

Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America

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Abstract

Cuticular hydrocarbon mixtures can be used to discriminate insect taxa. They have utility for determining phylogenetic relationships where they are independent characters with discrete states and represent a hierarchical distribution of shared, derived characters. We report inferred degrees of relatedness among the chemical phenotypes of *Reticulitermes* from PAUP (phylogenetic analysis using parsimony) analyses of cuticular hydrocarbon characters. One hundred and forty-one *Reticulitermes* colonies collected from California, Georgia, New Mexico, Arizona and Nevada were used. Initial maximum parsimony analyses sorted the 141 colonies into 26 chemical phenotypes. Subsequent analyses, using the ancestral species *Coptotermes formosanus* and *Heterotermes* sp. as outgroups, sorted *Reticulitermes* taxa into three major lineages, each characterized by a different set of dominant methyl-branched or unsaturated hydrocarbon components. *Reticulitermes* in lineage I have cuticular hydrocarbon mixtures with a preponderance of internally branched monomethylalkanes and 11,15-dimethylalkanes. Those in lineage II are defined by a preponderance of 5-methylalkanes and 5,17-dimethylalkanes. Taxa in lineage III are characterized by the predominance of olefins and a relative paucity of *n*-alkanes and methyl-branched alkanes. Bootstrap analyses and decay indices provided statistical support and robustness for these chemical-based relationships. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

The use of cuticular hydrocarbons as taxonomic characters to identify species of termites is not a new concept (Howard and Blomquist, 1982). The first report of cuticular hydrocarbons in a termite species, *Nasutitermes exitiosus* (Hill), was not done with taxonomy in mind (Moore,

1969). However, there is now an extensive body of unequivocal evidence that most termites have, with a few exceptions such as the case with *Macrotermes michaelseni* (Bagine et al., 1994), species-specific mixtures of cuticular hydrocarbons (Haverty et al., 1999a; Kaib et al., 1991).

The complete characterization of the cuticular hydrocarbon profile from the dampwood termite species, *Zootermopsis nevadensis* (Hagen) (Blomquist et al., 1979) [this species was originally misidentified as *Z. angusticollis* (Hagen)], was followed by the identification of four cuticular hy-

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drocarbon phenotypes for the three extant species in this genus (Haverty et al., 1988). Two hydrocarbon phenotypes were designated as subspecies, *Z. nevadensis nevadensis* and *Z. nevadensis nuttingi* (Haverty and Thorne, 1989). Three pairs of termite species, *R. flavipes* (Kollar) and *R. virginicus* (Banks), *Nasutitermes corniger* (Motschulsky) and *N. ephratae* (Holmgren), and *N. costalis* (Holmgren) and *N. ephratae* (Holmgren) were found to be distinguishable by their vastly different hydrocarbon mixtures (Haverty et al., 1990b; Howard et al., 1978, 1982, 1988). The characterization of cuticular hydrocarbon mixtures corroborated the recognition of three morphologically distinct and geographically restricted species of *Coptotermes* in Australia: *C. michaelsoni* Silvestri; *C. brunneus* Gay; and *C. dregghorni* Hill (Brown et al., 1994). A chemotaxonomic survey of species of the Australian harvester termite, *Drepanotermes Silvestri*, revealed 'an intriguing blend of difference and similarity' in cuticular hydrocarbon profiles; three morphologically distinct species had qualitatively similar hydrocarbon profiles, whereas sympatric pairs of species showed markedly different profiles (Brown et al., 1996). In studies of various species of the pantropical genus *Coptotermes*, the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, was easily separated from other species of *Coptotermes* using cuticular hydrocarbons (Haverty et al., 1991, 1992). An analysis of cuticular hydrocarbons from six species of *Odonotermes* in Kenya confirmed species specificity of cuticular hydrocarbon profiles, but resulted in an arrangement of the species that opposed the established division based on numerical analysis of morphometric data (Kaib et al., 1991). In an extensive study of the taxonomy of *Heterotermes* in southeastern Australia, cuticular hydrocarbons were used to define two termite species, *H. brevicatena* sp. n. and *H. longicatena* sp. n. from the *H. ferox* (Froggatt) complex (Watson et al., 1989).

Identification of the cuticular hydrocarbons from samples taken in recent surveys of the *Reticulitermes* from Georgia, California, Nevada, Arizona and New Mexico provides evidence that suggests there are numerous undescribed species or species complexes of *Reticulitermes* in North America (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). Published biogeographical accounts consistently report six extant species of *Reticulitermes* in North America (Nutting, 1990;

Weesner, 1970). Much of this biogeographical information on *Reticulitermes* was developed in the first half of the last century (Banks and Snyder, 1920; Banks, 1946; Light, 1934; Miller, 1949; Pickens, 1934a,b; Snyder, 1954). No one has thoroughly reexamined this biogeographical or taxonomic treatment of *Reticulitermes*, yet there is agreement among authors that the taxonomy of *Reticulitermes* from North America is desperately in need of revision (Nutting, 1990; Scheffrahn and Su, 1994; Weesner, 1970). Several species of North American *Reticulitermes* have been synonymized without a detailed taxonomic revision (*R. claripennis* Banks into *R. flavipes*; *R. humilis* Banks into *R. tibialis* Banks; and *R. tumiceps* Banks into *R. tibialis*) (Snyder, 1949). The lack of a rigorous, modern taxonomic study of this genus could explain our difficulty in identifying our collections of *Reticulitermes* from the states of Georgia, New Mexico, Arizona, Nevada, and California (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a) using keys based on morphology (Nutting, 1990; Scheffrahn and Su, 1994; Snyder, 1954; Weesner, 1965).

Three species, *R. virginicus* Banks, *R. hageni* Banks, and *R. flavipes*, are reported to occur in Georgia. These three species occur sympatrically throughout much of the eastern and southeastern areas of the United States; only *R. flavipes* occurs in the northern regions of the United States, including the New England states (Nutting, 1990; Weesner, 1970). Only *R. tibialis* is recognized from New Mexico, Arizona, and Nevada, and is reported to occur in the inland valleys and desert areas of California, through the Great Basin and the Great Plains, northward into Montana, and southward into the desert Southwest, extending to the tip of Baja California and inland areas of Mexico (Weesner, 1970). *R. hesperus* Banks is another species reported to occur in California (Nutting, 1990, Weesner, 1970). *R. hesperus* should be found in most of California except for the southern deserts, the San Joaquin Valley, and Sacramento Valley (Pickens, 1934a). *R. tibialis* either co-exists with *R. hesperus* or replaces it in the inland valleys of California (Light and Pickens, 1934; Pickens, 1934b; Weesner, 1970). A sixth species, *R. arenicola* Goellner, has been reported only from the sand dune areas along the southern shore of Lake Michigan in Indiana and Michigan (Weesner, 1970).

The phylogeny of termites has been established

with little modification over the past five decades (Ahmad, 1950; Krishna, 1970), yet there is little confidence in the relationships among species of many genera, especially *Reticulitermes*. Termites are notoriously 'lacking in ornamentation and furnish few if any of those satisfyingly definite differences... which facilitate specific diagnoses.... Further, termite species are extremely plastic and exhibit a wide range of (morphological) variation. Finally, the specific characters which are available express themselves almost entirely in the form of differences in range of size...' (Light, 1927). This morphological variation in *Reticulitermes* has led to confusing species diagnoses and has encouraged us to develop additional characters to gain a taxonomic understanding of this genus (Haverty and Nelson, 1997; Haverty et al., 1991, 1996a, 1999a).

If indeed there are undescribed species of *Reticulitermes*, then a robust phylogeny should prove beneficial for further biological studies to aid in species identification (Brooks and McLennan, 1991). Parsimony-based analyses are the preferred methods for determining degrees of relationship under the broadest range of conditions (Mishler, 1994). We hypothesize, as with termites in the genus *Odontotermes* (Kaib et al., 1991), cuticular hydrocarbons are homologous characters among *Reticulitermes* taxa. They represent hierarchical distributions of shared characters and are independent characters with discrete states. As such, hydrocarbon composition is an expression of a taxon's genotype and therefore, we can assume there is a genetic basis (Coyne et al., 1994; Dallerac et al., 2000; Takahashi, et al., 2001) for different hydrocarbon composition among species.

2. Materials and methods

2.1. Hydrocarbons and character states

Colonies of *Reticulitermes* previously used to characterize cuticular hydrocarbon chemical phenotypes (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a), as well as additional samples collected from Georgia and Maryland (Table 1), comprise the data set for this study. Cuticular hydrocarbons were identified by gas chromatography-mass spectrometry (GC-MS). We used the same assignment of capital letters to identify

chemical phenotypes of *Reticulitermes* and the shorthand nomenclature to identify hydrocarbons in the text and tables as previously described (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). The classes of hydrocarbons characterized were *n*-alkanes, alkenes, alkadienes, trienes, tetraenes, pentaenes, and methyl-branched alkanes. We did not locate and characterize the double bond positions for the olefins, therefore, they are distinguished only by their approximate equivalent chain length (ECL) values (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). Two hundred and thirty-three cuticular hydrocarbons were used as phylogenetic characters. They were identified from various *Reticulitermes* phenotypes (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a), the outgroup species *Coptotermes formosanus* Shiraki from Hawaii (Haverty et al., 1990a, 1996b), and *Heterotermes* sp. from Guana Island, British Virgin Islands (Haverty et al., 1997). No individual *Reticulitermes* taxon nor outgroup species contained all 233 hydrocarbons. Area counts of individual hydrocarbons were converted to percent of total hydrocarbons. These proportions were converted to discrete characters and coded as absent or not detected (0) or present in the following relative quantities: $\leq 0.3\% = 1$; $0.3-1.0\% = 2$; $> 1.0-3.0\% = 3$; $> 3.0-6.0\% = 4$; $> 6.0-10.0\% = 5$; $> 10-20\% = 6$; and $> 20\% = 7$.

2.2. Phylogenetic analyses

A series of parsimony analyses were performed to determine degrees of relatedness among *Reticulitermes* chemical phenotypes. A preliminary analysis was conducted on 141 individual *Reticulitermes* collections as an independent assessment of empirical sorting of *Reticulitermes* into groups. The analysis sorted the 141 disparate collections into the 26 chemical phenotypes as previously reported (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). Subsequent analyses of the ingroup and analyses using outgroup species, *Coptotermes formosanus* and *Heterotermes* sp., sorted *Reticulitermes* taxa into major groups suggesting three lineages. All analyses were conducted using the heuristic search algorithm of PAUP [Mac version 3.1.1 (Swofford, 1993)]. With a large data set of 26 phenotypes and 233 cuticular hydrocarbons, the algorithm does not guarantee finding the most parsimonious tree (MPT).

We selected *C. formosanus* and *Heterotermes* sp. as the outgroup species. We hypothesized that they represented true ancestral species based on the published phylogenies of their subfamilies, Coptotermitinae and Heterotermitinae, respectively (Ahmad, 1950; Krishna, 1970; Snyder, 1949). *Coptotermitinae* is considered the most primitive subfamily of the *Rhinotermitidae*, and *Coptotermes* is the single genus within the subfamily. The subfamily *Heterotermitinae* evolved from the *Coptotermitinae* and has only two genera, *Heterotermes* and *Reticulitermes*, with *Heterotermes* being the more primitive of the two genera (Ahmad, 1950; Krishna, 1970). Our designation of these outgroups was also supported by the discovery of hydrocarbons found almost exclusively in *Coptotermes* and *Heterotermes* sp. We identified unusual dimethyl- and trimethyl-branched alkanes from species of *Coptotermes* (Haverty et al., 1990a, 1991, 1992, 1996b) and other hydrocarbons present uniquely in *C. formosanus* and *Heterotermes* sp. populations (Haverty et al., 1990a, 1991, 1992, 1996b, 1997).

Four parsimony analyses were performed as follows: (1) The 26 ingroup phenotypes alone; (2) the ingroup with *C. formosanus*; (3) the ingroup with *Heterotermes* sp.; and (4) the ingroup with both outgroups. All analyses were performed using the mean percentage of each hydrocarbon for each of the phenotypes. Support was assessed for each cladogram by bootstrap consensus trees from 1000 replications (Felsenstein, 1985) and computing decay indices (Mishler, 1994). The decay index evaluates the relative support for a group by relaxing parsimony one step at a time and calculating strict consensus for all trees one step longer than the most parsimonious, then two steps longer, etc. The decay index for a group is the number of steps that can be added before that group loses its support (Mishler, 1994). Goodness-of-fit statistics, consistency index (CI) and retention index (RI) were calculated and reported in all figures. Consistency index (Kluge and Farris, 1969) measures the level of support for each tree. Consistency index will equal one when a data set explains the tree as well as possible. Retention index (Farris, 1989) measures the congruency of the characters to each other and the tree. Retention index will equal one when the characters in a data set are totally congruent with each other and the tree.

3. Results

3.1. Phylogenetic analyses

Our empirical assignment of 26 chemical phenotypes was supported in the analysis of all 141 *Reticulitermes* collections. This analysis using both outgroups produced a strict consensus tree from 1000 trees of equal parsimony (Fig. 1). Four colonies (CA-A' HC1, CA-A' HASPS, CA-A TB5 and GA-A ATH2) of the 141 collections did not cluster with their chemical phenotype, however, this did not alter the designations of 26 phenotypes.

Single MPTs were produced from analyses of the ingroup alone (Fig. 2a), with both outgroups combined (Fig. 2b), and with *C. formosanus* as a single outgroup (Fig. 3a). A strict consensus tree was constructed from four trees of equal parsimony in the single analysis with the closely related species, *Heterotermes* sp. (Fig. 3b). All analyses produced trees showing consistent clustering of phenotypes into three groups with phenotype CA-C as a single taxon separate from the clades. Each group can be defined by phenotypes with a preponderance of monomethylalkanes and dimethylalkanes or olefins (alkenes, alkadienes). Also, a close examination of the hydrocarbon mixtures within taxa suggests the relative importance of the predominant components for deriving relationships among taxa suggested in these parsimony analyses. The presence, abundance and consistency of similar hydrocarbons within a group of phenotypes suggest these are separate lineages.

In the unrooted tree, phenotypes AZ-D, CA-A, CA-A', GA-A, GA-AB and GA-B comprised lineage I (Fig. 2a). This group lost its support at two decay steps because phenotype CA-A was no longer supported as a member. Phenotype GA-C becomes a member of lineage I in analyses with both outgroups (Fig. 2b), with *C. formosanus* (Fig. 3a), and with *Heterotermes* sp. (Fig. 3b). It is a single taxon separate from all other group clusters. Phenotypes GA-A and GA-AB are always paired as sister taxa, and together form a sister group to GA-B. These three taxa form a group that does not lose its support until a decay index of four steps in all analyses (Fig. 2a, Fig. 3a,b) except with both outgroups (Fig. 2b) where the support is not lost until five steps. Phenotypes

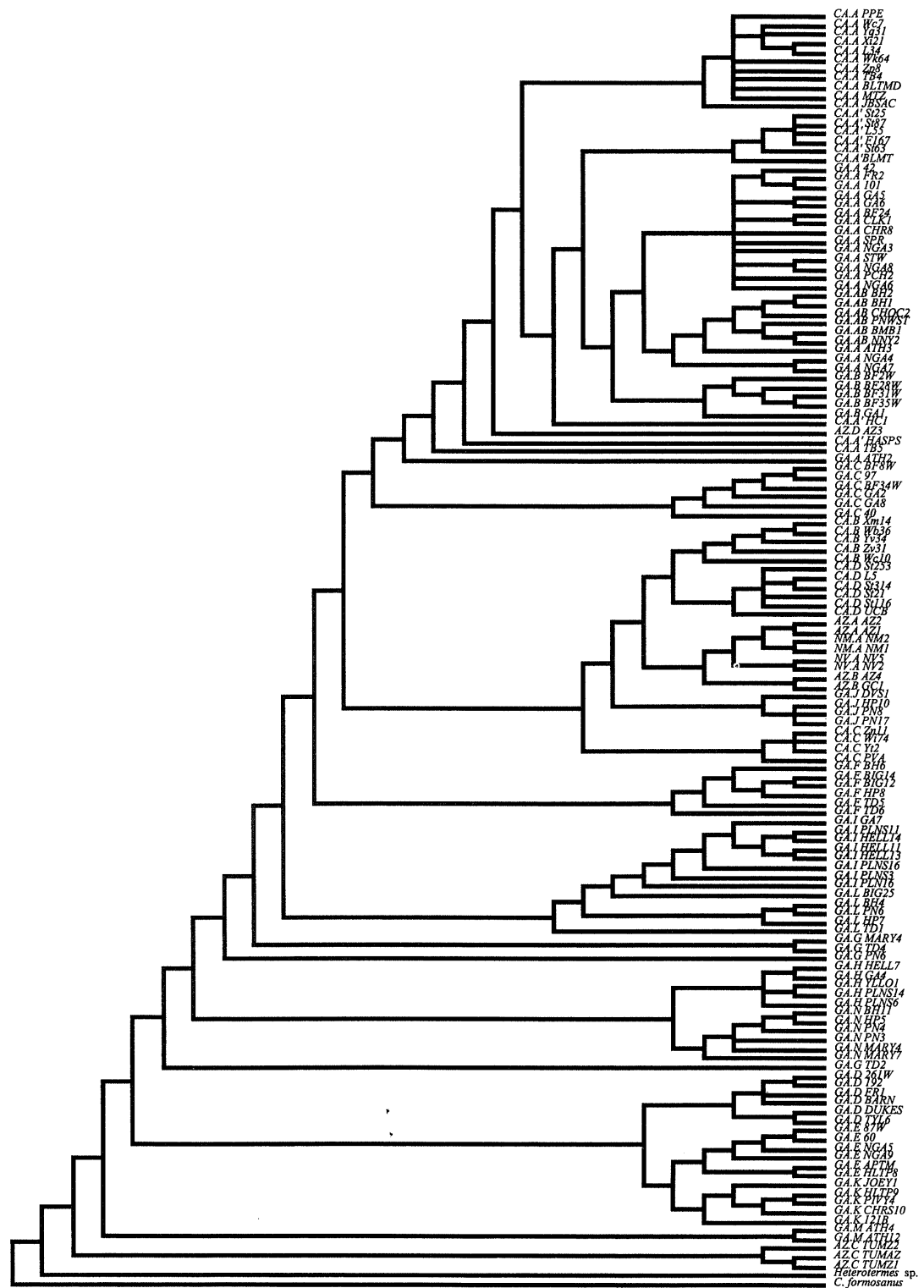


Fig. 1. Strict consensus tree of 1000 equal length most parsimonious trees (1636 steps each) based on analysis of cuticular hydrocarbons from 141 *Reticulitermes* colonies. The tree was generated with *Coptotermes formosanus* and *Heterotermes* sp. as outgroups. Length = 1636, consistency index = 0.420, and retention index = 0.579.

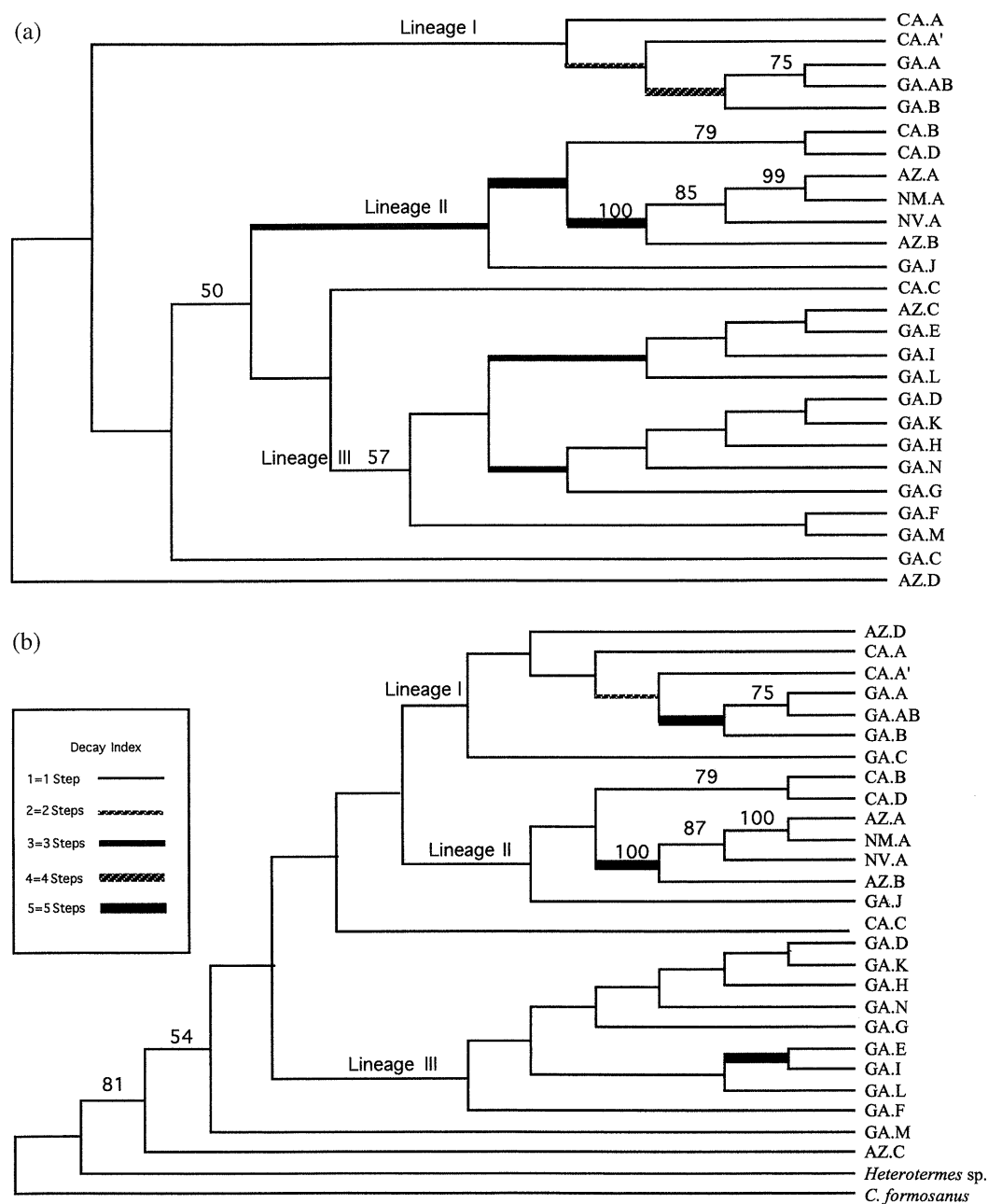


Fig. 2. Single most parsimonious trees of *Reticulitermes* chemical phenotypes based on analysis of cuticular hydrocarbons. (a) Tree generated without outgroups; length = 1471, consistency index = 0.422, and retention index = 0.517. (b) Tree generated with outgroups, *Coptotermes formosanus* and *Heterotermes* sp.; length = 1632, consistency index = 0.412, and retention index = 0.513. The decay indices, the number of steps that parsimony was relaxed before a particular clade lost its support, are indicated by branch thickness. The thick branches are better supported. Numbers above branches are bootstrap percentage values.

AZ-D, CA-A and CA-A' are separate from GA-A, GA-AB and GA-B as sister taxa in all analyses. The most strongly supported relationship in this lineage is GA-A and GA-AB. Their relationship has bootstrap values of 75% in both the ingroup and binary outgroup analyses (Fig. 2a,b), and 71

and 72% in *C. formosanus* and *Heterotermes* sp. analyses, respectively. Results from analyses of all samples collected from lineage I provide more evidence for their relationships (Fig. 4). The single collection of phenotype AZ-D and all collections of CA-A, CA-A', except TB5 (CA-A) and

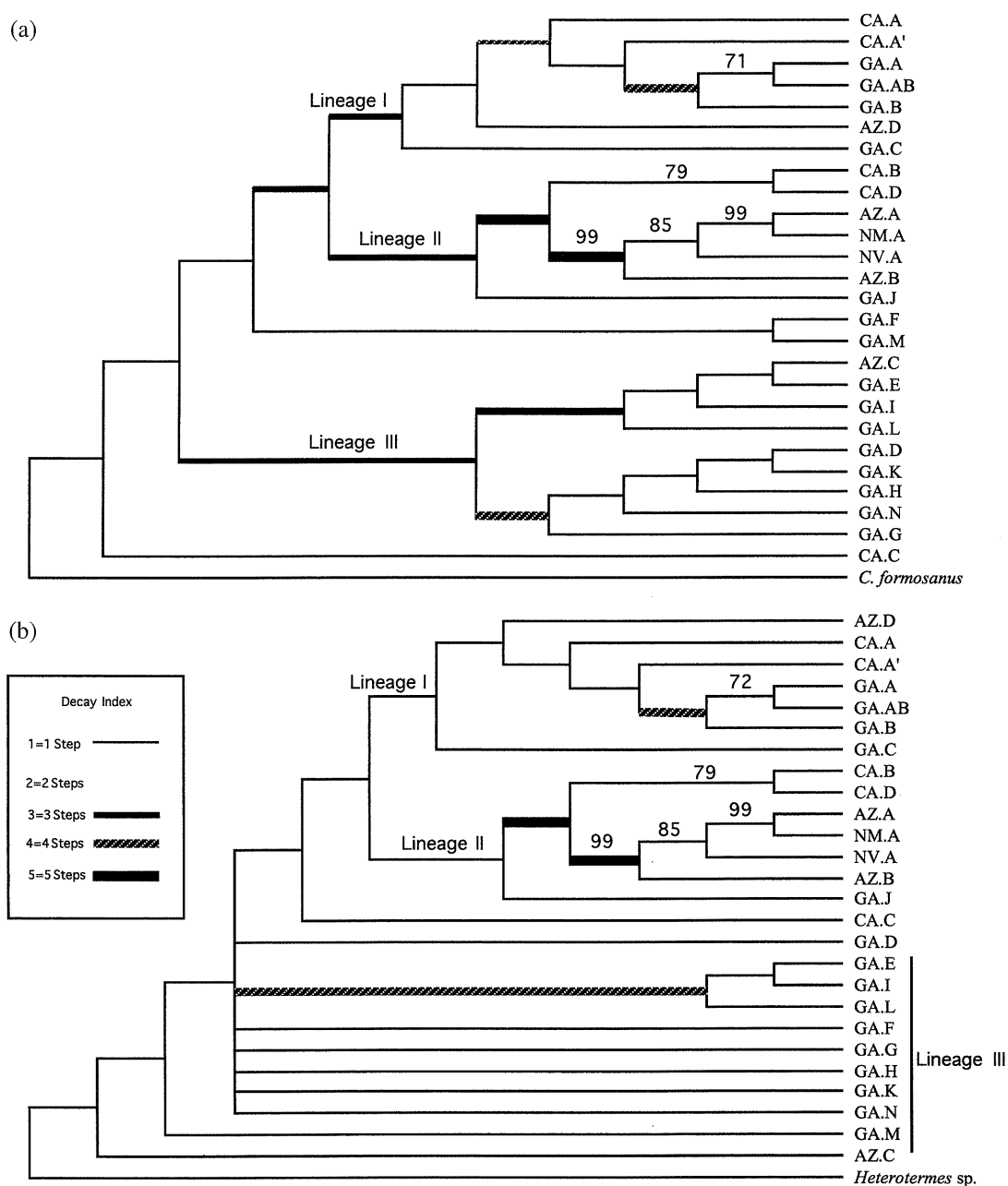


Fig. 3. (a) Single most parsimonious tree generated with *Coptotermes formosanus* as the single outgroup; length = 1559, consistency index = 0.418, and retention index = 0.56. The decay indices, the number of steps that parsimony was relaxed before a particular clade lost its support, are indicated by branch thickness. The thick branches are better supported. Numbers above branches are bootstrap percentage values. (b) Strict consensus of the four most parsimonious trees of *Reticulitermes* chemical phenotypes based on analysis of cuticular hydrocarbons. Tree generated with *Heterotermes* sp. as the single outgroup; length = 1563, consistency index = 0.424, and retention index = 0.514.

HC1 and HASPSP (CA-A'), separate as sister groups from GA-A, GA-AB and GA-B. The sample ATH2 (GA-A) is the only colony separated from all groups.

Phenotypes CA-B, CA-D, AZ-A, NM-A, NV-A,

AZ-B and GA-J form lineage II (Fig. 2a), which appears as a sister group to lineage I in all analyses using outgroups (Fig. 2b, Fig. 3a,b). Phenotype GA-J is consistently placed as a phenotype within lineage II in all analyses (Figs. 2

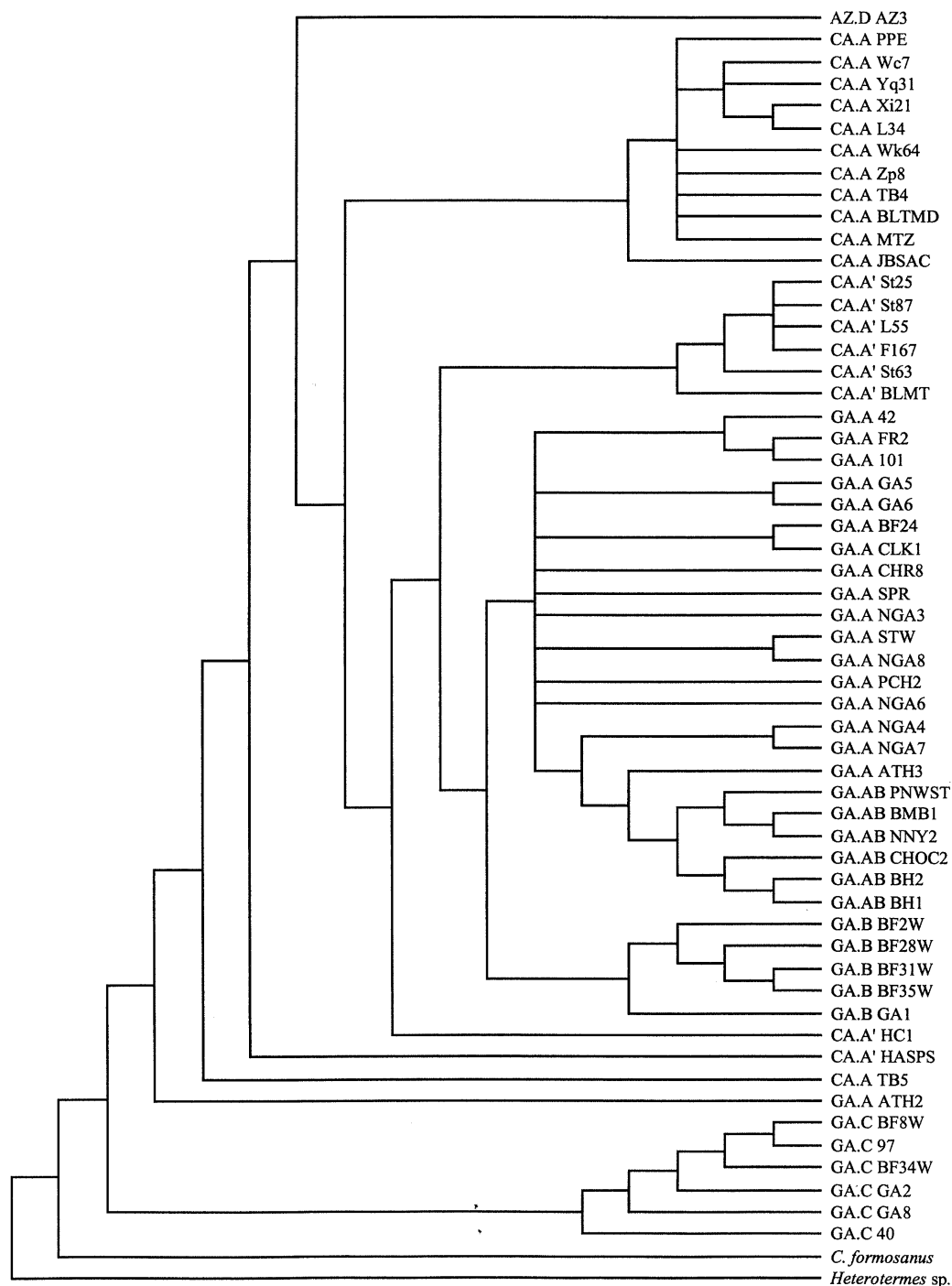


Fig. 4. Strict consensus tree from analysis of all individual samples in lineage I. Tree generated from 1000 trees of equal parsimony with both outgroups; length = 895, consistency index = 0.429, and retention index = 0.786.

and 3), but is not supported as a member of this lineage in bootstrap analyses. The predominant hydrocarbons for this phenotype would suggest that it belongs in lineage I, even though it pro-

duces traces of 5-meC25, 5-meC27, 5,15-dimeC25, 5,17-dimeC27 and 5,17-dimeC39 (Haverty et al., 1996a). It would appear by the placement of phenotype GA-J in all analyses that it is an inter-

mediate phenotype between lineages I and II. This cluster, minus GA-J, does not lose its support until a decay index of five steps in all analyses except with both outgroups (Fig. 2b) where the support is lost at two steps. All analyses support phenotypes CA-B and CA-D as closely related and a sister group to AZ-A, NM-A, NV-A, and AZ-B. The relationship of phenotypes CA-B to CA-D is supported 79% in bootstrap analyses. The relationship among phenotypes AZ-A, NM-A, NV-A and AZ-B has a bootstrap support of 100% in the ingroup and binary outgroup analyses, and 99% with each individual outgroup. Phenotype AZ-A is more closely related to NM-A (100% bootstrap value) than to NV-A and AZ-B.

Phenotypes AZ-C, GA-E, GA-I, GA-L, GA-D, GA-K, GA-H, GA-N, GA-G, GA-F and GA-M, comprise lineage III (Fig. 2a). Phenotypes GA-D, GA-K, GA-H, GA-N and GA-G maintain the same structure as a group and are a sister group to phenotypes AZ-C, GA-E, GA-I and GA-L in the ingroup (Fig. 2a) and *C. formosanus* trees (Fig. 3a). The same phenotypes minus AZ-C and GA-M represent lineage III in the tree with both outgroups (Fig. 2b). Phenotypes GA-E, GA-I and GA-L are the only taxa of lineage III that maintain their relationship as a group in the consensus tree with *Heterotermes* sp. (Fig. 3b). The relationship of all other taxa is unresolved. Phenotypes GA-F and GA-M appear as a separate group unrelated to lineages I, II and III in the *C. formosanus* analysis (Fig. 3a).

Because we have observed a similarity in classes of hydrocarbons among *Reticulitermes* chemical phenotypes in this study, it is significant to focus on the predominant cuticular hydrocarbons in each lineage and taxon-specific hydrocarbon differences. Also, a close examination of the hydrocarbon profiles within lineages indicates the relative importance of the predominant components for deriving relationships among taxa suggested in these parsimony analyses. All *Reticulitermes* phenotypes synthesize *n*-alkanes, 2- and 3-methylalkanes, and internally branched monomethylalkanes, but produce variable quantities of alkenes, dienes, trienes, dimethyl- and trimethylalkanes.

3.2. Hydrocarbons of *Reticulitermes* in lineage I

The predominant hydrocarbons in the cuticular hydrocarbon phenotypes that designate lineage I

are the 11-, 13-, 15-methylalkanes and 11,15-dimethylalkanes (Fig. 5). Qualitative differences in various hydrocarbons are taxon-specific diagnostic characters among members of this lineage. All phenotypes produce the same two positional isomers of the diene, C25:2, which have ECLs of 25.35 and 25.50 (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a), respectively. Phenotypes CA-A', GA-A, GA-AB and GA-B produce several positional isomers of trienes, mainly C25:3, (ECLs of 25.98, 26.07 and 26.47, respectively) which are diagnostic. Phenotype CA-A is the only phenotype in this lineage that does not synthesize trienes. All phenotypes, except GA-B and GA-C, have generally the same monomethylalkanes and dimethylalkanes. Phenotype GA-C is the only phenotype in lineage I that synthesizes monomethylalkanes and dimethylalkanes with carbon chains C29 and C31. Phenotype GA-B synthesizes the additional components 5-meC25, 7,11-dimeC33, 9,13-dimeC35, 5,17-dimeC35, 6,18-dimeC36, 5,17-dimeC36, 5,17-dimeC37, 6,18-dimeC38, 5,17-dimeC38, 5,17-dimeC39, 5,17-dimeC41, 5,17-dimeC43, 5,9,15-trimeC25, 5,9,17-trimeC35, 7,11,15-trimeC35 and 5,9,17-trimeC37 (Haverty et al., 1996a).

3.3. Hydrocarbons of *Reticulitermes* in lineage II

Lineage II is characterized by the presence of 5-methylalkanes and 5,*X*-dimethylalkanes at carbon chains C25–27, and the dominance of 5,17-dimethylalkanes at C33–43 (Fig. 6). Phenotypes are further characterized by variations in the presence and absence of monoenes, dienes and trienes. Phenotypes CA-B and CA-D synthesize abundant quantities of a homologous series of 5,17-dimethylalkanes (Haverty and Nelson, 1997). CA-B synthesizes the homologous series 5,17-dimeC25–5,17-dimeC29, and 5,17-dimeC33–5,17-dimeC43. Phenotype CA-D possesses the same homologous series, except 5,17-dimeC29 and 5,17-dimeC33 are not present. 5,17-DimeC31 was not detected in either phenotype. Phenotypes CA-B and CA-D also synthesize the 5,9,17-trimethylalkanes with parent carbon chains of C25 (CA-D only), C27, C35, C37 and C39 (CA-B only). Most unusual is the presence of 5,17- and/or 6,18-dimethylalkanes with even number parent chains in both CA-B and CA-D (Haverty and Nelson, 1997). Dimethylalkanes are synthesized in smaller quantities in phenotypes AZ-A, NM-A,

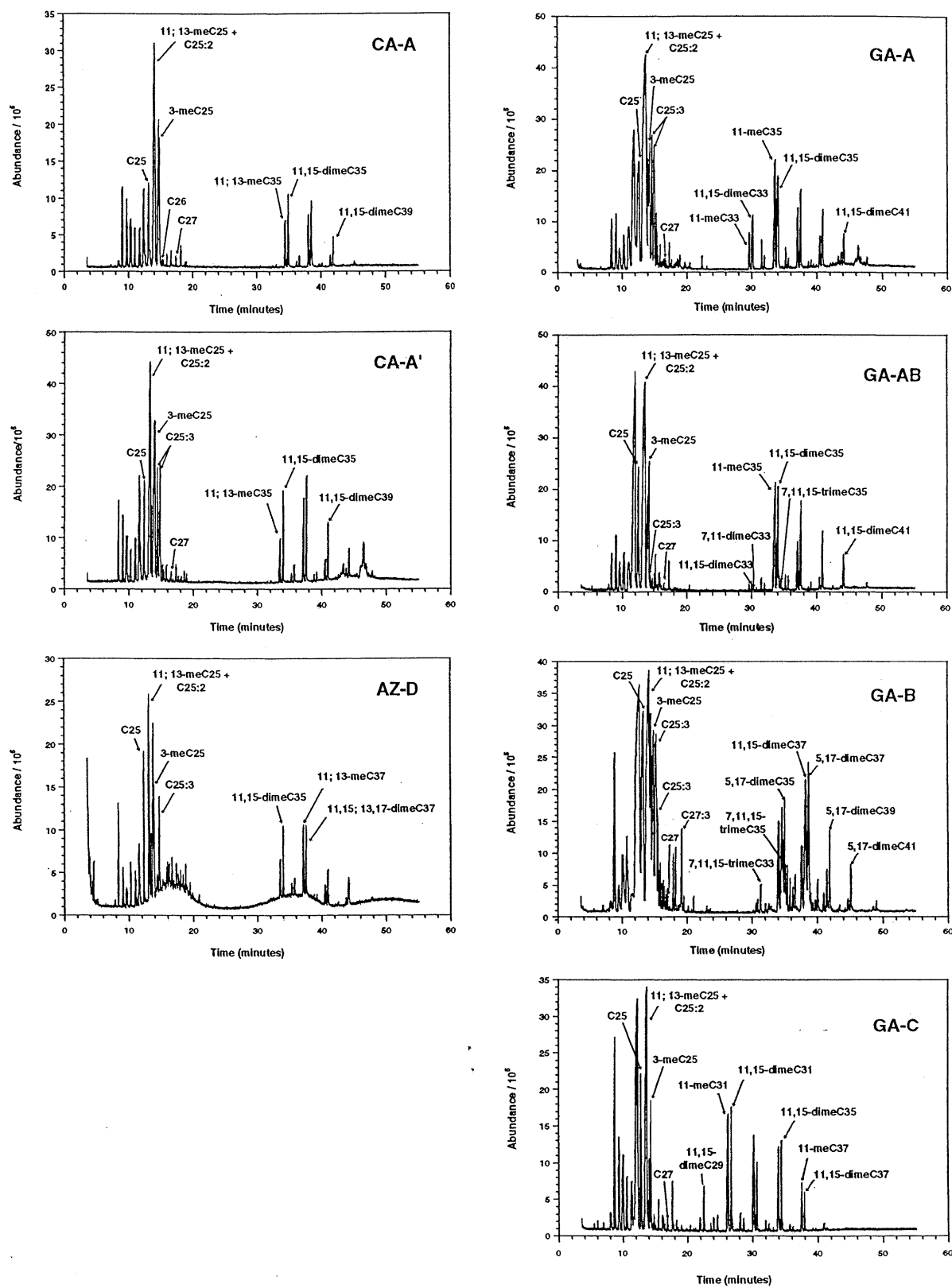


Fig. 5. Total ion chromatograms of cuticular hydrocarbons from *Reticulitermes* chemical phenotypes representing lineage I.

NV-A and AZ-B than in phenotypes CA-B and CA-D (Haverty and Nelson, 1997; Haverty et al., 1999a). The absence of olefins in CA-B and their presence in CA-D (C23:1, C25:3, C27:2 and C27:3) provides further discrimination between these two phenotypes. Phenotypes AZ-A, AZ-B, NM-A and NV-A possess a number of the same olefins in varying amounts, however, the C37:2 and C39:2 isomers are completely absent in AZ-B (Fig. 6). Phenotype GA-J produces much less of the diagnostic 5-methyl- and 5,X-dimethylalkanes than the other member of this group. All colonies of phenotype GA-J are separate from the other taxa in the analysis of individual *Reticulitermes* collections of lineage II.

3.4. Hydrocarbons of *Reticulitermes* in lineage III

Alkenes and alkadienes are the predominant hydrocarbons in members of lineage III (Fig. 7). All taxa synthesize olefins in excess of 50% of their total hydrocarbon (Haverty et al., 1996a, 1999a). Hydrocarbon profiles of these phenotypes also contain numerous internally branched monomethyl- and dimethylalkanes. As there is great diversity in the production of positional isomers of olefins among taxa in this group, we conducted a separate analysis with all individual collections (Fig. 8). There is a separation between groups consisting of phenotypes GA-D, GA-F, GA-G, GA-N, GA-H and GA-K producing major quantities of heptacosadiene (C27:2) and phenotypes GA-E, GA-I and GA-L producing no C27:2. The phenotypes that synthesize C27:2 are subdivided into three more clusters. Phenotype GA-D, the only phenotype that does not produce significant (< 0.3%) amounts of any C29 olefins (nonacosene, nonacosadiene and nonacosatriene), is a separate group from the remaining C27:2 producers. Of the phenotypes that do synthesize significant amounts of various isomers of C29:2, GA-F, GA-G and GA-N, form a separate group from their sister taxa, GA-H and GA-K, according to the variety of positional isomers of C27:X and C29:X, as well as the diversity of methylalkanes. Two groups each composed of a single phenotype, AZ-C or GA-M, are separate from the other taxa in this lineage. These two phenotypes have unique characteristics that separate them from the other taxa. AZ-C is the only phenotype with a homologous series of both 5,17-dimethylalkanes and dienes at C27–45. GA-M produces an abundance

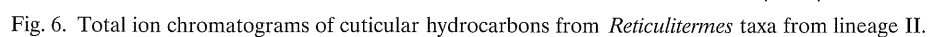
(> 20%) of nonacosatriene (C29:3) at ECL 29.00 not present in any other taxon.

3.5. Hydrocarbons of outgroups

The putative phylogeny of termite families (Krishna, 1970) describes *Coptotermes* and *Heterotermes* as genera that evolved before the genus *Reticulitermes*. Of the 233 hydrocarbons characterized in this study, *C. formosanus* produced a total of 46 and *Heterotermes* sp. produced 30. Eight components are unique to *C. formosanus* and nine are found only in *Heterotermes* sp. (Fig. 9). The hydrocarbon mixtures of *C. formosanus* specimens contain several methyl-branched hydrocarbons that are rarely identified in any insect species: methylalkanes with branches separated by one methylene group (Nelson et al., 1980; Thompson et al., 1981).

C. formosanus synthesizes a 13,15- and 15,17-dimethylalkane series. The most abundant of these dimethylalkanes is 13,15-dimeC29 (13%; coelutes with 11-, 13-, and 15-mec29) (Haverty et al., 1990a, 1991, 1992, 1996b). Produced in smaller amounts (< 3% of the total) are 13,15-dimeC30, 13,15-dimeC31, 15,17-dimeC31, 15,17-dimeC37, 15,17-dimeC39, 15,17-dimeC41 and 15,17-dimeC43 (Fig. 9). Homologs of these rare dimethylalkanes occur in only a few *Reticulitermes* phenotypes. Phenotype GA-C produces traces of the abundant 13,15-dimeC29 found in *C. formosanus*. Phenotypes GA-J and GA-N also produce 15,17-dimeC39, and 15,17-dimeC41 occurs in phenotype GA-J. Several phenotypes synthesize a 11,13-dimethylalkane: phenotype CA-C is the only one to produce 11,13-dimeC27; GA-A and GA-AB produce trace amounts of 11,13-dimeC33; phenotypes GA-H, GA-I, and GA-N produce 11,13-dimeC35 and phenotype GA-J is the lone producer of 11,13-dimeC37. *C. formosanus* also synthesizes the only trimethylalkane, 13,15,17-trimeC29, with methyl branches separated by one methylene group.

The unique components synthesized in *Heterotermes* sp. were all internally branched 9,X-dimethylalkanes (Haverty et al., 1997). *Heterotermes* sp. specimens produced a continuous 9,17-dimethylalkane series from C27 to C30, and a 9,19-dimethylalkane series from C28 to C30. They also produced 9,21-dimeC29 and 9,21-dimeC31. These dimethylalkanes characterized in *Heterotermes*, have not been identified in the hydrocarbon pro-



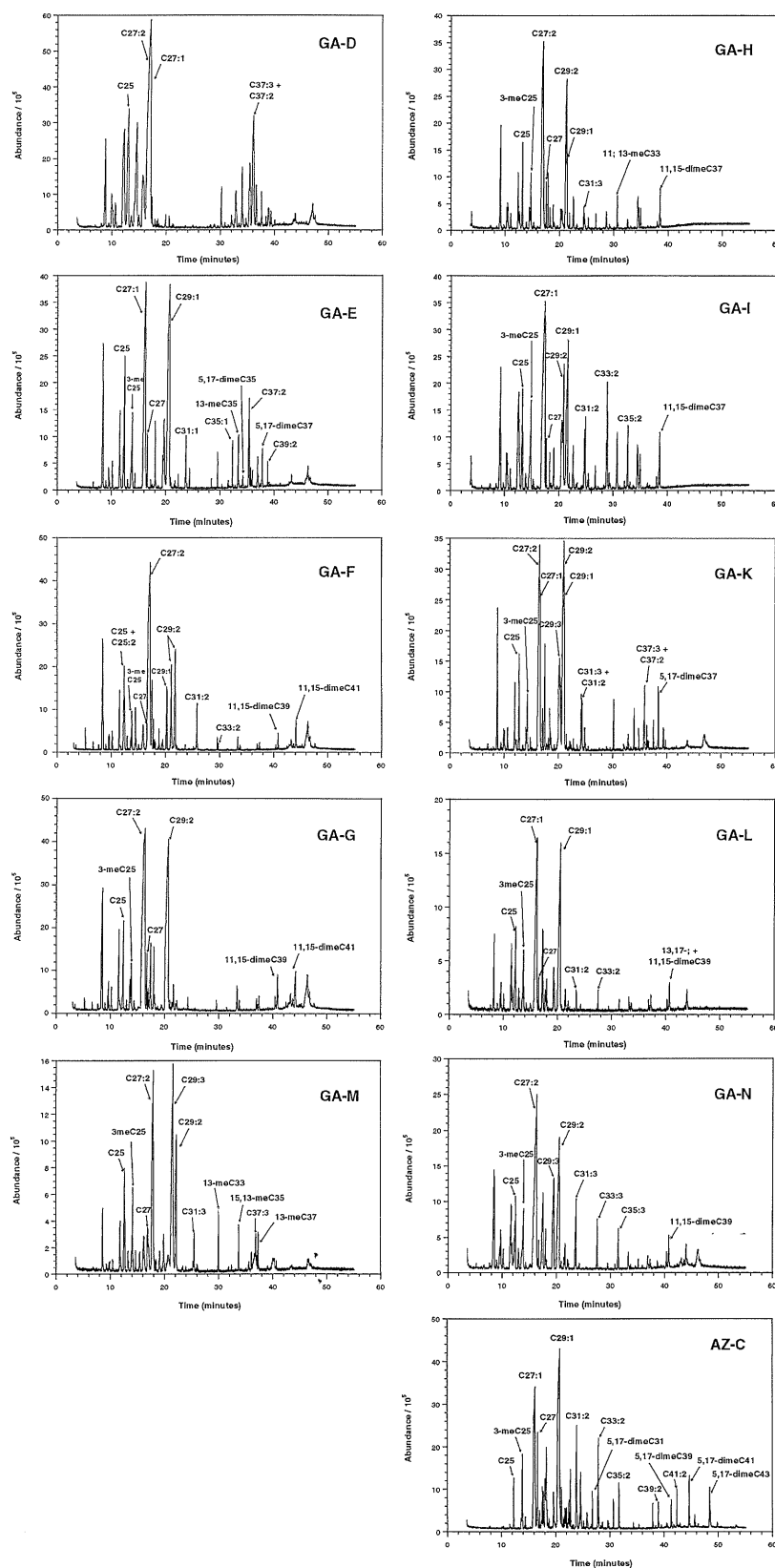


Fig. 7. Total ion chromatograms of cuticular hydrocarbons from *Reticulitermes* taxa from lineage III.

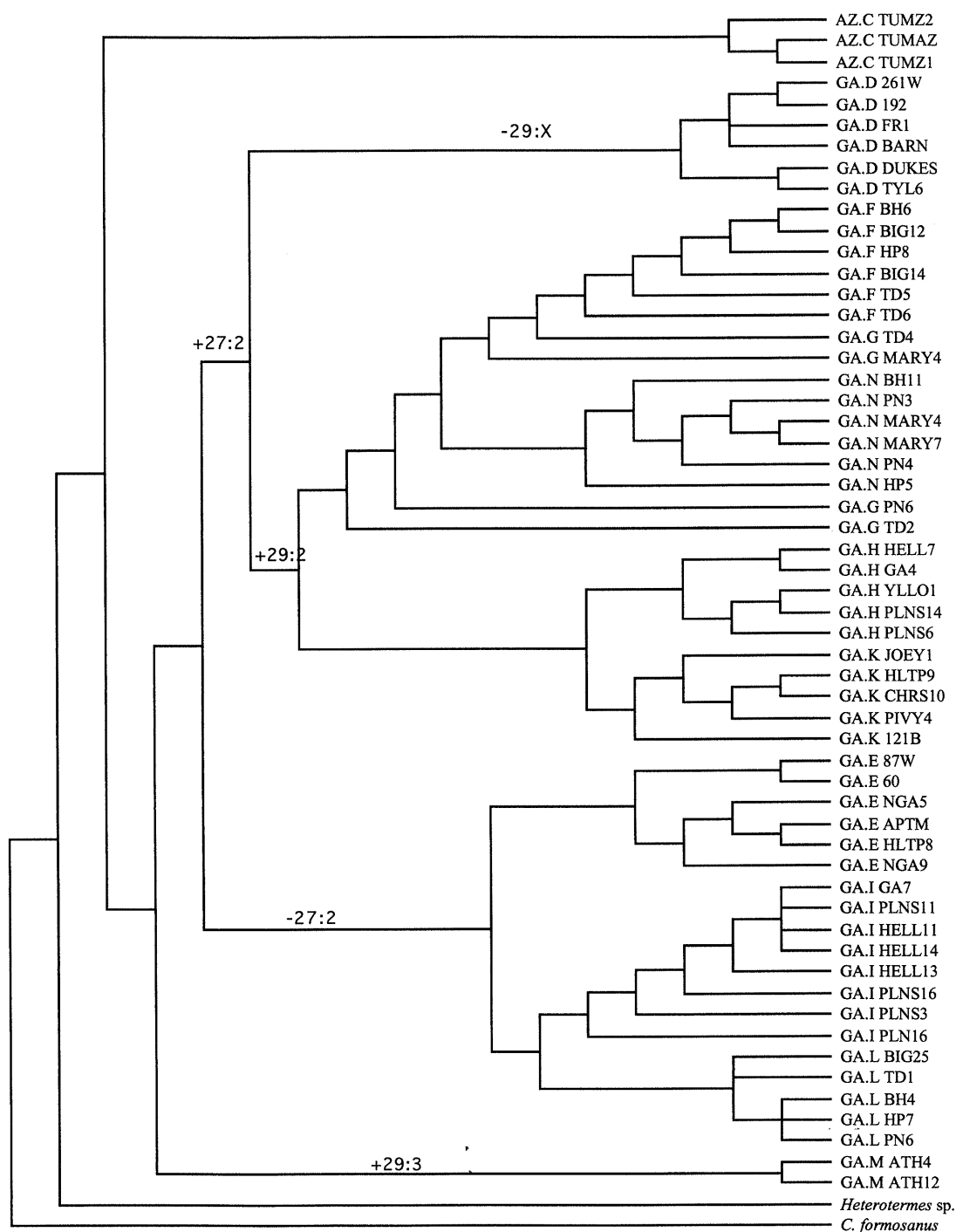


Fig. 8. Strict consensus tree from analysis of all individual samples in lineage III. Tree generated from 60 trees of equal parsimony with both outgroups, length = 1420; consistency index = 0.370, and retention index = 0.744. Numbers above branches are the codes for alkenes and alkadienes.

files of *Coptotermes* or *Reticulitermes* reported thus far (Brown et al., 1990, 1994; Clément et al., 1985; Haverty and Nelson, 1997; Haverty et al., 1990a, 1991, 1992, 1996a,b, 1999a, Howard et al., 1978, 1982).

4. Discussion

A significant amount of variation exists in the cuticular hydrocarbon profiles of *Reticulitermes* collected from sympatric and allopatric geographical locations in the United States (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). We believe this variation in *Reticulitermes* taxa represents species groups, undescribed species and/or subspecies. Takematsu and Yamaoka (1999) reported another example of multiple phenotypes in *Reticulitermes* from Japan and neighboring countries. They propose that the nine cuticular hydrocarbon phenotypes of termites in their study represent different taxa. Their study and ours differ from the case of the soil-burrowing cockroach, *Macropanesthia rhinoceros* Saussure in Australia (Brown et al., 2000). Brown and colleagues argue that cuticular hydrocarbon phenotype differences among *M. rhinoceros* populations did not represent different species, but geographical variation much greater than usually was seen within a species. However, biological information such as body weight, intraphenotype agonism and soldier defense secretions, supports the conclusion that cuticular hydrocarbon phenotypes represent distinct taxa (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a,b; Nelson et al., 2001). In a study evaluating agonistic behavior, Haverty et al. (1999b) discovered that pairings of 10 work-

ers each from different hydrocarbon phenotypes resulted in immediate aggression 61.5% of the time and consistently high mortality after 24 h. Nelson et al. (2001) recently characterized soldier defense secretions from *Reticulitermes* collected from the same geographical regions as in this study. Soldier defense secretion phenotypes have been characterized from 24 of the 26 cuticular hydrocarbon phenotypes. These data lend support to the conclusion that there are numerous sibling species of *Reticulitermes*.

Our initial parsimony analysis was an independent assessment of the sorting of *Reticulitermes* colonies into repeatable chemical phenotype groups. This study supports the empirical selection of 26 chemical phenotypes of *Reticulitermes* (Haverty and Nelson, 1997; Haverty et al., 1996a, 1999a). All subsequent analyses agree on the division of chemical phenotypes into three major clusters, each with a dominant set of cuticular hydrocarbons. Do the relationships described by parsimony analyses in this study indicate lineages among *Reticulitermes*?

The current taxonomy of *Reticulitermes* recognizes three species in Georgia: the sympatric species *R. flavipes*; *R. virginicus*; and *R. hageni*. Two species, *R. flavipes* and *R. virginicus*, as identified by morphological criteria, are represented by four of the seven chemical phenotypes in the 11,15-dimethylalkane clade (Figs. 2 and 3). We consistently identify GA-A, GA-AB and GA-B phenotypes by morphological criteria as *R. flavipes*; Georgia phenotype GA-C is always identified as *R. virginicus* (Haverty et al., 1996a). This identity is consistent with the differentiation of *R. flavipes* from *R. virginicus* by mtDNA analyses of these phenotypes (Jenkins et al., 2000). Only two

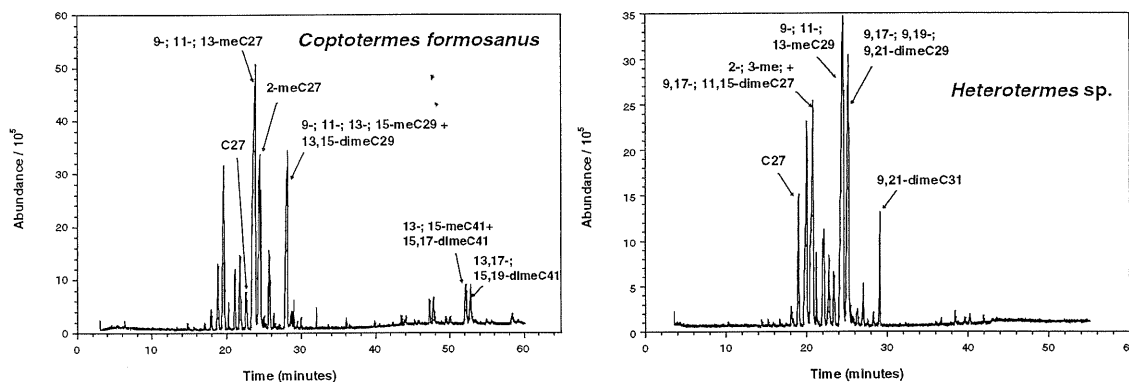


Fig. 9. Total ion chromatograms of cuticular hydrocarbons from *C. formosanus* and *Heterotermes* sp.

species, *R. hesperus* and *R. tibialis*, have been reported from California (Weesner, 1970). In the purportedly exclusive range of *R. hesperus*, all of our samples always keyed to *R. tibialis* by soldier morphology (Haverty and Nelson, 1997). Five different cuticular hydrocarbon phenotypes were characterized from our collections of *Reticulitermes* in northern California. We identified phenotypes CA-A, CA-B and CA-C, from Placerville, California, and phenotypes CA-A' and CA-D in Marin County, California, and various other locations in northern California. However, only phenotypes CA-A and CA-A' are characterized in parsimony analyses as belonging to lineage I. It is not yet clear which of these phenotypes is representative of either *R. hesperus* or *R. tibialis* (Haverty and Nelson, 1997).

Our termite collections in Nevada, Arizona and New Mexico, according to current biogeographical information, should all be *R. tibialis* (Weesner, 1970). We identify six chemical phenotypes (AZ-A, NM-A, NV-A, AZ-B, AZ-C and AZ-D) from these states. One of these, the chemically distinct AZ-C, has been collected only at higher elevations in central and southern Arizona (Haverty et al., 1999a), which, according to published information, suggests to us that it is likely *R. tumiceps* Banks as described by Banks and Snyder (1920). Lineage II contains phenotypes identified as *R. tibialis* (AZ-A, NM-A, and NV-A). We were not able to confirm the identity of phenotypes CA-B, CA-D and AZ-B as *R. tibialis*, but our analyses were unequivocal in the association of all of these phenotypes as members of the 5,17-dimethylalkane cluster. Soldiers of GA-J key to *R. hageni* based on size of the soldier and geographic location.

Collections of phenotypes GA-D, GA-K, GA-H, GA-G, GA-E, GA-I, GA-L, GA-J, GA-F, GA-M and GA-N from Georgia were all identified as *R. hageni* based on soldier morphology. However, alates from collections of GA-E and GA-F keyed to *R. virginicus* and GA-D, was identified by alate morphology as *R. hageni* (Haverty et al., 1996a). All of these phenotypes were consistently sorted as members of the group, lineage III, whose chemical profiles are dominated by unsaturated hydrocarbons.

We are sensitive to the errors in making phylogenetic assumptions from only a few outstanding characters (Krishna, 1970). However, as with morphological characters, insect species that are

closely related should be more similar to each other in production of hydrocarbon components. The synthesis of a preponderance of a particular dimethylalkane within a group of taxa surely indicates relatedness. The evidence that exists on biosynthetic pathways for cuticular hydrocarbons, from a limited number of insects other than *Reticulitermes*, suggests that variation in parent chain lengths, patterns of unsaturation, and methyl branch positions is the result of the activities of different enzymes (Chase et al., 1990; Chu and Blomquist, 1980; Dwyer et al., 1981; Juarez et al., 1992; Mpuru et al., 1996). The evidence is unequivocal for insects studied to date that internal branches in alkanes with multiple methyl branches arise from propionate in the form of methylmalonyl-CoA elongation units (Blomquist et al., 1980; Dwyer et al., 1981; Nelson, 1993). The elongation unit can be derived from succinate (Blomquist et al., 1980), branched-chained amino acids (Dillwith et al., 1982) or odd-chained fatty acids (Voet and Voet, 1995). Species specificity in spacing between methyl branches occurs when a given species controls at what point propionate is incorporated in the elongating hydrocarbon chain (Nelson, 1993). Insertion of propionate in the elongating hydrocarbon chain gives rise to taxaspecific spacing between methyl branches (Blomquist et al., 1980; Chase et al., 1990; Chu and Blomquist, 1980; Nelson, 1993). The regulation of this insertion is unknown, although in the German cockroach, *Blattella germanica*, it appears to be regulated by a microsomal fatty acid synthetase in integumental tissue (Juarez et al., 1992).

Many of the differences in hydrocarbon mixtures of social insects are due to the variability of double bond positions in olefins (Nelson and Blomquist, 1995). Yet there is no known biosynthetic information on the insertion of double bonds in alkenes. There is limited information on the elongation of oleic and linoleic acids in the production of (Z)-9 and (Z,Z)-6,9 double bond positions in insects (Blomquist et al., 1982; de Renobales et al., 1987; Wang et al., 1982). However, there is recent molecular-genetic data for *Drosophila melanogaster* that directly associates the variation in the double bond positions in unsaturated hydrocarbons among males and females with a desaturase gene (Dallerac et al., 2000). Takahashi et al. (2001) identified the nucleotide changes governing this variation in the double bond position. We have identified unsatu-

rated hydrocarbons in 12 phenotypes with tremendous variation in the number and apparent position of double bonds. They form a large group, with subgroups, that we classify as lineage III in this study. We identified 10 of these phenotypes as *R. hageni* based on soldier morphology.

The genera *Heterotermes* and *Reticulitermes* are represented in the fossil record. *Heterotermes* has been identified in amber from Chiapas, Mexico, and *Reticulitermes* is represented by a fossil species in the Eocene Baltic amber and Miocene Florissant of Colorado (Krishna, 1970). This is significant because we did not identify 5,17-dimethylalkanes in the wood roach, *Cryptocercus* sp. (unpublished data), but they are species-specific characters in *Zootermopsis* (Haverty et al., 1988). *Cryptocercus* and *Zootermopsis* are ancestral to all three genera examined this study. *Cryptocercus* sp. does, however, produce a series of 5-methylalkanes. The predominance of unsaturated compounds with 1–4 double bonds, almost exclusive to lineage III of the *Reticulitermes* phenotypes, is also indicative of derived characters. With the exception of C37:1 and C39:1 in *Heterotermes* sp., both ancestral species are devoid of unsaturated hydrocarbons. We have, however, identified unsaturated hydrocarbons in another *Coptotermes* species, *C. acinaciformis* (Haverty et al., 1991, 1992).

The unique hydrocarbons in *C. formosanus* and *Heterotermes* sp. most likely represent derived characters for these species. Six of the 10 unusual dimethylalkanes with one methylene group between methyl branches are unique to *C. formosanus*, as is the only trimethylalkane, 13,15,17-trimethylC27. *C. formosanus* produces only 13,15- and 15,17-dimethyl homologs. This type of dimethylalkane has been identified in very few insects (Nelson, 1993; Nelson et al., 1980; Thompson et al., 1981). We asked several of the authorities who have conducted research on hydrocarbon biosynthesis (Gary Blomquist, Dennis Nelson, Desiree Vanderwel, personal communication), if synthesis of a dimethylalkane with only a methylene bridge is a primitive biosynthetic pathway. To date no one has an answer.

Few insect chemotaxonomic studies using hydrocarbons use parsimony assessments of the phylogeny of the taxa under investigation. To our knowledge, only one other study of cuticular hydrocarbons with termites (Kaib et al., 1991) employed a phylogenetic analysis. The authors

encouraged future investigators to examine cuticular hydrocarbon profiles as an important step in understanding the evolution in particular insect groups and to provide the basis for an independent hypothesis of evolution in termites. The bootstrap analyses and decay indices function to tell us how often we define a group of taxa as well supported. The bootstrap values in this study imply that we could not conclude there are three lineages of *Reticulitermes* because the 95% criterion of statistical confidence (Felsenstein, 1985) was not attained to earn support for all relationships. If we accept that criterion, only one cluster of lineage II, phenotypes AZ-A, NM-A, NV-A and AZ-B, attained this level of significance. We are not inclined to accept this conclusion because the consistency of the appearance of three major groups in these parsimony analyses. The computed decay indices in this study are conservative, sensitive measures of relative support that consistently measure three major groups of phenotypes.

We must conclude here that the relationships discovered with parsimony analyses provide strong evidence when coupled with other biological information, unpublished and published (including the large data set on the biosynthesis of hydrocarbons), that *Reticulitermes* appears to possess three lineages whose distributions are not confined to a particular geographical region in the US. Cuticular hydrocarbons and additional chemical characters such as soldier defense secretions, should allow further discrimination of morphotypes and could lead to identification of a different set of discrete character states for species determination. Careful evaluation of all potential characters, morphological, chemical as well as molecular in a total analysis (Mishler, 1994) should be the next step in determining the phylogenetic placement of *Reticulitermes* taxa in the *Rhinotermitidae*.

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