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Phylogenetic analyses of mtDNA sequences corroborate taxonomic designations based on cuticular hydrocarbons in subterranean termites

Kirsten A. Copren [a,b,*], Lori J. Nelson [b], Edward L. Vargo [c], Michael I. Haverty [b]

[a] *Center for Population Biology, Department of Entomology, 1 Shields Ave, University of California, Davis, CA 95616, USA*
[b] *Chemical Ecology of Forest Insects, Pacific Southwest Research Station, Forest Service, USDA, P.O. Box 245, Berkeley, CA 94701, USA*
[c] *Department of Entomology, Campus Box 7613, North Carolina State University, Raleigh, NC 27695-7613, USA*

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Abstract

Cuticular hydrocarbons (CHCs) are valuable characters for the analysis of cryptic insect species with few discernible morphological characters. Yet, their use in insect systematics, specifically in subterranean termites in the genus *Reticulitermes* (Isoptera: Rhinotermitidae), remains controversial. In this paper, we show that taxonomic designations in *Reticulitermes* from California (USA) suggested in light of differences among CHC phenotypes are corroborated by phylogenetic analyses using mtDNA sequences. Analyses based on CHC phenotypes and supported, in part, by behavioral and ecological differences have suggested the presence of more species than the two currently recognized: *R. hesperus* Banks and *R. tibialis* Banks. We analyze a 680 base pair fragment of the mitochondrial DNA cytochrome oxidase (COII) gene from 45 new (21 collection localities) and two previously recorded samples of *Reticulitermes* from California using parsimony and maximum likelihood methods. Both methods result in trees with highly similar topologies. Bootstrapping indicates support for six clades of *Reticulitermes*, and corroborates groupings based on cuticular hydrocarbons. One of the clades, *R. hesperus*, is already recognized in California, while four clades appear to be previously undescribed taxa. Although identification of the final clade is inconclusive, it includes a sample putatively identified as *R. tibialis*. Therefore, using phylogenetic analyses we corroborate chemical characters used to identify taxa, associate a chemical phenotype with a previously described species, and provide additional support for undescribed taxa of *Reticulitermes*.

Keywords: Western subterranean termite; Cryptic species; Cuticular hydrocarbons; COII; Phylogenetics; *Reticulitermes*

1. Introduction

Reticulitermes is a genus of economically important subterranean termites found in temperate climates of the Holarctic (Weesner, 1970). In forest ecosystems they are important decomposers of woody materials, and are the dominant termites in North America. Proper identification of species is imperative for proper control of these insects in urban settings and for understanding their role in forest ecosystems. However, the taxonomy of North American *Reticulitermes* is problematic and in need of revision (Nutting, 1990; Scheffrahn and Su, 1994; Weesner, 1970). Much of the taxonomic and biogeographical information on *Reticulitermes* spp. was developed in the first half of the last century and needs revision using modern phylogenetic and taxonomic methods (Banks, 1946; Banks and Snyder, 1920; Light, 1934; Miller, 1949; Pickens, 1934a,b; Snyder, 1954).

* Corresponding author. Present address: Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 6035, Faculté des Sciences et Techniques, Parc Grandmont, 37200 Tours, France. Fax: +1 330 247 36 73 56.
E-mail address: kcopren@hotmail.com (K.A. Copren).

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The present state of taxonomy using morphological keys on western *Reticulitermes* is not adequate for proper identification of species. Samples of subterranean termite colonies collected in the field typically consist of a few to dozens of workers, a small percentage of soldiers, and, rarely, alates (winged reproductives). The available keys to species, based on the morphology of soldiers and alates (Banks and Snyder, 1920; Snyder, 1954; Weesner, 1965), are difficult to use and unreliable. If only workers are found, the keys are not useful. Even when soldiers and alates are found together in the same sample, the keys can be equivocal. For example, some collections of termites in Georgia (USA) have soldiers within the size range for *R. hageni* Banks, yet alates from the same sample key to *R. virginicus* Banks (Haverty et al., 1996). For *Reticulitermes* spp. from the Pacific Coastal states, Weesner (1965) did not even attempt a key to the soldiers. Nutting (1990) did include a key to soldiers of *Reticulitermes*, but it is based on the original descriptions of Banks and Snyder (1920) and subsequent synonymies by Snyder (1949), and therefore does not add new, easily useable information. According to the most recently published biogeographical information, only two species of *Reticulitermes*, *R. hesperus* Banks and *R. tibialis* Banks, occur in California (Nutting, 1990; Weesner, 1970). However, morphological keys used on samples throughout northern California keyed all soldiers to *R. tibialis* (Haverty and Nelson, 1997).

Due to the problems with the available keys, Haverty et al. (1996, 1999b, 2000) and Haverty and Nelson (1997) examined the suitability of chemical characters, cuticular hydrocarbons, to distinguish taxa (CHCs). CHCs have aided in the identification of cryptic species in a number of arthropod species including ticks, bark beetles, mosquitoes, and grasshoppers (Buckley et al., 2003; Estrada-Peña et al., 1994; Horne and Priestman, 2002; Page et al., 1997). Typically, qualitative differences in many hydrocarbons coupled with large quantitative differences in a few are argued to be species level variation, whereas quantitative variation in the same hydrocarbons is considered population level variation (Estrada-Peña et al., 1994; Haverty and Nelson, 1997; Page et al., 2002).

Variation in CHC profiles has been shown to be polygenically inherited and important for reproductive isolation in *Drosophila* spp. (Coyne et al., 1994; Dallerac et al., 2000; Takahashi et al., 2001). However, CHCs may also show environmental variation, especially in social insects where CHC profiles can vary depending on nesting materials, food, and diet (Liang and Silverman, 2000; Page et al., 1991). Because of the possible environmental variation of CHCs, their use in taxonomy has been controversial. However, we argue that few studies have systematically attempted to quantify the levels (qualitative versus quantitative) of variation to test the use of cuticular hydrocarbons as taxonomic characters. Previous studies in *Reticulitermes* have shown that qualitative differences in large numbers of cuticular hydrocarbons coupled with large quantitative differences in other hydrocarbons likely represent species level differences and, therefore, can be useful for taxonomic purposes.

The evidence suggesting species-specific mixtures of cuticular hydrocarbons in termites is extensive (Page et al., 2002 and references cited within). In *Reticulitermes* in the western United States repeatable qualitative (presence/absence) and quantitative differences, collectively grouped into CHC phenotypes have been found suggesting the presence of more taxa than currently recognized. Thus far eight CHC phenotypes have been identified in California. Five have been found west of the Sierra Nevada, two in coastal southern California, and one east of the Sierra Nevada (Haverty and Nelson, 1997, unpublished results). If all phenotypes indicate separate species, then there may be at least eight species of *Reticulitermes* in California.

Ecological, behavioral, and additional chemical differences among samples of the CHC phenotypes have supported the suggestion that they represent distinct taxa. Haverty et al. (2003) reported alate flight times differing between two hydrocarbon phenotypes, supporting reproductive isolation between these two phenotypes. Dramatic and unambiguous, interphenotype aggression has been shown among several of the phenotypes studied (Delphia et al., 2003; Haverty et al., 1999a). Workers from colonies possessing different hydrocarbon phenotypes always fight, and the aggression is rapidly expressed. In contrast, when colonies of the same phenotype are paired, aggression is neither consistently rapid nor apparent (Copren, 2004; Delphia et al., 2003; Haverty et al., 1999a). Aggression has been used as a correlate for reproductive isolation under the argument that strong aggression among colonies would prevent mating. In European *Reticulitermes* species the reproductive isolation proposed based on aggression was later supported by chemical and genetic analyses (Clément et al., 2001). Furthermore, separate chemical characters, soldier defensive secretions, have also been found to correlate with CHC phenotypes in western *Reticulitermes* (Nelson et al., 2001). The only information lacking in western North American *Reticulitermes* is phylogenetic confirmation that CHC phenotypes represent monophyletic taxa, which we address in this paper.

We chose to use the cytochrome oxidase II (COII) region of mitochondrial DNA (mtDNA) to examine the putative species complex of *Reticulitermes* in California, previously proposed in the light of the differentiation revealed by CHC phenotypes. This marker has been the most successful and widely used in identifying *Reticulitermes* species (Austin et al., 2002; Jenkins et al., 1999, 2000, 2001; Miura et al., 1998). Other markers such as the nuclear ITS2 were phylogenetically uninformative in *Reticulitermes* (Jenkins et al., 2001; Uva et al., 2004). Phylogenetic analysis of the COII gene supported

currently recognized species designations of *Reticulitermes* in the southeastern states of the United States and corroborated CHC phenotypes as distinct taxa (Jenkins et al., 1999, 2000). A recent phylogeny built using this gene examined 21 species and subspecies of *Reticulitermes* from three continents. It provided support for the separation of Turkish *R. lucifugus* from European members of the species as well as identifying other samples to known species (Austin et al., 2002). Because of its previous use and success with *Reticulitermes*, we chose COII for phylogenetic analysis of *Reticulitermes* samples from California (USA). In this paper, we describe qualitative and quantitative differences in 54 cuticular hydrocarbons. We hypothesize that the combined differences, known as CHC phenotypes, represent monophyletic taxa of *Reticulitermes*. Using phylogenetic analyses of the COII gene, we test this hypothesis.

2. Materials and methods

2.1. Collection information

The original hydrocarbon phenotypes were identified from two field sites, one in Marin County and one in El Dorado County (Haverty and Nelson, 1997). Due to previous and ongoing work most of our samples came from these two field sites (Delphia et al., 2003; Getty et al., 2000a,b; Haverty and Nelson, 1997; Haverty et al., 1999a,b, 2000, 2003; Nelson et al., 2001; Page et al., 2002). Furthermore, two of the phenotypes, CA-B and CA-C, have not yet been found outside of El Dorado County (Fig. 1). The remaining samples were collected to examine the distribution of *Reticulitermes* CHC phenotypes throughout California. The 45 total samples collected and their locations are reported in Table 1, including topotype samples. To correlate CHC phenotypes with species designations, we collected topotype samples (samples were collected from the type locality). The type specimen of *R. hesperus* was reported from Little Bear Lake, now known as Lake Arrowhead, in the San Bernardino Mountains of California, USA (Snyder, 1949). We have collected multiple samples from the vicinity of Lake Arrowhead, found them all to be the same phenotype (SC-A), and thus have designated this phenotype as the CHC phenotype of *R. hesperus* (Haverty et al., 2003). Voucher specimens have been submitted to the Essig Museum at the University of California, Berkeley.

2.2. Designations of cuticular hydrocarbon phenotypes

Methods for isolation and identification of cuticular hydrocarbons using gas chromatography–mass spectrometry methods can be found in Haverty et al. (1996). Cuticular hydrocarbon phenotypes of *Reticulitermes* have been identified and discussed in detail in previous

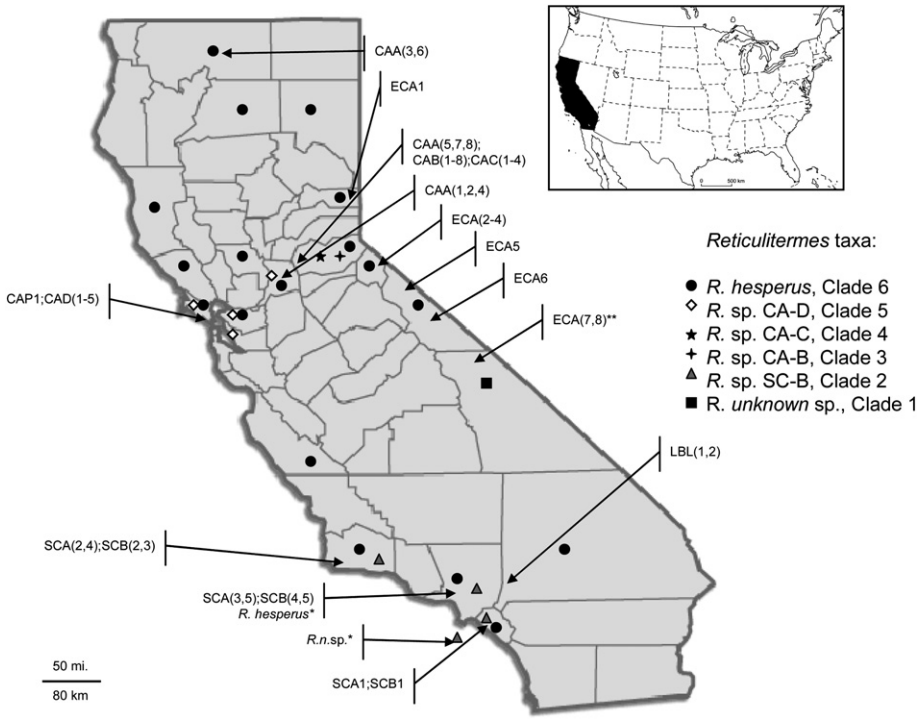

Fig. 1. Approximate distribution of *Reticulitermes* taxa in California (USA). Symbols indicate the taxon identified by cuticular hydrocarbon phenotype has been found in the county, reported either in this study or previously in Haverty and Nelson (1997). Labels and arrows indicate the approximate localities of the specimens from Table 1 sequenced for this study. Forty-five new samples were collected from 21 localities. *Sequence from Austin et al. (2002). **These samples had the same cuticular hydrocarbon phenotype as *R. hesperus*, but the mtDNA sequences fell into a separate clade.

Table 1
Collection localities for 45 *Reticulitermes* samples from 21 different collection localities in California (USA)

Label	Hydrocarbon phenotype	County	Elevation (m)	Latitude (°N)	Longitude (°W)	GenBank Accession No.
CAA1	CA-A	Yolo	14	38.54509	121.73798	AY623459
CAA2	CA-A	Yolo	14	38.54509	121.73798	AY623434
CAA3	CA-A	Siskiyou	1091	41.27919	122.07452	AY623464
CAA4	CA-A	Yolo	14	38.54509	121.73798	AY623477
CAA5	CA-A	El Dorado	828	38.74211	120.74406	AY623478
CAA6	CA-A	Siskiyou	1312	41.18742	121.72513	AY623465
CAA7	CA-A	El Dorado	828	38.74211	120.74406	AY623450
CAA8	CA-A	El Dorado	828	38.74211	120.74406	AY623462
CAP1	CA-A′	Marin	828	38.74211	120.74406	AY623457
CAB1	CA-B	El Dorado	828	38.74211	120.74406	AY623453
CAB2	CA-B	El Dorado	828	38.74211	120.74406	AY623474
CAB3	CA-B	El Dorado	828	38.74211	120.74406	AY623442
CAB4	CA-B	El Dorado	828	38.74211	120.74406	AY623452
CAB5	CA-B	El Dorado	828	38.74211	120.74406	AY623451
CAB6	CA-B	El Dorado	828	38.74211	120.74406	AY623460
CAB7	CA-B	El Dorado	828	38.74211	120.74406	AY623472
CAC1	CA-C	El Dorado	828	38.74211	120.74406	AY623461
CAC2	CA-C	El Dorado	828	38.74211	120.74406	AY623475
CAC3	CA-C	El Dorado	828	38.74211	120.74406	AY623473
CAC4	CA-C	El Dorado	828	38.74211	120.74406	AY623470
CAD1	CA-D	Marin	7	38.10888	122.57244	AY623458
CAD2	CA-D	Marin	7	38.10888	122.57244	AY623456
CAD3	CA-D	Marin	7	38.10888	122.57244	AY623449
CAD4	CA-D	Marin	7	38.10888	122.57244	AY623455
CAD5	CA-D	Marin	7	38.10888	122.57244	AY623454
SCA1	SC-A	Orange	26	33.66688	117.81804	AY623463
SCA2	SC-A	Santa Barbara	19	34.44649	119.83879	AY623443
SCA3	SC-A	Los Angeles	115	34.08567	117.96108	AY623447
SCA4	SC-A	Santa Barbara	19	34.44649	119.83879	AY623448
SCA5	SC-A	Los Angeles	86	34.0565	118.23845	AY623446
SCB1	SC-B	Orange	26	33.66688	117.81804	AY623471
SCB2	SC-B	Santa Barbara	19	34.44649	119.83879	AY623467
SCB3	SC-B	Santa Barbara	29	34.40715	119.51702	AY623466
SCB4	SC-B	Los Angeles	86	34.0565	118.23845	AY623468
SCB5	SC-B	Los Angeles	115	34.08567	117.96108	AY623469
LBL1	SC-A	San Bernardino	1587	34.25836	117.16826	AY623444
LBL1	SC-A	San Bernardino	1587	34.25836	117.16826	AY623445
ECA1	CA-A	Sierra	1857	39.46537	120.23232	AY623436
ECA2	EC-A	Alpine	1734	38.77362	119.82806	AY623437
ECA3	EC-A	Alpine	1865	38.7254	119.79903	AY623438
ECA4	EC-A	Alpine	1777	38.64631	119.72595	AY623439
ECA5	EC-A	Mono	1775	38.50212	119.4779	AY623440
ECA6	EC-A	Mono	2124	38.17667	119.22431	AY623441
ECA7	EC-A	Inyo	2600	37.24015	118.59368	AY623435
ECA8	EC-A	Inyo	2888	37.21932	118.60673	AY623476

Samples and cuticular hydrocarbon phenotypes are labeled as described in text. Prefixes indicate general geographic location of the hydrocarbon phenotype, e.g., CA, California; SC, southern California; EC, eastern California. The label "LBL" (Little Bear Lake) indicate the topotype samples. Approximate locations are depicted in Fig. 1.

studies (Haverty et al., 1996, 1999c; Haverty and Nelson, 1997; Nelson et al., 2001; Page et al., 2002). A CHC "phenotype" comprises the relative quantities of various hydrocarbon components. As new samples of termites are analyzed, if their overall chemical profile displays qualitative or significant quantitative differences from those previously examined, they are given a new "phenotype" name. We describe our nomenclature below.

The publication describing the five phenotypes from northern California (Haverty and Nelson, 1997) used a single letter designation for each phenotype (A, A′, B, C, D). However, the prefix CA- was added in subsequent publications to avoid confusion with other CHC phenotype names such as GA-A from Georgia (USA). Phenotypes were designated with a letter in the order they were discovered in each location where we collected samples. Thus, there is not necessarily a correspondence between phenotypes with the same letter suffix, such as CA-A and GA-A. Samples collected in southern California are indicated with the prefix "SC-" and those collected east of the Sierra Nevada are given the prefix "EC-." The use of the location/state prefix does not preclude the

existence of a given phenotype in a different location; it is merely our naming convention. With information from multiple character sets we aim to replace these phenotype names with definitive species names.

We have included a table of the most diagnostic hydrocarbons in Table 2 for eight CHC phenotypes: CA-A, CA-A′, EC-A, SC-A, SC-B, CA-B, CA-C, and CA-D. A phylogram showing the similarities among the phenotypes obtained as described in Page et al. (2002) is shown in Fig. 2. This tree was obtained using an UPGMA cluster analysis based on the chemical similarity between samples. This was found by converting the relative quantity of each cuticular hydrocarbon (percent of total hydrocarbon) into a discrete character (Page et al., 2002). Five chemical groups are apparent, and we hypothesize that these five groups represent separate taxa. We compare these groupings with those obtained by phylogenetic analysis.

Cuticular hydrocarbon phenotypes CA-A, CA-A′, EC-A, and SC-A are qualitatively and quantitatively very similar. We hypothesize these differences are geographic variation of the same taxon. None of the other CHC phenotypes (CA-B, CA-C, CA-D, and SC-B) share the same level of similarity (Table 2, Fig. 2), and we hypothesize these represent separate, independent taxa. A complete discussion of the similarities and differences among CA-A, CA-A′, CA-B, CA-C, and CA-D can be found elsewhere (Haverty and Nelson, 1997). Of these phenotypes, CA-A, CA-B, and CA-C are sympatric, as are CA-A, CA-A′ and CA-D, and SC-A and SC-B (Fig. 1).

2.3. Mitochondrial DNA

Sequences were extracted from workers in colonies that had been previously assigned a hydrocarbon phenotype. A segment of the cytochrome oxidase II (COII) region of mtDNA was sequenced using universal primers, A-t Leu and B-t Lys, described by Liu and Beckenbach (1992). A-t Leu begins is found in the COI gene (also called TL2-J-3037: 5′-ATGGCAGATTAGTGCA ATGG-3′) and B-t Lys (also called TK-N-3785: 5′-GTTTAAGAGACCAGTACTTG-3′) at the end of the COII gene, the result being the amplification of almost the entire COII region. Amplification of this region was performed on two individuals per colony. Reaction volumes were 50.0 µl, containing approximately 20 ng of genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.06 U Biolase *Taq* polymerase (Bioline), and 1 pmol each primer. PCR was performed using the following program: initial denaturation step at 94 °C (2 min) followed by 35 cycles of 94 °C (1 min), 50 °C (1 min), and 70 °C (2 min) with a final extension step at 70 °C (5 min).

Amplified products were PCR purified using the QIAquick PCR purification kit (QIAGEN). Both forward and reverse sequencing reactions were performed using 1× ABI reaction mixture (ABI Prism DRhodamine Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) and 0.3 pmol of either primer A-t Leu or B-t Lys. Samples were then run through CENTRI-CEP columns (Princeton Separations), dried using a speed-vac and run on an ABI 377 Automated Sequencer.

2.4. Phylogenetic analyses

Samples of the same COII region were taken from GenBank from putative *Reticulitermes* species in North America (Austin et al., 2002; Jenkins et al., 2001; Ye et al., 2004) to ensure that our samples were not the same as the other described species (Table 3). There have been few phylogenetic studies on western subterranean termites, so to be thorough we included all samples previously recorded from California emphasizing those putatively identified as the two recognized species: *R. hesperus* and *R. tibialis*. The type locality of *R. tibialis* is not in California (Snyder, 1949), but this species has been reported to occur in the inland valleys, deserts in the southeastern counties, and the eastern Sierra Nevada, although there is considerable disagreement among authors as to its precise distribution (Nutting, 1990; Pickens, 1934b; Weesner, 1970). Therefore, we have included a sequence that was putatively identified as *R. tibialis*, collected in Cochise County, Arizona (USA) (GenBank AF525355). We have also included an unidentified *Reticulitermes* species collected in California on Catalina Island, California (listed as *R. new* sp., GenBank AF525342) (Austin et al., 2002). A sample of *Coptotermes formosanus* Shiraki (GenBank AF525317) was taken from GenBank to be used as the outgroup taxon. *C. formosanus* was selected as the outgroup because we hypothesized that it is one of the true ancestral species based on the published phylogenies of the subfamilies of Rhinotermitidae. Coptotermitinae is considered the most primitive subfamily of the Rhinotermitidae and *Coptotermes* is the single genus within the subfamily (Ahmad, 1950; Krishna, 1970; Snyder, 1949).

Sequences were aligned with ClustalW (Thompson et al., 1994) and adjusted manually. Phylogenetic reconstructions were calculated with PAUP* 4.0b10 (Swofford, 2001). Maximum parsimony (MP) analyses were performed (heuristic searches with random stepwise addition and TBR branch swapping options). The robustness of the strict consensus tree was examined after 1000 bootstrap replicates (Felsenstein, 1985). The phylogenetic relationships were also investigated using maximum likelihood (ML) analyses (heuristic searches with simple stepwise addition) based on the Tamura and Nei (1993) model of substitution chosen by Modeltest 3.5 (Posada and Crandall, 1998) with $\langle\alpha\rangle = 0.2579$ (TrN + G) and four rate categories. The Akaike criterion

Table 2
Diagnostic hydrocarbons (54 total) showing main qualitative differences and largest quantitative differences among phenotypes

		Cuticular hydrocarbon phenotype							
Clade number:		6	6	1,6	6	2	3	4	5
Associated taxon:		*hesperus*	*hesperus*	*hesperus*	*hesperus*	New	New	New	New
Hydrocarbon	ECL	CA-A	CA-A′	EC-A	SC-A	SC-B	CA-B	CA-C	CA-D
Olefins									
C25:1	24.70	+	+	+	+	tr		+	
C27:1	26.70							++	
C25:2	24.75			tr		+		+	tr
C25:2	25.35	+++	++	+++	+				
C25:2	25.50	+	+	+	+				
C27:2	26.70					tr		++	
C27:2	27.35	+	+	tr	tr			+	
C25:3	25.98		++	+	+	+			
C25:3	26.07		++	++	++	tr			+
Terminally branched methylalkanes									
2-meC23		+	+	+	+	tr		+	+
6-meC24						tr	tr		+
5-meC24						tr	tr		+
3-meC24		+	+	+	+	+			
5-meC25						+++	++		++
6-meC26						+	+		+
5-meC27						+	++		+
Internally branched methylalkanes									
9; 11-meC23		++	+	+	+			tr	+
11; 12-meC24		+	+	+	+		tr	+	+
7-meC25						++	+	+	+
11; 13-meC27		+	+	+		+	++	+++	+
7-meC27						+	+	tr	tr
11; 13; 15; 17-meC33							+		
11; 13; 15; 17-meC35		+	+	+	+		+	+	+
11; 13; 15; 17; 19-meC39		+	+	+	+		+	+	+
11; 13; 15; 17-meC41		tr	tr	tr	tr			+	+
11; 13; 15; 17; 19-meC43								+	
Dimethylalkanes									
9,11-dimeC23					+				
9,13-dimeC23					+				
9,13-dimeC24					+				
9,11-dimeC25					+				
9,13-dimeC25		++	++	+++	++				+
7,17-dimeC25						+			
5,17; (5,15; 5,9)-dimeC25						++	+		+++
6,18-dimeC26						+	+		+
5,17-dimeC26						+	+		tr
4,16-dimeC26						tr	+		tr
1,15-dimeC27		tr	tr	tr				++	
5,17-dimeC27						+++	+++		+
5,17-dimeC33						++	+		
11,15; 13,17-dimeC35		+	++	++	+			+	tr
5,17-dimeC35						++	++		+
11,15; 13,17-dimeC37		++	++	++	++			+	+
5,17; 5,15-dimeC37						+	++		+
11,15; 13,17-dimeC39		+	+	+	++			+	+
5,17-dimeC39						+	+		+
11,15; 13,17-dimeC41		+	+	tr	tr			+	+
5,17-dimeC41						+	+		++
11,15; 13,17; 15,19-dimeC43			tr	tr	+			+	+
5,17-dimeC43						+	+		+
Trimethylalkanes									
5,9,15-trimeC25						+			+
5,9,17-trimeC27						+	+		tr
5,9,17-trimeC35							+		tr

Table 2 (*continued*)

		Cuticular hydrocarbon phenotype							
Clade number:		6	6	1,6	6	2	3	4	5
Associated taxon:		*hesperus*	*hesperus*	*hesperus*	*hesperus*	New	New	New	New
Hydrocarbon	ECL	CA-A	CA-A′	EC-A	SC-A	SC-B	CA-B	CA-C	CA-D
5,9,17-trimeC37							+		tr
9,11,13-trimeC25					+				

Symbols represent the following ranges of percent of total hydrocarbon: 0, not detected; tr, <0.3%; +, 0.3–3%; ++, >3–10%; +++, >10%. ECL, Equivalent chain length (approximate). Similarities among phenotypes are depicted in Fig. 2. Phenotypes CA-A, CA-A′, EC-A, and SC-A are highly similar and are hypothesized to be geographic variations of the same taxon. The remaining phenotypes (CA-B, SC-B, CA-C, and CA-D) are less similar and are hypothesized to be separate taxa. Clade numbers of phenotypes and associated taxa were determined as in text, and see Fig. 3.

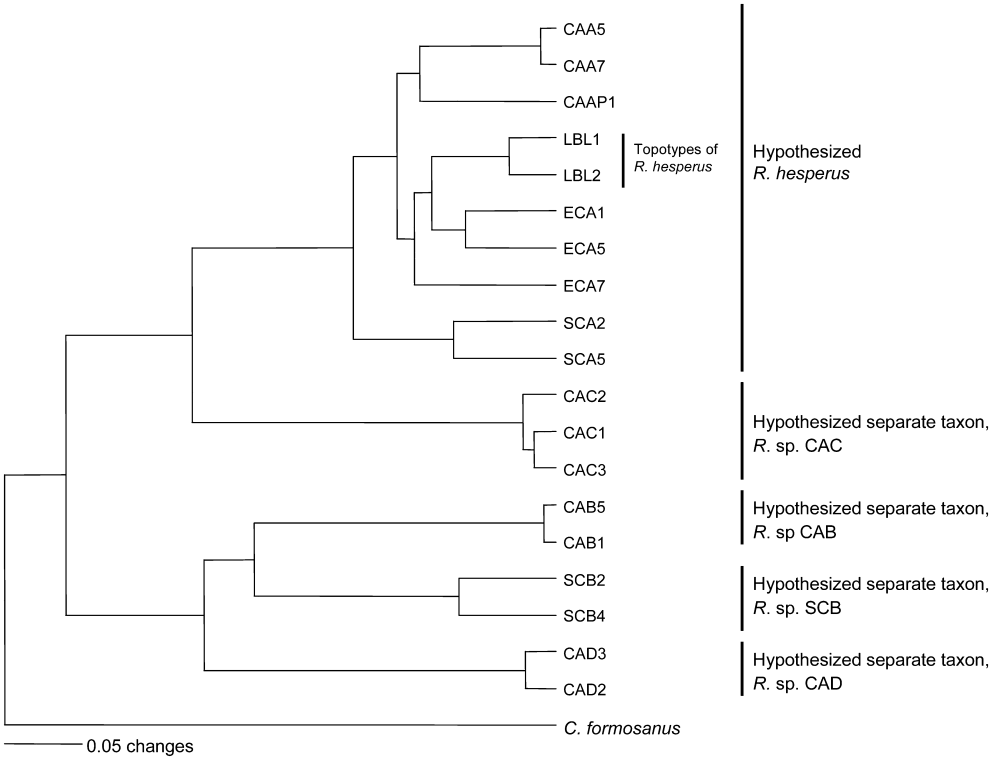

Fig. 2. UPGMA phylogram showing similarities among the chemical distances of different hydrocarbon phenotypes presented in this paper. Five chemical clades are shown, one of which is hypothesized to represent *R. hesperus*.

Table 3
Mitochondrial COII sequences of termite species from the United States taken from GenBank

Species	Location of sample	GenBank Accession No.
R. tibialis Banks[a]	Cochise Co, AZ	AF525355
R. new sp.[a]	Catalina Island, CA	AF525342
R. hesperus Banks[a]	Los Angeles County, CA	AF525329
R. virginicus Banks[b]	Wheatefield, IN	AY168205
R. flavipes (Kollar)[a]	West Lafayette, IN	AY168210
R. hageni Banks[c]	Georgia	AY027478
Coptotermes formosanus Shiraki[a]	Galveston, TX	AF525317

[a] Austin et al. (2002).
[b] Ye et al. (2004).
[c] Jenkins et al. (2001).

was not used to choose the substitution model. The robustness of the ML tree was examined with 500 bootstrap replicates performed using PHYML (Guidon and Gascuel, 2003).

3. Results

The sequence alignment resulted in 680 characters, of which 124 (18%) were parsimony informative, and 96 (14%) were variable but parsimony uninformative. Of the parsimony informative characters, 66 were third codon positions. Both MP and ML resulted in trees with highly similar topologies identifying six terminal clades highlighted in Fig. 3 that largely support the groups

proposed based on CHC phenotypes. The MP heuristic search resulted in six most parsimonious trees. All had the same six terminal clades, but minor differences within these clades. All trees had a length of 412, consistency indices (CI) of 0.624, and retention indices (RI) of 0.887. Removing third codon positions from the analysis did not change the deeper branches of the MP or ML trees (data not shown). Because removing the third codon positions did not affect the deeper branches and both the CI and RI indices are reasonably "large" for heuristic measures, we conclude that the COII region contains useful phylogenetic information for the taxonomic level we are examining. The MP strict consensus tree is reported in Fig. 3. The ML heuristic search resulted in an almost identical topology to the trees found using MP, except for a single difference. In the ML tree the GenBank *R. new* sp. falls within the clade of SC-B samples, but it is the sister group to the SC-B samples in the MP consensus tree. The six important monophyletic clades are labeled 1–6 on each tree according to their appearance in the phylogenies, and are discussed in numerical order (Fig. 3).

Clade (1) includes the putative *R. tibialis* sample and ECA7 and ECA8 (MP: 70% bootstrap support, ML: 100%). Clade (2) includes all phenotype SC-B samples along with the GenBank *R. new* sp. from Catalina Island (MP: 68% bootstrap support, ML: 50%). Clade (3) includes all phenotype CA-B samples (MP: 100% bootstrap support, ML: 100%). Clade (4) includes all phenotype CA-C samples (MP: 100% bootstrap support, ML: 100%). Clade (5) includes all phenotype CA-D samples (MP: 99% bootstrap support, ML: 99.8%). Clade (6) (MP: 99% bootstrap support, ML: 99.8%) includes all phenotype CA-A samples, all phenotype SC-A samples, the phenotype CA-A′ sample, the phenotype EC-A samples 1–6, as well as the topotypes of *R. hesperus* (LBL1&2).

4. Discussion

The parsimony and maximum likelihood methods produced trees with almost identical topologies, and bootstrapping supported six terminal clades of *Reticulitermes* (Fig. 3). The six terminal clades separate samples

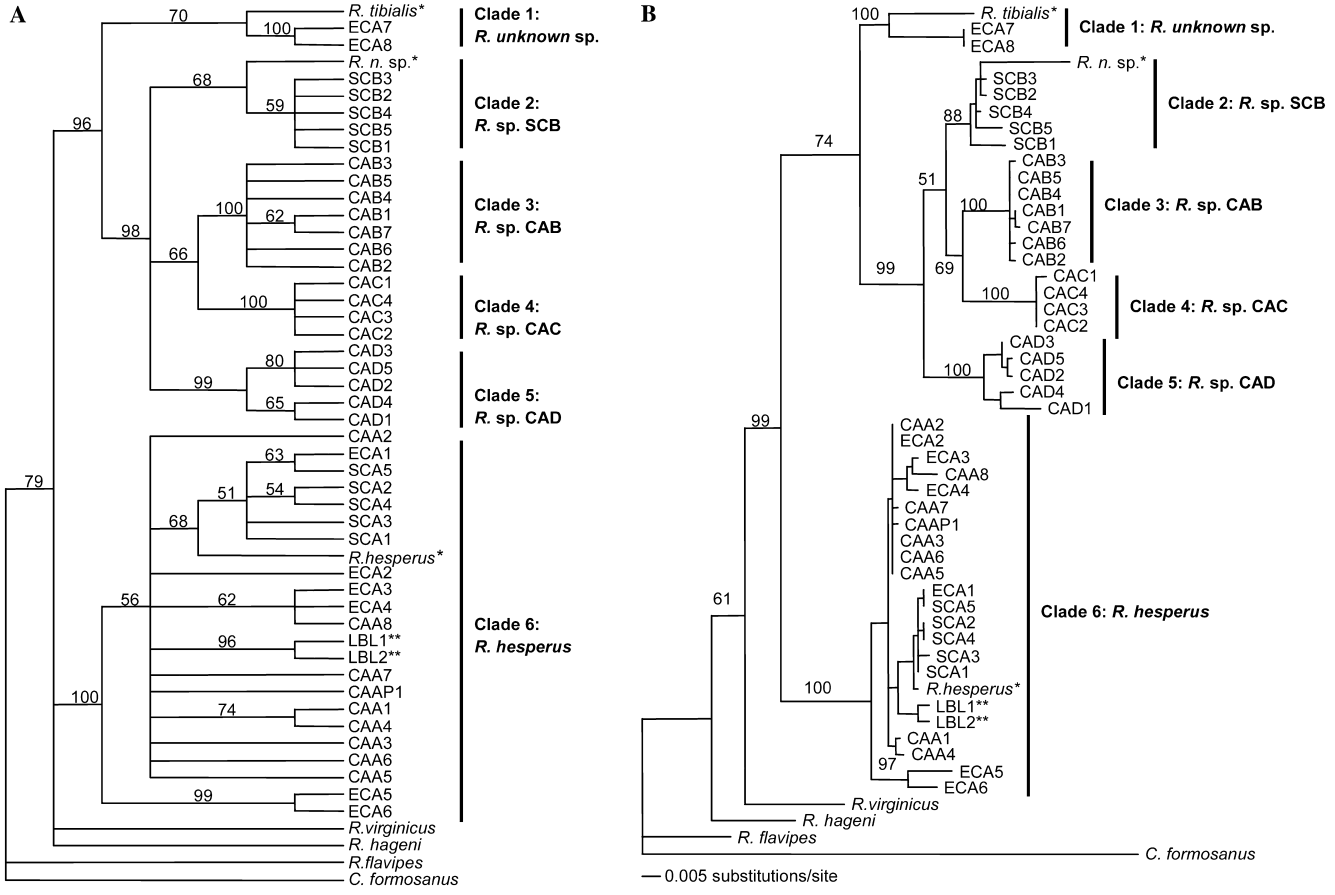

Fig. 3. Phylogenetic trees of *Reticulitermes* obtained from 680 bp of the cytochrome oxidase II (COII) gene of mitochondrial DNA. (A) Strict parsimony consensus of six trees of 412 changes. Bootstrap values for 1000 replicates are listed above the branches supported ⩾50%. (B) Maximum-likelihood phylogram constructed using the Tamura and Nei (1993) model of sequence evolution. Bootstrap values for 500 replicates are listed above branches supported ⩾50%. *Sequence from Austin et al. (2002). **Topotype samples of *R. hesperus*.

from different CHC phenotypes into monophyletic groups, with the exception of the EC-A phenotype found in clade (1) and (6). These six clades, with the exception of two samples (ECA7 and ECA8) support our hypothesis that taxa determined by CHC phenotypes are monophyletic. Clades 2–5 together form a monophyletic lineage and are discussed first, followed by Clade 6 and lastly, Clade 1.

Together Clades 2–5 form a well-supported lineage. The resolution of the relationship among Clades 2–5 is less well resolved, specifically in the MP consensus tree. However, there is strong support for each clade (2–5) within this larger group. Clade 2 consists of all the samples from the SC-B phenotype from five different populations located along coastal southern California. This phenotype occurs sympatrically with the SC-A phenotype. The unknown species in Austin et al. (2002) from Catalina Island, California, fell into the SC-B clade. We would predict that the cuticular hydrocarbons for this sample would be similar to the SC-B phenotype, although we do not have those data available for verification. This sample is genetically distinct from the rest of the SC-B samples, which would be expected of an isolated island population. Clade 2 has the weakest bootstrap support on both ML and MP trees. The hydrocarbons are most closely related to samples of the CA-B phenotype, yet it is the sister taxon to both the CA-B (Clade 3) and CA-C (Clade 4) samples in this phylogeny. Further analyses examining soldier defensive chemicals is underway to gain further insight into the SC-B clade and hopefully clarify its relationship to Clades 3 and 4 (Nelson and Haverty, unpublished data).

Clade 3 (CA-B samples) and Clade 4 (CA-C samples) are well-supported with bootstrap values of 100%. Samples of these phenotypes come from a single location because CA-B and CA-C, sympatric with CA-A in this location (Clade 6), have not been found outside of El Dorado County (Fig. 1). Colonies of the CA-B and CA-C phenotypes are rare in comparison to colonies of the CA-A phenotype in this community (Haverty and Nelson, 1997). Behavioral differences support the genetic and chemical separation of these three sympatric phenotypes. Individuals respond aggressively to workers of different phenotypes within 5 min and mortality is high, whereas aggressive interactions between workers from separate colonies of the same phenotypes on average are observed within one hour and mortality is lower (Copren, 2004; Delphia et al., 2003; Haverty et al., 1999a).

Clade 5 consists of all CA-D samples. This phenotype is sympatric with both CA-A and CA-A′, but is phylogenetically distinct in this study. The genetic and chemical separation of the CA-A/A′ phenotype from CA-D has been supported with aggressive behavior as described above for CA-A/B/C (Delphia et al., 2003; Haverty et al., 1999a). In addition, a recent study reported differences in alate flight periods. Flights in the spring were exclusively of the CA-A/A′ phenotype, while flights in the fall were exclusively CA-D phenotypes, supporting reproductive isolation between the two phenotypes (Haverty et al., 2003). The distribution of the CA-D phenotype appears to be mostly confined to coastal areas in northern California (Haverty and Nelson, 1997) (Fig. 1).

We designate Clade 6 as *R. hesperus* because it contains the topotype samples. Therefore, the *R. hesperus* clade includes all the CA-A, CA-A′, and SC-A samples as well as the EC-A samples in and north of Mono County, CA (ECA 1-6, Fig. 1). Cuticular hydrocarbon phenotypes CA-A, CA-A′, EC-A, and SC-A are qualitatively and quantitatively very similar. They are distinguished primarily by differences in the quantities of certain olefins (Table 2). The topotype samples (labeled LBL) have hydrocarbons that are very similar to the CA-A/A′ phenotypes, and have quantities of the diagnostic olefins intermediate to these phenotypes, suggesting that CA-A/A′ is *R. hesperus* (Haverty et al., 2003). This supports our hypothesis that the relatively slight variations in cuticular hydrocarbons among the "A" phenotypes (CA-A, CA-A′, EC-A, and SC-A) (Table 2, Fig. 2) are geographical variations of the same taxon. This is true except for the EC-A phenotype which has samples that are not found within the *R. hesperus* clade (Clade 6). The samples ECA5 and ECA6 are similar to one another and found within the *R. hesperus* clade, but they are genetically different from the rest of the samples in this clade. As ECA5 and ECA6 are found to the east of the Sierra Nevada and the remaining samples are found to the west, this relationship is consistent with limited gene flow among populations of a geographically separate species. Intriguingly, two samples of the EC-A phenotype do not fall into the *R. hesperus* clade. ECA7 and ECA8 fall into a separate clade (1) that is most closely related to the sequence of the *R. tibialis* GenBank sample from Arizona.

Haverty et al. (1999c) identified a hydrocarbon phenotype from Coconino County in northern Arizona (USA) called AZ-D that is quantitatively most similar to the EC-A phenotype reported here. This phenotype was found recently in Clark County, Nevada (USA), the southernmost county closest to Arizona (Nelson and Haverty, unpublished data). However, this phenotype was relatively rare in extensive collections of *Reticulitermes* performed in Arizona (Haverty et al., 1991, 1999c, unpublished results). Our results show ECA7 and ECA8 to be most closely related to the *R. tibialis* GenBank sample from Arizona, which is consistent with a relationship between our EC-A and AZ-D hydrocarbon phenotypes. However, we have no knowledge of the hydrocarbon phenotype of the *R. tibialis* GenBank sample to enable us to verify this.

The position of the *R. hesperus* clade suggest that *R. hesperus* is the ancestral taxon of this group. *R. hesperus* is the most common species encountered in California and has the largest distribution for all samples studied in California (Fig. 1) (Haverty and Nelson, 1997, unpublished). If distribution reflects the age of the taxon, the wide distribution of *R. hesperus* is consistent with the phylogenetic data suggesting it is the ancestral group for the taxa presented here. The paraphyly of the "A" phenotypes (CA-A/A′, EC-A, and SC-A) also suggests that Clade 1, which contains ECA-7 and ECA-8, evolved from *R. hesperus*. Furthermore, the more localized distribution of the other taxa suggest that they have speciated from, or are in the process of speciating from, *R. hesperus*. MP analysis excluding third codon positions retained three of the six major clades here: 1, 6, and 2–5 were collapsed into a single clade (data not shown). This information supports the hypothesis that *R. hesperus* is the ancestral taxon and that the other hydrocarbon phenotypes, i.e., taxa, evolved from *R. hesperus*.

Caution is warranted in determining which phenotype is *Reticulitermes tibialis*. Austin et al. (2002) identified *R. tibialis* from Arizona using keys based on morphology of soldiers and alates. Yet it has been argued that the keys themselves are problematic (Haverty and Nelson, 1997). The sample from the Austin et al. (2002) study groups together in our phylogeny with the eastern California samples which are similar to the AZ-D phenotype suggesting a relationship between the eastern California samples and the Arizona samples. It has been suggested that *R. tibialis*, itself, may be a species complex (Szalanski et al., 2003). Therefore, we do not attempt to identify this clade. Further sampling from the type locality and genetic analysis is required to determine the species distribution of *R. tibialis* and whether or not ECA7 and ECA8 are related or separate taxa.

Extensive morphological analysis has been attempted on western North American *Reticulitermes* with little success, and may not provide information adequate for future keys (Haverty, unpublished results). Chemical and genetic analyses have provided better results. In fact, this work supports elevating the CHC phenotypes CA-B, CA-C, CA-D, and SC-B to new taxa using the diagnostic hydrocarbons shown in Table 2. However, the lack of monophyly for the "A" phenotype underscores that taxonomic designations based on hydrocarbons alone may not correctly identify all species. *R. hesperus* is chemically similar to the two samples ECA-7 and ECA-8, but genetically quite different. Therefore, with *Reticulitermes*, multiple character sets are required to adequately describe species, as we have used here.

In conclusion, *Reticulitermes hesperus* (Clade 6) consists of hydrocarbon phenotypes CA-A, CA-A′, SC-A, and the samples of EC-A that fall within this clade. Future identification of which EC-A samples that fall within the *R. hesperus* clade may be based on cuticular hydrocarbons and specific mitotypes as Szalanski et al. (2003) have proposed for other *Reticulitermes* species. For the remaining phenotypes that were monophyletic, CA-B, CA-C, CA-D, and SC-B, evidence is strongest for identifying CA-D as a species separate from *R. hesperus* based on the differences of reproductive flight times (Haverty et al., 2003). We feel that it is premature to conclusively identify SC-B, CA-B, and CA-C as separate species. However, we have tentatively labeled them as new, putative species to ease taxonomic confusion (Fig. 1). Future genetic and chemical studies will more closely examine the species status of these taxa to determine whether they are independent species or simply subspecies of *R. hesperus*.

This phylogenetic study illustrates that cuticular hydrocarbons can be useful taxonomic characters in some groups of insects. Our findings support groupings of putative taxa identified based on qualitative and quantitative differences in CHC profiles. Cuticular hydrocarbons, by themselves, may not be enough to describe a new species, yet are an excellent first grouping strategy for taxa with few distinguishing morphological characters. This method gave us the first clue that there are more species of *Reticulitermes* in California than currently recognized. The genetic data largely support the groupings found using cuticular hydrocarbons, and suggest elevating some of these phenotypes to separate taxa. Further studies using genetic, morphological, and additional chemical analyses should aid in understanding the taxonomy and phylogenetics of this genus in North America.

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