaxonomic phe-

nograms and mtDNA cladograms demonstrating congruence. They were distinct from all known congeners: *R. flavipes*, *R. virginicus*, and *R. hageni*. As was observed in the present evaluation, their results also had difficulty discerning *R. malletei* from *R. hageni* and *R. virginicus* based on morphology and cuticular hydrocarbons alone, instead demonstrating the strength of their mtDNA sequence data to discern this group from other congeners. Jenkins *et al*. (2000) concluded that these two populations, although from disparate locations, constituted a discrete taxon and that they were more related phylogenetically to each other than to either *R. virginicus* or *R. hageni*, postulating that they may represent a saltational speciation event (Mayr 1954, Carson 1968, Templeton 1980). Future genetic evaluation of the samples from Jenkins *et al*. (2000) would be nice to confirm species identities with existing specimens of *R. malletei* that have been more recently procured.

Our comparison of these species applying the 16S rRNA gene also provides an additional view into the species isolation of *R. malletei* from other North American species in this genus. Maximum Parsimony analysis shows strong bootstrap support for the separation of *R. malletei* from *R. flavipes* and other known *Reticulitermes* groups (Fig 4). Furthermore, Maximum Likelihood analysis also supports both their relationship among and between other *Reticulitermes* (Fig. 5). Unlike previous studies which focused on *Reticulitermes* from the south central United States (Austin *et al*. 2004a, b, c), inclusion of taxa from throughout the United States demonstrates that our sequence data strongly support most existing morphological-based descriptions of species for Nearctic *Reticulitermes.*

To be certain that the samples we have found were not introduced species, we compared our samples to other *Reticulitermes* in our database, and found no polytomy with other described species, including disjunct populations from Europe (Austin, unpublished data). Coupled with cuticular hydrocarbon and morphometric measurements subjected to discriminant analysis, we propose that the weight of evidence for this cryptic species provides sufficient justification for the first confirmed description of a new *Reticulitermes* termite in the United States in over 75 years. Future studies which employ molecular methods of identification may confirm Clément *et al*.'s (1986a) supposition that this species is found widely across the United States. In Georgia, near Athens, we found that between a third to half of the colonies collected were *R. malletei* (Bagnères, unpublished data). The abundance of *R. malletei* was equally true in Delaware (Whitney-King *et al*. 2007). The extent of damage incurred to structures from this species is presently unknown and future studies should be encouraged. Samples or *R. malletei* from South Carolina were procured from in-ground termite monitoring stations on the Clemson University campus. We speculate that their likely participation as urban pests may resemble those of other *Reticulitermes* species such as *R. hageni* or *R. virginicus*. In a recent genetic study in North Carolina, the application of both mtDNA and microsatellite DNA used to investigate breeding structure of *Reticulitermes* species from two distinct forests, also demonstrated the incongruence among populations of *R. hageni* and *R. malletei* (Vargo and Carlson 2006) further supporting the species isolation of the two. Strong population structure in *R. hageni* may contribute to its sympatric isolation from *R. malletei* in North Carolina forests. Vargo and Carlson (2006) described the two distinct populations occupying Duke and Schenck Forests as a single species (*R. hageni*), applying *R. hageni sensu stricto* to the Schenck Forest population and *R. n. sp*. (= *R. malletei*) to the Duke Forest population until the formal description could be written.

At the time of the original chemical analysis (Bagnères PhD thesis 1989), *R. malletei* was the only species found with trienes, confirmed by infra red analysis conducted in R.W.Howard's lab in 1989). However, subsequent authors have found these kinds of components (Page *et al*. 2002, Jenkins *et al*. 2000), but never at these high quantities, with more than 60% unsaturated components. The presence of dimethyl branched alkanes were not found, which is rare in *Reticulitermes* termites. Soldiers also possessed only one sesquiterpene, a known defensive substance which is particularly frequent in other *Reticulitermes* analysis (Clément *et al*. 2001). These two characteristics appear to be specific to this taxa. The PCA analysis (Fig. 2) shows the clear separation of *R. malletei* with *R. flavipes*, and the European species *R. lucifugus* and *R. grassei* (both western Palearctic *Reticulitermes* species). Inclusion of *R. flavipes*, (= *R. santonensis*) (see Jenkins *et al*. 2001 and Austin *et al*. 2005b) was incorporated into this evaluation. Axis one (32.0% of the variation) seems to separate

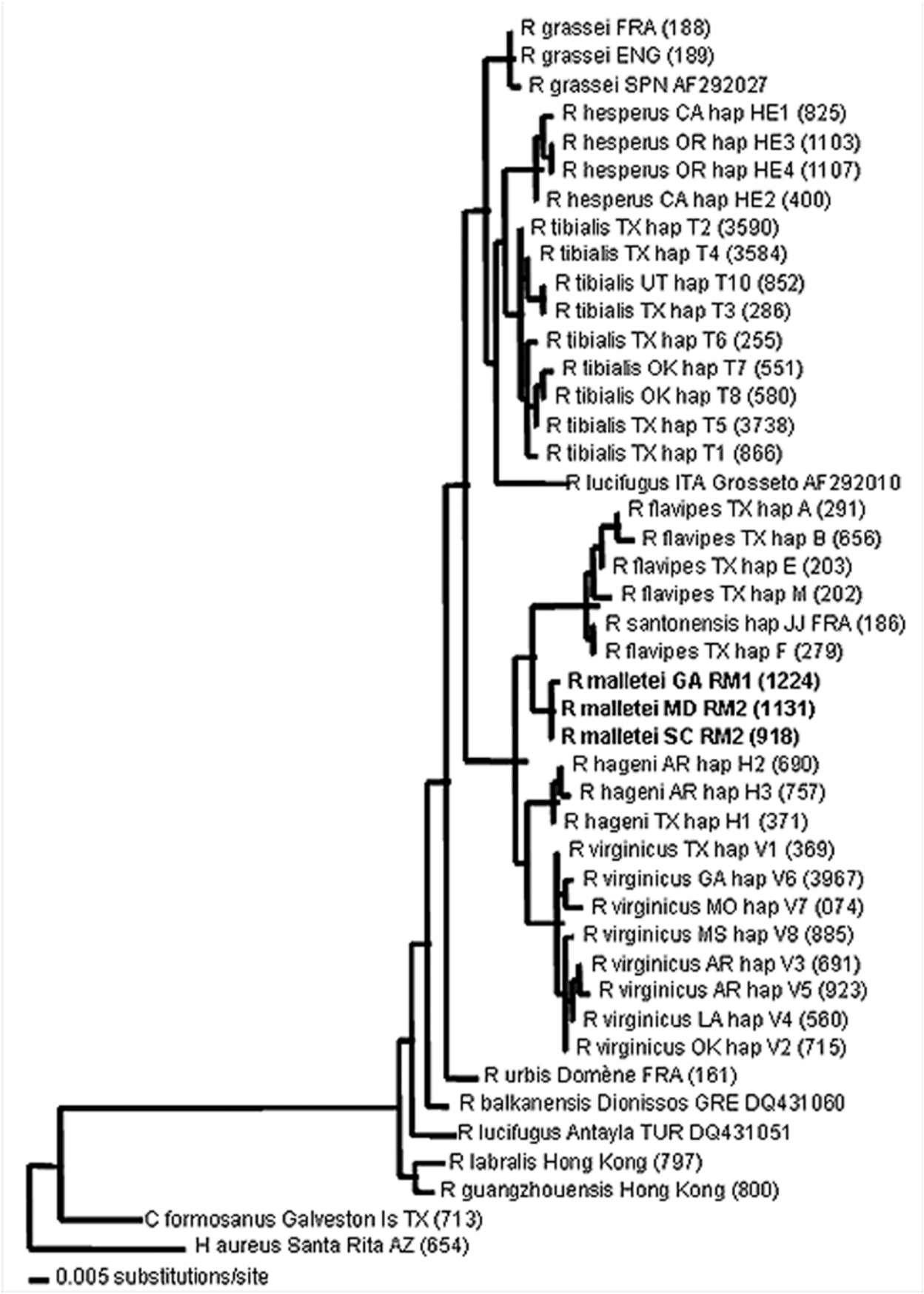


**FIGURE 10.** Topology obtained by maximum-likelihood analysis based on the HKY85 model (see text). Log L =-1582.31587.

American (on the negative part of the axis) and European species (one the positive part). *R. malletei* seems to be chemically very homogenous, which differs from *R. lucifugus and R. grassei* (Figures 2 and 8). Likewise, we find that soldier pronota of *R. malletei* appear to be very homogenous as well, with the same general shape (Fig. 4) throughout their described distributions. However, smaller sized soldiers from some populations can confound accurate identification of this group if samples are not also genetically evaluated. Labral shape and size was most similar to *R. hageni* (Fig. 6).

Applying the biometric index of Bagnères *et al*. (1990), the resulting index was calculated to be 4.97 for *R. malletei*: [AB] = 0.60 ± 0.03, [BC] = 0.51 ± 0.05, and [L] = 4.48 ± 0.13 (n = 5). This index clearly delimited *R. malletei* from either *R. flavipes*, *R. grassei*, or *R. lucifugus* (Figures 2 and 3). There was no statistically significant intra-specific variation detected with this index for *R. malletei*, but differences were detected from all other groups for [AB], [BC], and [L], respectively. It could be that the *R. malletei* samples from Athens, GA, were more locally adapted or homogenous than with other *R. malletei* that could be found from other states. Genetic evidence suggests the presence of at least two distinct lineages in Delaware (Whitney-King *et al*. 2007), and both are observed to share a sister clade to *R. flavipes* from several Atlantic states (e.g., populations from Georgia, Maryland, and South Carolina in Figs. 9 and 10).

Results from ethological tests performed by Clément *et al*. (1986a) demonstrated chemical isolation of *R. malletei* from both *R. flavipes* and *R. virginicus*. Subsequent assays conducted in Athens, GA, in 1987 substantiated low intra-specific aggression (Ag index = 10.90, n =11) versus high inter-specific aggression (Ag index = 60.93) from 23 independent pairings of *R. malletei* with *R. flavipes* (Bagnères, 1989). While the subsequent evaluation of these tests with other sympatric species of *R. hageni*, and *R. virginicus* would be desirable, it is also unrealistic. One of the more interesting casual observations of *R. malletei* is that when inspecting wooden stakes in Delaware from Sentricon® (Dow AgroSciences, Indianapolish, IN) stations, *R. malletei* were observed to completely crust over their food sources with soil, more than generally observed with other *Reticulitermes* species, exhibiting a slight behavioral occupational difference for this species. This may be at least a slight behavioral difference that has allowed this species to coexist with other sympatric *Reticulitermes*, occupying slightly different ecological niches (Houseman *et al*. 2001, Green *et al*. 2006) and yet remaining discrete. However, it should be a specific goal to evaluate all sympatric populations residing in areas where this species is known to occur. Although sometimes unrealistic, our genetic evaluations provided significant insight into the relationship of this cryptic group to its congeners. There is clear evidence that isolation of *R. malletei* occurs from a genetic, chronological (alate swarming dates), morphological, ethological, and chemical basis, constituting a discrete "new" species in the Nearctic *Reticulitermes*.

Revision of the genus *Reticulitermes* has been needed for decades. Preliminary investigations by Banks and Snyder (1920), Snyder (1954), Weesner (1965), and now Messenger (2003) have provided crucial information for our knowledge concerning the distribution and occurrence of these economically important species in the United States. Only through extensive collecting and surveying, and now the application of more robust non-morphological examinations will we begin to fully realize that many cryptic species (like *R. malletei*) are still waiting to be properly described. The scope and nature of their destructive feeding preferences (e.g. structural timbers or attractiveness to termite baiting regimes) still need to be evaluated, and now aided through modern identification techniques such at mitochondrial markers, we can identify problematic species and the areas they inhabit which have gone previously undetected.

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