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Phylogenetic analyses of *Reticulitermes* (Blattodea: Rhinotermitidae) from California and other western states: multiple genes confirm undescribed species identified by cuticular hydrocarbons

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Subterranean termites in the genus *Reticulitermes* Holmgren 1913 are among the most economically important wood-destroying pests in the western United States. Yet, there remains uncertainty regarding the taxonomy and biology of the species in this genus. The 2 species described as having distributions in this region are the western subterranean termite, *Reticulitermes hesperus* Banks, and the arid land subterranean termite, *Reticulitermes tibialis* Banks. Taxonomic studies utilizing cuticular hydrocarbon (CHC) profiles, agonistic behavior, flight phenology, and mitochondrial DNA (mtDNA) suggested that *R. hesperus* is a species complex comprised of 2 or more sympatric, yet reproductively isolated species. To further delineate these taxa, we examined multiple genes from samples of *Reticulitermes* collected in the western United States. Alates collected after recent spring and fall mating flights, as well as previously collected workers, were subjected to CHC phenotyping and DNA sequence analyses that targeted mitochondrial cytochrome oxidase subunit II (COII), mitochondrial 16S rRNA, and nuclear Internal Transcribed Spacer 1 and 2 (ITS1 and 2). Phylogenetic analyses conducted also included published sequences of other putative western *Reticulitermes* species. Results suggest that at least 5 species of *Reticulitermes* may be present in California and that *Reticulitermes* in Arizona consistently group into multiple clades, including samples previously identified as *R. tibialis* in a sister clade. These analyses further support the species status of qualitatively different CHC phenotypes and that alates swarming in spring vs. fall are reproductively isolated species.

Key words: 16S, COII, cryptic species, ITS1, ITS2

Introduction

The unresolved taxonomy of the *Reticulitermes* species complex in the western United States has been the subject of numerous studies using different approaches, such as chemotaxonomy (Haverty and Nelson 1997, Haverty et al. 1999a, 2003, Nelson et al. 2001, 2008, Page et al. 2002), phylogenetics (Austin et al. 2002, 2008, Copren et al. 2005, McKern et al. 2006, 2007, Szalanski et al. 2006, Tripodi et al. 2006, Copren 2007), and behavioral traits (Haverty et al. 1999b, 2003, Getty et al. 2000, Delphia et al. 2003). Despite their ubiquity and economic importance, subterranean termites are challenging to study due to their cryptic habits and complex biology. The traditional morphological approach has been largely unsuccessful due to inadequate and outdated keys (Weesner 1965, Nutting 1990, Scheffrahn and Su 1994, Hostettler et al. 1995, Haverty and Nelson 1997, Ye et al. 2004, Brown et al. 2005, Nelson et al. 2008). Studies employing other methods have improved our understanding, yet the taxonomy of

western *Reticulitermes* has not been fully elucidated. The most visible individuals in a subterranean termite colony are the winged, sexually mature adults produced seasonally for dispersal and reproduction. In California, these swarming events occur in spring, usually on clear sunny afternoons after periods of rainfall, and similarly in the fall and winter, on sunny days following substantial, soaking rains (Pickens 1934a, Weesner 1956, 1970). Previous research has shown that this bimodal flight phenology may indicate a species complex, with 2 or more sympatric species reproductively isolated by disparate swarming seasons. Qualitatively distinct cuticular hydrocarbon (CHC) profiles have been characterized for populations of the 2 temporally separate flight seasons (Haverty et al. 2003), and the mitochondrial cytochrome oxidase II (COII) gene provides further evidence that these CHC phenotypes are likely separate species (Copren et al. 2005).

Early literature describes *R. hesperus* as the predominant species in California, with a widespread distribution, including populations in British Columbia, Oregon, Washington, Idaho, Nevada, and northern Baja California (Banks and Snyder 1920, Light 1934, Pickens 1934a, Snyder 1954, Weesner 1970). The type locality of this species is Little Bear Lake (now known as Lake Arrowhead) in the San Bernardino Mountains in southern California (Krishna et al. 2013). According to Pickens (1934a, b), *R. hesperus* is sympatric with *R. tibialis* Banks in the inland valleys of California, but only *R. tibialis* is found in the desert areas of California and the Great Basin. The known distributions of these 2 species have almost certainly changed since these early descriptions were published, and evidence of the presence of other species in the western region has since been discovered. The existence of at least 4 undescribed taxa in California was indicated by analysis of CHC data and COII phylogenies (Haverty and Nelson 1997, Copren et al. 2005, Nelson et al. 2008). *Reticulitermes flavipes* (Kollar) has been reported in California, Nevada (Austin et al. 2005, Su et al. 2006, Tripodi et al. 2006) and Oregon (McKern et al. 2006). Szalanski et al. (2006) and Tripodi et al. (2006) presented evidence for an undescribed species of *Reticulitermes* found in British Columbia, Idaho, Oregon, Nevada, and California based on mitochondrial 16S rRNA (16S) sequences. McKern et al. (2006, 2007) reported finding a colony of *R. hageni* Banks in Oregon, identified by sequencing the 16S gene. In addition, Su et al. (2006) and Tripodi et al. (2006) each reported evidence of 2 possible undescribed species of *Reticulitermes* collected in California, and McKern et al. (2007) reported 2 samples of an unknown species (*R.* n. sp. 2) in the state of Washington based on phylogenetic analysis of the 16S gene. These findings have fueled an interest in unraveling the taxonomy of *Reticulitermes* in California and other western states.

We assembled an array of samples for phylogenetic analysis, consisting of newly collected alates from 2 separate flight periods and voucher samples of workers from earlier studies, all identified by CHC phenotype (Haverty and Nelson 1997, 2007, Haverty et al. 1999a, Nelson et al. 2001, 2008, Page et al. 2002, Copren et al. 2005). The correspondence of CHC phenotypes and COII phylogeny has been demonstrated for samples from California (Copren et al. 2005). However, most of the published phylogenies that include western *Reticulitermes* are based on 16S sequences with no associated CHC information. The purpose of the study reported here was (i) to obtain mitochondrial (mt) and nuclear (nu) DNA sequences of alates from both spring and fall flight periods and from workers of several different CHC phenotypes, (ii) to better delineate the species status of these phenotypes, and (iii) to allow comparisons to previously published phylogenies. Since the morphological separation of these taxa has been problematic, we hope that robust chemical and genetic evidence will aid in the detection of undescribed and/or cryptic species, and lead to identification of diagnostic morphological characters. It is also our aim to address incorrect or misleading species designations that exist in the literature and genetic databases, particularly with regard to *R. hesperus*. We present here genetic and chemical markers for *R. hesperus* as well as evidence of multiple undescribed species in California.

Materials and Methods

Collection of Termites

Samples of *Reticulitermes* were collected from both residential sites and field locations in California. Alates were collected from spring and fall/winter flights in the San Francisco Bay Area and Riverside (Table 1). Termites were frozen at −20 °C as soon as possible

Table 1. Locality information for new collections in California

Sample ID	Collection location	Date collected	Caste	GenBank Accession No.		
				COII	16S	ITS1 and 2
Rh01	Richmond, Contra Costa Co.	2 Dec 2019	Alate	OR130250	OR183599	OR231771
Rh02	Hayward, Alameda Co.	16 Dec 2019	Worker	OR130251	OR183600	OR231772
Rh03	Richmond, Contra Costa Co.	2 Dec 2019	Alate	OR130252	OR183601	OR231773
Rh04	Hayward, Alameda Co.	16 Dec 2019	Worker	OR130253	OR183602	OR231774
Rh06	Alameda, Alameda Co.	16 Dec 2019	Worker	OR130254	OR183603	OR231775
Rh08	Diablo Foothill Regional Park, Contra Costa Co.	24 Apr 2020	Alate	OR130255	OR183604	OR231786
Rh09	Richmond, Contra Costa Co.	21 Sep 2020	Alate	OR130256	OR183605	OR231776
Rh10	Bolinas, Marin Co.	29 Nov 2019	Alate	OR130257	OR183606	OR231777
Rh11	Lafayette, Contra Costa Co.	14 Nov 2020	Alate	OR130258	OR183607	OR231778
Rh11b	Martinez, Contra Costa Co.	14 Nov 2020	Alate	OR130259	OR183608	OR231779
RH12	Lafayette, Contra Costa Co.	14 Dec 2020	Alate	OR130260	OR183609	OR231780
Rh13	San Mateo, San Mateo Co.	15 Nov 2020	Alate	OR130261	OR183610	OR231781
Rh14	Alameda, Alameda Co.	25 Jan 2021	Alate	OR130262	OR183611	OR231782
Rh15	Martinez, Contra Costa Co.	10 Apr 2021	Alate	OR130263	OR183612	OR231783
Rh16	Martinez, Contra Costa Co.	10 Apr 2021	Alate	OR130264	OR183613	OR231784
Rh17	Martinez, Contra Costa Co.	11 Apr 2021	Alate	OR130265	OR183614	OR231785
IPM18	Lafayette, Contra Costa Co.	17 Apr 2021	Alate	OR130243	OR183592	OR231764
RhMT21	Martinez, Contra Costa Co.	24 Jan 2019	Alate	OR130266	OR183615	OR231787
RhUCR	University of California Riverside, Riverside Co.	22 Jan 2021	Alate	OR130267	OR183616	OR231788

after collection. Voucher specimens of workers from previous chemotaxonomic studies that had been stored in 80% ethanol were also used in this study (Table 2). The authors have retained voucher specimens in either 80 or 100% ethanol.

Characterization of CHC Mixtures

Frozen termites were brought to room temperature before extraction in hexane. CHC extraction methods were identical to those reported in Nelson et al. (2008). Chemical analysis was performed using an Agilent 7890/5977 gas-chromatograph/mass spectrometer (GC–MS) with MassHunter Acquisition software version B.07.05.2479. The GC–MS was equipped with an Agilent/J&W HP-1MS (part # 19091S-933) fused silica capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). The method employed split injection (split ratio of 25:1) and a temperature program of 200 to 320 °C at 3 °C min^{-1} with a final hold of 5 min. Electron impact mass spectra were obtained at 70 eV. Verification of CHC phenotypes utilized Agilent MSD ChemStation version F.01.03.2357.

DNA Extraction

After hexane extraction, termite specimens were preserved in 100% ethanol and used for DNA sequencing. DNA was extracted from individual termites using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Table 2. Voucher specimens of workers used in phylogenetic analysis

Sample ID	CHC phenotype	Date collected	Collection location	Reference publication[a]	GenBank accession no.		
					COII	16S	ITS1 and 2
NSCA-10	CA-A	13 Dec 2001	Santa Ynez, Santa Barbara Co., CA	Haverty and Nelson 1997	OR130246	OR183595	OR231767
YK20	CA-A	3 Apr 2001	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130279	OR183628	OR231800
Yq29	CA-A	3 Apr 2001	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130280	OR183629	OR231801
Yw25	CA-A	31 Jul 2000	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130284	OR183633	OR231805
Wc10	CA-B	31 Jul 2000	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130276	OR183625	OR231797
YD16	CA-B	26 Jun 2001	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130278	OR183627	OR231799
Yq30	CA-B	26 Jun 2001	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130281	OR183630	OR231802
Yv34	CA-B	31 Jul 2000	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130283	OR183632	OR231804
Wi74	CA-C	31 Jul 2000	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130277	OR183626	OR231798
Yt2	CA-C	26 Jun 2001	Placerville, El Dorado Co., CA	Haverty and Nelson 1997	OR130282	OR183631	OR231803
St244	CA-D	30 Apr 2001	Novato, Marin Co., CA	Haverty and Nelson 1997	OR130274	OR183623	OR231795
IR-73	SC-A	3 Jun 2001	Irvine, Orange Co., CA	Nelson et al. 2008	OR130244	OR183593	OR231765
SCA-126	SC-A	28 Aug 2000	Baldwin Park, Los Angeles Co., CA	Nelson et al. 2008	OR130270	OR183619	OR231791
SCA-115	SC-A	2 Aug 2000	Lake Arrowhead, San Bernardino Co., CA (Type locality of *R. hesperus*)	Nelson et al. 2008	OR130268	OR183617	OR231789
SCA-124	SC-A	22 Aug 2000	Los Angeles, Los Angeles Co., CA	Nelson et al. 2008	OR130269	OR183618	OR231790
SCA-138	SC-B	28 Aug 2000	Carpenteria, Santa Barbara Co., CA	Nelson et al. 2008	OR130272	OR183621	OR231793
SCA-148	SC-B	16 Oct 2000	Rialto, San Bernardino Co., CA	Nelson et al. 2008	OR130273	OR183622	OR231794
NSCA-17	SC-B	23 Jul 2002	Santa Maria, Santa Barbara Co., CA	Nelson et al. 2008	OR130247	OR183596	OR231768
SCA-127	SC-B	28 Aug 2000	Baldwin Park, Los Angeles Co., CA	Nelson et al. 2008	OR130271	OR183620	OR231792
MR-4	SC-B′	30 Apr 1998	Motte Rimrock Reserve, Riverside Co., CA	Nelson et al. 2008	OR130245	OR183594	OR231766
AZ-00-105B	AZ-B	11 Jul 2000	Kaibab National Forest, AZ	Haverty et al. 1999a	OR130241	OR183590	OR231762
UT-03-6	AZ-B	30 Sep 2003	Canyonlands, UT	Haverty et al. 1999a	OR130275	OR183624	OR231796
AZ-99-58	AZ-C	29 Jul 1999	On Hwy 180 nr. Luna, NM	Haverty et al. 1999a	OR130242	OR183591	OR231763
NV-00-5	AZ-D	12 Jul 2000	Mt Charleston, Clark Co., NV	Haverty et al. 1999a	OR130248	OR183597	OR231769
NV-00-6	AZ-D	12 Jul 2000	Mt Charleston, Clark Co., NV	Haverty et al. 1999a	OR130249	OR183598	OR231770

[a]Publication in which the CHC phenotype was first described.

Phylogenetic Analysis

Sequences from 2 mitochondrial loci (cytochrome oxidase subunit II (COII) and 16S rRNA genes), and 2 nuclear regions (ribosomal DNA Internal Transcribed Spacer 1 and 2 [ITS1 and 2]), were used to reconstruct phylogenies. Portions of the COII, 16S rRNA genes, ITS 1 and 2 were amplified using primers listed in Supplementary Table S1 following the PCR conditions described below. PCR mixtures consisted of 1–2 µl of template DNA, 0.2 µM primer pair, 12.5 µl of K0171 PCR Master Mix (ThermoFisher Scientific, MA), and ddH_2O to make 25 µl reactions. PCR conditions included a 3-min denaturation step at 95 °C, 35 cycles of 95 °C (30 s), 50–57 °C (30 s), 72 °C (1 min), and a final extension phase of 7 min at 72 °C (Annealing temperature adjusted as in Supplementary Table S1). Products were purified with GeneJET PCR (ThermoFisher Scientific, MA) and quantified with an Epoch 2 spectrophotometer (BioTek, San Clemente, CA). Samples were sent to Retrogen Inc. (San Diego, CA) for Sanger sequencing (Applied Biosystems DNA Analyzer 3730xl). Assembly and analysis of sequence data were done using Sequencher 4.9 (GeneCodes). Obtained sequences were aligned using MUSCLE as implemented in MEGA 11 with default settings (Tamura et al. 2021). Additional sequences from *Reticulitermes* species taken from GenBank were included in the analyses (Supplementary Tables S2–S4). The maximum likelihood phylogenetic analyses were reconstructed using IQ-TREE web server http://iqtree.cibiv.univie.ac.at/ (Trifinopoulos et al. 2016) under the following settings: ultrafast bootstrap with 10,000 iterations; maximum correlation coefficient = 0.99; single branch test with the use of the approximate Likelihood-Ratio Test (SH-aLRT); best substitution model and partitions estimated in PartitionFinder version 2.1.1 (Lanfear et al. 2016); and other default options. The same setting was used in COII, 16S, and ITS phylogenies and a joined phylogeny (phylogeny based on concatenated sequence of COII, 16S, and ITS).

Species Delimitation

To test the species delimitation scenarios proposed by the joined phylogeny, we conducted an A10 analysis in the program BPP 4.6.2 (Yang 2015) using multi-locus data set (COII, 16S, ITS1 and 2). BPP method uses the multispecies coalescent model to calculate the posterior probability of competing species delimitation models in a Bayesian framework while accounting for incomplete lineage sorting due to ancestral polymorphism and gene-tree conflicts (Yang and Rannala 2010, 2014, Rannala and Yang 2013). We ran 2 types of rjMCMC-guided species delimitation (A10) analyses using BPP, each with 2 prior settings. Setting 1, Algorithm 0, with $\varepsilon = 2$, θ prior = Inv-gamma [3, 0.004], τ prior = Inv-gamma [3, 0.002]; Setting 2, Algorithm 0, with $\varepsilon = 2$, θ prior = Inv-gamma [3, 0.04], τ prior = Inv-gamma [3, 0.02]; Setting 3, Algorithm 1, with $\alpha = 2$ and $m = 1$, θ prior = Inv-gamma [3, 0.004], τ prior = Inv-gamma [3, 0.002]; Setting 4, Algorithm 1, with $\alpha=2$ and $m=1$, θ prior = Inv-gamma [3, 0.04], τ prior = Inv-gamma [3, 0.02]. We conducted sampling every 5 generations for a total of 100,000 samples, discarding the first 10,000 samples as burn-in. Tracer v 1.7.2 was used to ensure convergence of all parameters (ESS > 200).

Results

Characterization of CHC Mixtures

Alates from spring flights (April) were all characterized as CHC phenotype CA-A or CA-A′, while alates from fall and winter flights (September to January) were all characterized as CHC phenotype CA-D (Table 3). Collections were made from the same localities in Lafayette and Martinez, CA, in both spring and fall, confirming the sympatry of these CHC phenotypes (Tables 1 and 3). One collection of alates made on the UC Riverside campus on 22 Jan2021, was determined to be CHC phenotype SC-B′. These alates were collected from an in-ground monitoring station prior to taking flight.

Phylogenetic Analysis

Mitochondrial phylogenies

Phylogenetic analysis results based on the COII gene (680 bp) were consistent with CHC groupings (Fig. 1). Phenotypes CA-A, CA-A′, and SC-A clustered together, as they did in Copren et al. (2005)

Table 3. Cuticular hydrocarbon phenotypes for new collections of *Reticulitermes* in California

Sequence ID	Collection location	Caste	Flight season	Date collected	CHC phenotype
Rh01	Richmond, Contra Costa Co.	Alate	Fall/Winter	12/2/2019	CA-D
Rh02	Hayward, Alameda Co.	Worker	N/A	12/16/2019	CA-D
Rh03	Richmond, Contra Costa Co.	Alate	Fall/Winter	12/2/2019	CA-D
Rh04	Hayward, Alameda Co.	Worker	N/A	12/16/2019	CA-D
Rh06	Alameda, Alameda Co.	Worker	N/A	12/16/2019	CA-D
Rh08	Diablo Foothill Regional Park, Contra Costa Co.	Alate	Spring	4/24/2020	CA-A′
Rh09	Richmond, Contra Costa Co.	Alate	Fall/Winter	9/21/2020	CA-D
Rh10	Bolinas, Marin Co.	Alate	Fall/Winter	11/29/2019	CA-D
Rh11	Lafayette, Contra Costa Co.	Alate	Fall/Winter	11/14/2020	CA-D
Rh11b	Martinez, Contra Costa Co.	Alate	Fall/Winter	11/14/2020	CA-D
Rh12	Lafayette, Contra Costa Co.	Alate	Fall/Winter	12/14/2020	CA-D
Rh13	San Mateo, San Mateo Co.	Alate	Fall/Winter	11/15/2020	CA-D
Rh14	Alameda, Alameda Co.	Alate	Fall/Winter	1/25/2021	CA-D
Rh15	Martinez, Contra Costa Co.	Alate	Spring	4/10/2021	CA-A
Rh16	Martinez, Contra Costa Co.	Alate	Spring	4/10/2021	CA-A′
Rh17	Martinez, Contra Costa Co.	Alate	Spring	4/11/2021	CA-A′
IPM18	Lafayette, Contra Costa Co.	Alate	Spring	4/17/2021	CA-A
RhMT21	Martinez, Contra Costa Co.	Alate	Fall/Winter	1/24/2019	CA-D
N/A[a]	Alameda, Alameda Co.	Alate	Fall/Winter	10/19/2020	CA-D
N/A[a]	San Mateo, San Mateo Co.	Alate	Fall/Winter	11/15/2020	CA-D
RhUCR	University of California Riverside, Riverside Co.	Alate	Fall/Winter	1/22/2021	SC-B′

[a]Not available as PCR unsuccessful but data point included to present flight dates for phenotype CA-D.

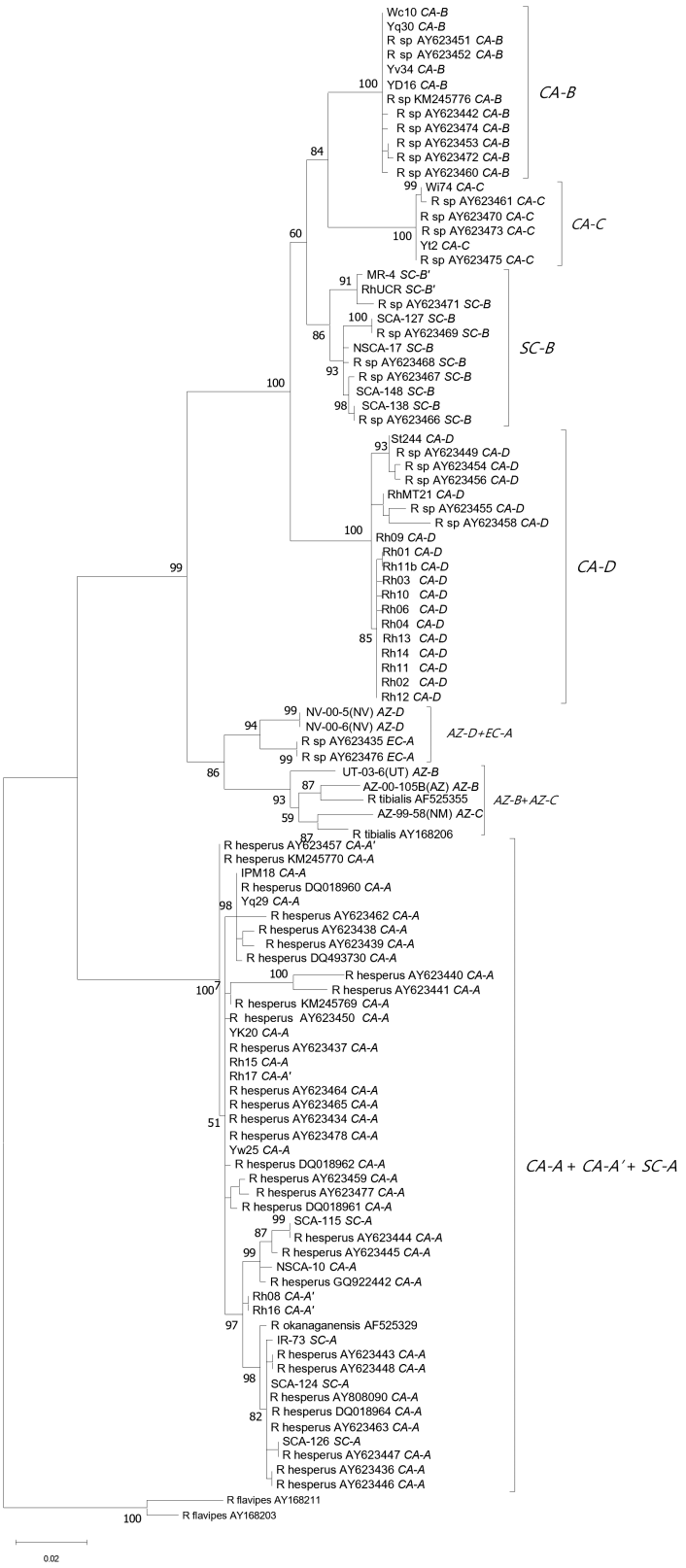

Fig. 1. Maximum likelihood phylogeny of *Reticulitermes* based on partial COII gene (680 bp). Numbers above branches indicate ultrafast bootstrap values calculated by IQ-TREE. The tip labels indicate the sample identity as labeled in GenBank, followed by the cuticular hydrocarbon (CHC) type in italics. For samples not collected from California, the location is denoted by state abbreviation in brackets following the sample identity.

where they were designated as the *R. hesperus* clade based on distribution and congruence with the topotype samples (Copren et al. 2005, Nelson et al. 2008). These 3 CHC phenotypes are quite similar; the main distinction being consistent differences in the relative quantities of 2 pentacosatrienes (Nelson et al. 2008). These 3 CHC phenotypes can be considered geographic variants of 1 species

(*R. hesperus*) and are hereafter referred to as CA-A. The *R. hesperus* sequences from Dedeine et al. (2016), Su et al. (2006), Yashiro and Matsuura (2007), and Zhang et al. (2011) cluster with our CA-A samples, suggesting this is the correct species designation. One sequence, AF525329 (Austin et al. 2002), initially designated as *R. hesperus*, clustered with CA-A, however, this sequence was renamed in the Genbank database by Tripodi et al. (2006) (Supplementary Table S2). The sequences used in our analyses that were taken from GenBank are labeled in the Figures and Tables with the species designation given by the submitting authors.

There was considerable genetic divergence (average pairwise distance = 7.8%) among samples of CA-A and CA-D. They are likely distinct species. CHC phenotypes CA-B, CA-C, and SC-B each formed separate clades (Fig. 1). The 2 samples of SC-B′ clustered with SC-B (Fig. 1). Phenotype SC-B′ has thus far been found in only 2 locations, the University of California, Riverside campus, and Motte Rimrock Reserve, both in Riverside Co., CA.

Four CHC phenotypes, EC-A from Inyo Co., California (Copren et al. 2005), AZ-B from southern Utah and northeastern Arizona, AZ-C from southeastern Arizona, and AZ-D from southern Nevada (Haverty and Nelson 2007) cluster most closely with sequences identified as *R. tibialis* in GenBank. One of the *R. tibialis* sequences is from Cochise Co., Arizona and another is from W. Lafayette, Indiana (Fig. 1, Supplementary Table S2). These results are consistent with results from Copren et al. (2005) using the COII gene.

Sequencing results based on the 16S gene (383 bp) were also consistent with groupings based on CHC results and COII phylogeny (Fig. 2). CA-B, CA-C, and CA-D formed separate clades, and CA-A, CA-A′, and SC-A clustered together (Fig. 2). However, in this analysis the 2 SC-B′ samples, MR-4 and RhUCR, fell outside of the SC-B group (Fig. 2). Similar to the COII results, the CHC phenotypes from collections in the southwestern US (AZ, NM, NV, and UT) clustered with a sequence from sample from W. Lafayette, IN, identified as *R. tibialis* in GenBank.

When we compared the published *Reticulitermes* sp. 16S sequences with our dataset, sequences of the 16S gene labeled in GenBank with the subjectively invalid name *R. okanaganensis* published by Szalanski et al. (2006), Tripodi et al. (2006), and Tai et al. (2015) clustered with our CA-A samples. Sequences of the 16S gene attributed to *R. hesperus* published by Austin et al. (2005), Tripodi et al. (2006), and Tai et al. (2015) clustered with our CA-D samples. However, COII and 16S sequences attributed to *R. hesperus* submitted by Su et al. (2006), Yashiro and Matsuura (2007), Zhang et al. (2011), and Dedeine et al. (2016) cluster with our CA-A samples. These incongruent results may be explained due to the misidentification of certain specimens submitted to the database, as detailed in Nelson et al. (2008). Similarly, samples identified as *R. hesperus* in GenBank (DQ389198, DQ389199, DQ389204, and DQ389207) clustered with samples with CHC phenotypes CA-C. These samples were identified as *R. hesperus* by Tripodi et al. (2006) by comparison with the mitochondrial 16S haplotypes HE1-4, which were incorrectly assigned to *R. hesperus* by Szalanski et al. (2006). The benefit of correcting this misidentified species label is that the range of CA-C has now been expanded to include San Joaquin, Sacramento, and Nevada counties of California. Prior to the report by Tripodi et al. (2006) we had only collected CA-C from Placerville in Eldorado County, CA.

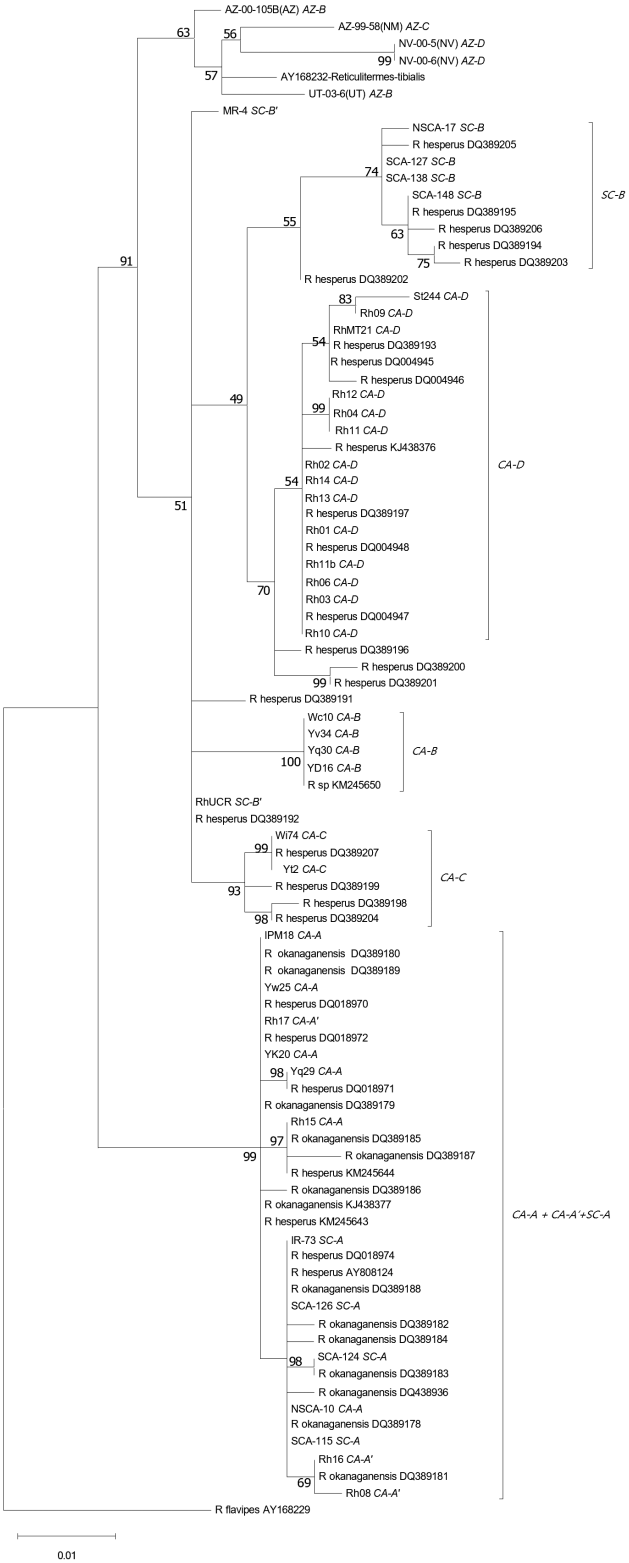

Fig. 2. Maximum likelihood phylogeny of *Reticulitermes* based on partial 16S gene (383 bp). Numbers above branches indicate ultrafast bootstrap value calculated by IQ-TREE. The tip labels indicate the sample identity as labeled in GenBank, followed by the cuticular hydrocarbon (CHC) type in italics. For samples not collected from California, the location is denoted by state abbreviation in brackets following the sample identity.

Nuclear phylogeny

In general, the topology of COII phylogeny is partially supported by nuDNA phylogeny (ITS1 and 2; 831 bp): the separation of CA-A, CA-B, CA-C, CA-D, and SC-B (Fig. 3). However, most SC-A individuals shared identical nuDNA sequences (ITS1 and 2) with SC-B individuals (Fig. 3). The incongruence between mtDNA- and nuDNA-based phylogeny could result from a low mutation rate of the nuDNA sequences, incomplete lineage sorting, or introgression/hybridization.

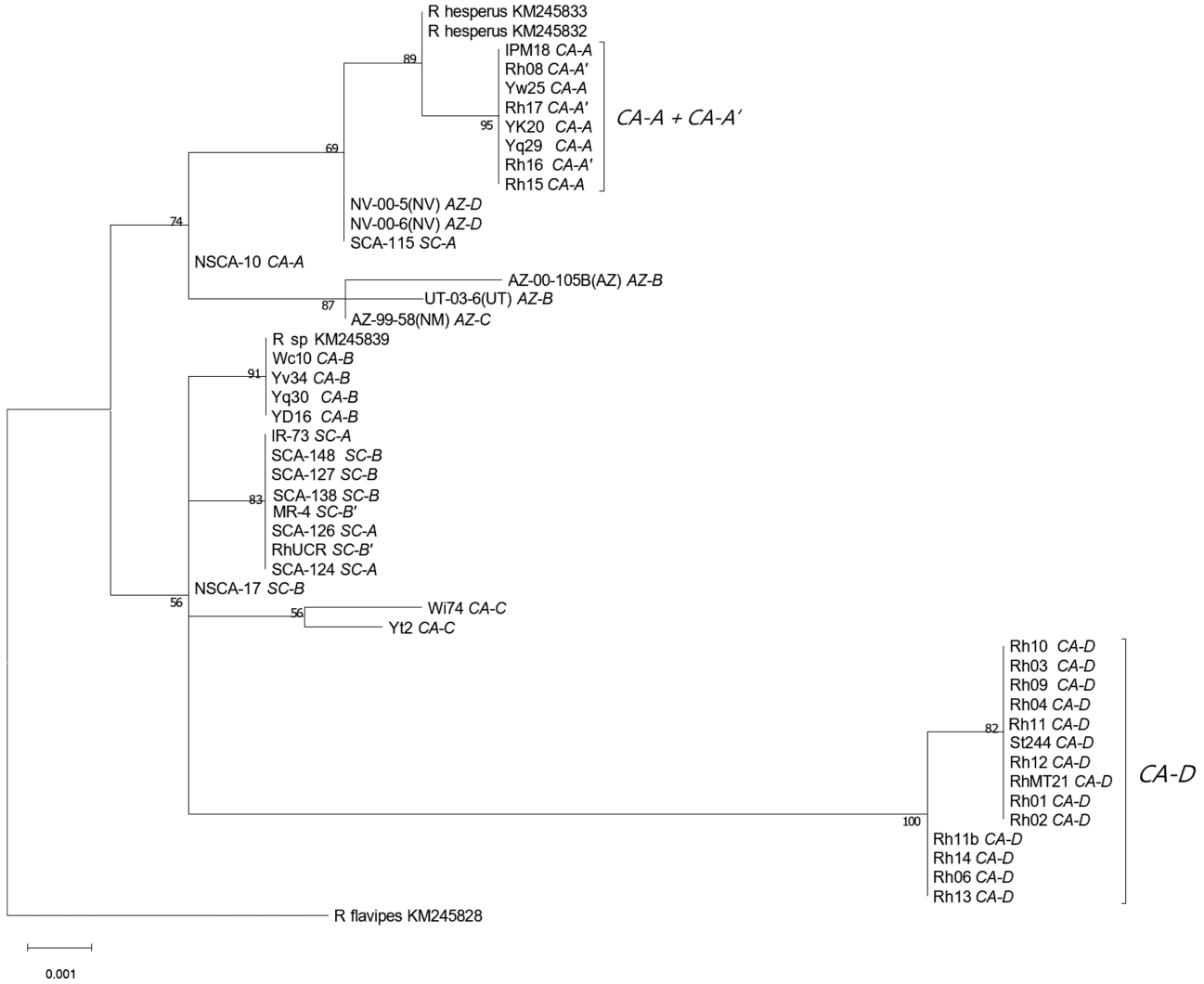

Fig. 3. Maximum likelihood phylogeny of *Reticulitermes* based on concatenated ITS 1 and 2 sequences (831 bp). Numbers above branches indicate ultrafast bootstrap value calculated by IQ-TREE. The tip labels indicate the sample identity as labeled in GenBank, followed by the cuticular hydrocarbon (CHC) type in italics. For samples not collected from California, the location is denoted by state abbreviation in brackets following the sample identity.

Joined phylogeny and species delimitation

Distinct CHC phenotypes form well-supported monophyletic clades (bootstrap values > 70) in the joint phylogeny (16S, COII, ITS1 and 2, 1,885 bps), including the separation of CA-A, CA-B, CA-C, CA-D, SC-B, and Arizona phenotypes (AZ-B, AZ-C, and AZ-D) (Fig. 4). The support for the monophyly of the CA-B, CA-C, CA-D, and SC-B clades is robust (bootstrap values = 99). However, the support for the monophyly of the combined clades (CA-B + CA-C + CA-D) is relatively low (bootstrap values = 56), which may be attributed to the rapid divergence of these 3 clades and the SC-B clade, potentially leading to complexities such as incomplete lineage sorting that lower phylogenetic resolution.

Based on the joined phylogeny, we subsequently organized samples into 7 candidate species, which formed the foundational units for species delimitation analyses (BPP analyses): CA-A, CA-B, CA-C, CA-D, SC-B, Arizona phenotypes, and outgroup (refer to Fig. 4). BPP results indicated that the 7 candidate species were delineated as distinct lineages, regardless of the combinations of prior settings used (Supplementary Table S5). This finding suggests that these CHC phenotypic groups strongly support the existence of valid biological species.

Discussion

The colonies that produce alates in the spring (CHC phenotype CA-A) versus the fall and winter (CHC phenotype CA-D) are undoubtedly different species, evidenced by differences in chemical characters, genetics, and temporal reproductive isolation. The CHC results reaffirm those of Haverty et al. (2003), where flights of phenotypes CA-A and CA-A′ occurred from February to May, and flights of phenotype CA-D occurred from September to January with a larger geographic sampling. Agonistic behavior between CHC phenotypes has been demonstrated (Haverty et al. 1999b) and could present another barrier to inter-breeding.

In addition to the 2 species represented by CHC phenotypes CA-A and CA-D, genetic evidence supports at least 3 other undescribed species in California represented by CHC phenotypes CA-B, CA-C, and SC-B. Alates have never been observed for phenotypes CA-B or CA-C so we have no information on the timing of their flight seasons. Nelson et al. (2008) did report that alates of phenotype SC-A (=CA-A) were collected in the month of March in years 2001 and 2002, and alates of SC-B were collected in September and November of 2001. Alates of SC-B′ were collected on 22 Jan 2021. Phenotypes SC-B′ and

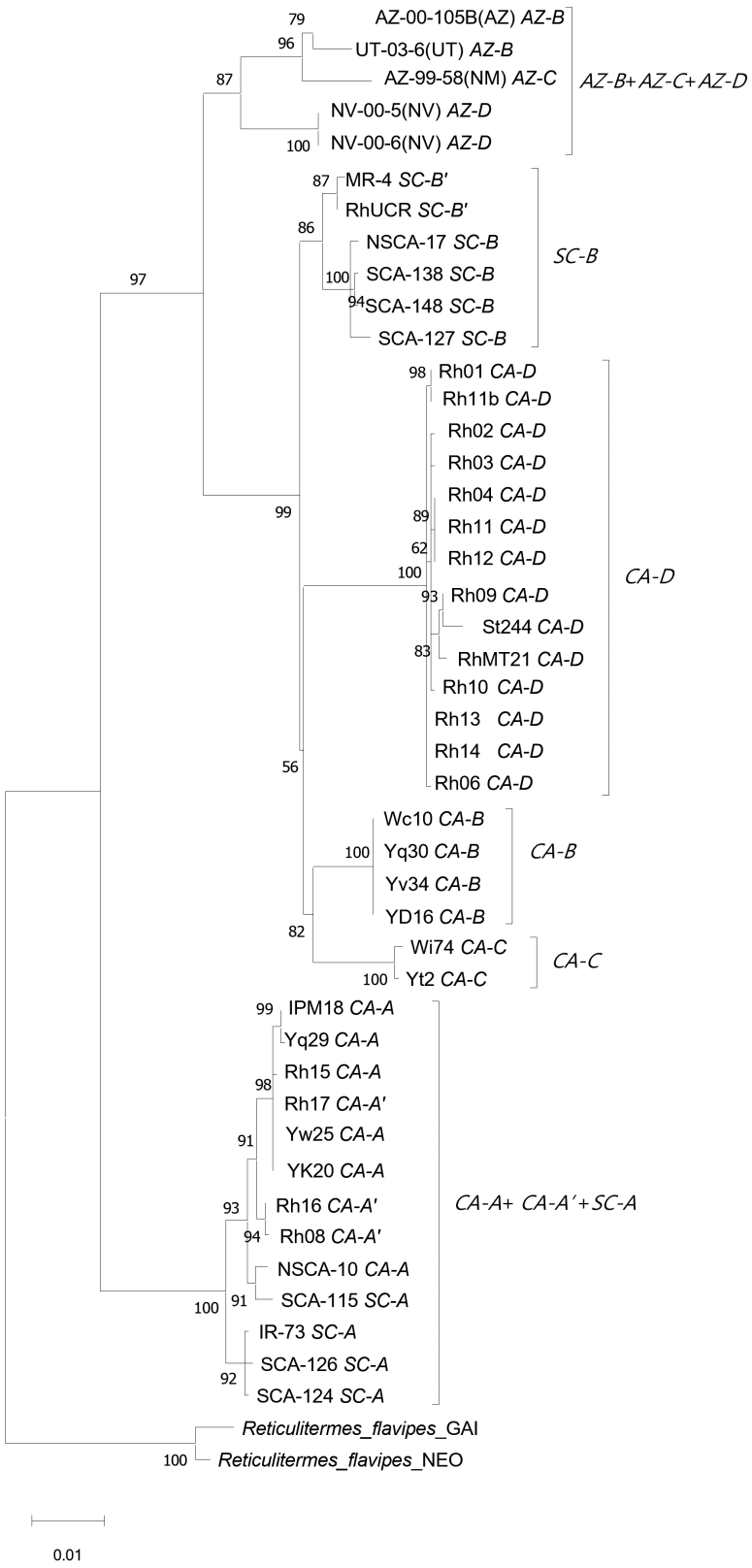

Fig. 4. Maximum likelihood phylogeny of *Reticulitermes* based on concatenated COII, 16S, ITS1 and 2 sequences (1,885 bp). Numbers above branches indicate ultrafast bootstrap value calculated by IQ-TREE. The tip labels indicate the sample identity as labeled in GenBank, followed by the cuticular hydrocarbon (CHC) type in italics. For samples not collected from California, the location is denoted by state abbreviation in brackets following the sample identity.

SC-B have very similar CHC profiles, SC-B′ having higher quantities of internally branched mono- and dimethyl alkanes with chain lengths of 35 to 39 carbons, and smaller amounts of 5,17-dimeC27 compared to SC-B (Nelson et al. 2008). This is consistent with SC-B′ occupying a more xeric habitat and producing a greater quantity of the longer chained methyl branched alkanes that better protect against water loss (Nelson et al. 2023). The COII gene does not separate these 2 phenotypes, but 16S phylogeny indicates some differentiations.

So far, no reliable, definitive morphological characters have been discovered that differentiate these species of western *Reticulitermes*. Differences in overall size and soldier head dimensions easily separate the larger CA-A and CA-D from the smaller CA-B and CA-C phenotypes (Haverty and Nelson 1997). However, further discrimination still needs to be resolved. CHC profiles seem to be the most informative phenotypic characteristics currently available to separate these undescribed species.

Based on topotype information and clustering with multiple *R. hesperus* sequences in GenBank, we contend that the spring flights represent *R. hesperus* (CA-A), and the fall and winter flights represent a different species (CA-D), which has yet to be formally described. Several of the 16S sequences from GenBank used in this paper did not provide locality information associated directly with the accession number (Supplementary Table S3). Two of the reference publications (Szalanski et al. 2006, Tripodi et al. 2006) listed locality information (including latitude and longitude) by haplotype but did not provide the accession number for individual samples, nor was the locality included in the GenBank entry. Ideally, the exact locality would be associated with each accession number, as the geographic range for a given haplotype can be quite expansive.

The lack of consensus in our 16S phylogeny emphasizes the uncertainty of species identification and the potential for circular reasoning and compounding of errors when assigning species based solely on similarity to a GenBank sequence. Johnson and Forschler (2022) recognized this in their survey of *Reticulitermes* in Georgia, cautioning that many sequences deposited in GenBank lack taxonomic support, such as associated morphological, behavioral, or chemical characters. Wu et al. (2019) also noted some misidentifications of GenBank sequences of Asian *Reticulitermes* species. Incorrect or dubious species identification of GenBank accessions is common in insect genera with complex taxonomy (Pentinsaari et al. 2020). Some of the GenBank sequences included here that were in the *R. hesperus* clade were labeled in the database as *R. okanaganensis* (Supplementary Tables S2 and S3), and that name should be considered a junior subjective synonym of *R. hesperus*. Previous phylogenetic analyses using COII by Copren et al. (2005) and Lim and Forschler (2012) show sequence AF525329 (Austin et al. 2002) falling in a clade with other *R. hesperus* specimens, although it was labeled in Lim and Forschler (2012) with the aforementioned synonym as revised by Tripodi et al. (2006). Similarly, some GenBank sequences appearing in the CA-D clade were mislabeled in the database as *R. hesperus* (Supplementary Tables S2 and S3).

Another obvious conclusion from the research presented here is that there are likely additional species to be clarified by further phylogenetic analysis of the CHC phenotypes collected in eastern California, Nevada, Arizona, Utah, and New Mexico. Copren et al. (2005) identified 2 genotypes that were associated either with GenBank COII sequences attributed to *R. tibialis* or *R. hesperus*, but strongly separate with bootstrap values of 100. Haverty and Nelson (2007) identified 5 distinct CHC phenotypes in 4 southwestern states that are different from those in California. Phylogenetic analyses of vouchers from these collections would be helpful in determining whether these CHC phenotypes also represent valid biological species.

The understanding of *Reticulitermes* taxonomy in the western United States is best addressed by using multiple methods in order to avoid errors that may occur when relying on a single character. Mitochondrial DNA sequencing is very informative, but we demonstrate here that without associated chemical or behavioral characteristics, some published inaccuracies in species identifications would have been overlooked. We present COII, 16S, and ITS 1 and 2 sequences along with CHC phenotypes for *R. hesperus* and at least 4 other undescribed species present in California.

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Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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