



Household and Structural Insects

Genetic Analysis of Formosan Subterranean Termite (Blattodea: Rhinotermitidae) Populations in California

Shu-Ping Tseng,¹ Jason Boone,² Lowell Boone,² Natalee King,³ Siavash Taravati,⁴ Dong-Hwan Choe,^{1,○} and Chow-Yang Lee^{1,5,○}

¹Department of Entomology, University of California, 900 University Avenue, Riverside, CA 92521, ²BODS Pest Control, 33877 Begonia Pl, Murrieta, CA 92563, ³Target Speciality Products, 15415, Marquardt Avenue, Santa Fe Springs, CA 90670, ⁴UC Cooperative Extension, Los Angeles County, 700 W. Main Street, Alhambra, CA 91801, and ⁵Corresponding author, e-mail: chowyang.lee@ucr.edu

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Abstract

A new infestation of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae), was discovered in Canyon Lake, Riverside County, California. We used three mitochondrial DNA (COI, COII, and 16S) and seven polymorphic microsatellite markers to characterize the genetic relationship of the colony with two other colonies that were collected in 1992 and 2018 in La Mesa, San Diego County. Maximum likelihood phylogeny of *C. formosanus* based on concatenated COI and COII sequences revealed that the two La Mesa populations (CA01 and CA02) and the Canyon Lake population (CA03) were from different maternal lineages. Based on the 14 COII haplotypes of *C. formosanus* found world-wide, CA01 and CA02 belonged to a haplotype widely distributed across the United States, while CA03 was grouped under a haplotype predominantly found in Asia. Microsatellite allele frequencies across all loci for both La Mesa populations were relatively similar, but significant genetic differences were found between CA02 and CA03 colonies ($F_{ST} = 0.24$; $Dest = 0.30$; $G''_{ST} = 0.55$; $P < 0.01$).

Key words: mitochondrial DNA, ribosomal DNA, microsatellite marker, haplotype, phylogenetics

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki is one of the world's most invasive pest species (Su 1990, Lowe et al. 2000, Rust and Su 2012, Evans et al. 2013). It is believed to have originated from southern China and Taiwan (Chouvenc et al. 2016) based on a higher level of genetic variability and presence of inquiline insects (Kistner 1985, Li et al. 2009, Evans et al. 2013). It has spread beyond the eastern Asian region (Mori 1987, Vargo et al. 2003, Chen et al. 2020) to other parts of the world, including the United States (Su 2003, Vargo et al. 2006, Fang et al. 2008, Evans et al. 2013), Grand Bahama (Jones et al. 2017), and more recently in Israel (Scheffrahn et al. 2020). *Coptotermes formosanus* is one of the most commonly intercepted termites in the United States (Blumenfeld and Vargo 2020).

In the United States, the earliest records of *C. formosanus* was in Oahu, Hawaii in 1907 (Swezey 1914, Bess 1970), in San Francisco in 1927 (Jacobsen 1927), in Charleston, South Carolina in 1957, Houston, Texas in 1965, and in Lake Charles and New Orleans, Louisiana in 1966 (Beal 1987, Chambers et al. 1988). Until recently,

it has established in Hawaii, Texas, Florida, Mississippi, Alabama, Tennessee, North Carolina, South Carolina, Georgia, Louisiana, and California (Woodson et al. 2001, Evans et al. 2013, Scheffrahn et al. 2020). A recent study reveals that both bridgehead effect and multiple introductions play vital roles in the establishment of *C. formosanus* in the United States, where initial introduction in Hawaii was originated from at least two distinct events. Later, the Hawaiian population served as the multiple introduction source and was introduced to the southeastern United States (Blumenfeld et al. 2021). Florida's population was most likely introduced from both Louisiana/Texas and southcentral China (Blumenfeld et al. 2021).

In February 1992, *C. formosanus* was discovered in La Mesa, San Diego County in southern California (Atkinson et al. 1993). The colony was estimated to be 8–10 yr old and was baited with hexaflumuron bait in July 1993 (Haagsma et al. 1995). Still, newer infestations ensued, and the infested houses were either treated with termiticides, baited, or fumigated (Rust et al. 1998). Due to the lack of funding, the baiting program was discontinued in 1997.

Approximately 10 yr later, *C. formosanus* infestation was rediscovered in La Mesa by a pest management professional (PMP 2018). This infestation location was about 0.5 km from the original location in 1992, and the colony was treated and assumed eliminated. Additional inspections in 2018, however, revealed an active infestation about 100 m from the original infestation in 1992.

In June 2020, an infestation of *C. formosanus* was discovered in a house in Canyon Lake, Riverside County, California. Canyon Lake is an upscale gated home community on Canyon Lake reservoir, located in the hill area between Menifee and Lake Elsinore (33.69°N, 117.26°W; elevation = 426 m). It is surrounded by native oakwood lands and coastal scrub with 30.48 cm of rainfall per year. The alates were swarming indoors, and the infestation caused considerable damage to the exterior walls, indoor wall panels, and the floor of the second-story bathroom (Supp Fig. 1 [online only]). Alates and soldier termites were collected before the infested house was fumigated with sulfuryl fluoride. This new infestation was located approximately 104 km from the previous infestation in La Mesa.

Knowledge of the invasion source and routes provides important information for developing practical management strategies against invasive species (Estoup and Guillemaud 2010). Population genetic analyses of the introduced populations could provide insights into the invasion source (Blumenfeld et al. 2021). In this study, we used mitochondrial DNA and microsatellite markers to examine 1) the mtDNA haplotype of California colonies compared to other known haplotypes of *C. formosanus* and 2) the genetic relationship of the Canyon Lake colony of *C. formosanus* to that of the earlier La Mesa colonies (that were collected in 1992 and 2018).

Materials and methods

Termite Samples

Fresh samples (alates and soldiers) from the Canyon Lake infestation (CA03, Fig. 1) were collected by C.-Y.L. from the infested house and

kept in absolute ethanol. They were identified as the Formosan subterranean termite, *C. formosanus*, based on a pair of setae on each side of the soldier's fontanelle. The La Mesa samples from 1992 (CA01) and 2018 (CA02, approximately 0.5 km from the original location in 1992) were collected by M.K. Rust earlier and were kept in 80% ethanol (Fig. 1).

DNA Extraction and mtDNA Sequencing

Three individuals from each colony were used in mtDNA analyses. DNA was extracted from individual soldier termites using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Portions of the cytochrome oxidase subunit I (COI), the cytochrome oxidase subunit II (COII), and 16S rRNA genes were amplified using primers listed in Supp Table S1 (online only) following the PCR conditions described below. PCR mixtures contained 1–2 µl of template DNA, 0.2 µM of each primer, 12.5 µl PCR Master Mix (Cat# K0171, Thermo Scientific, MA, USA), and ddH₂O (25 µl reactions volume in total). PCR conditions included an initial denaturation step at 95°C (3 min) followed by 35 cycles of 95°C (30 s), 52°C (30 s), 72°C (1 min), and a final extension phase at 72°C (7 min). The PCR amplicons were purified using the GeneJET PCR purification kit (Cat# K0701, Thermo Scientific, MA). The amount of DNA in the purified PCR products was measured using Epoch spectrophotometer (BioTek Epoch, VT). The purified PCR products were sent to Retrogen Inc (CA) for Sanger sequencing for both directions on an Applied Biosystems 3730xl DNA analyzer. Sequence data were assembled by using Sequencher 4.9 (GeneCodes). MtDNA sequences obtained in this study (Accession numbers MW558103-MW558105 for COI; MW558362-MW558364 for COII; MW558113, MW558114 for 16S) were deposited in GenBank.

Phylogenetic and Network Analysis

We obtained identical mtDNA sequences from the same colony. Therefore, we only used one sequence per colony for the following

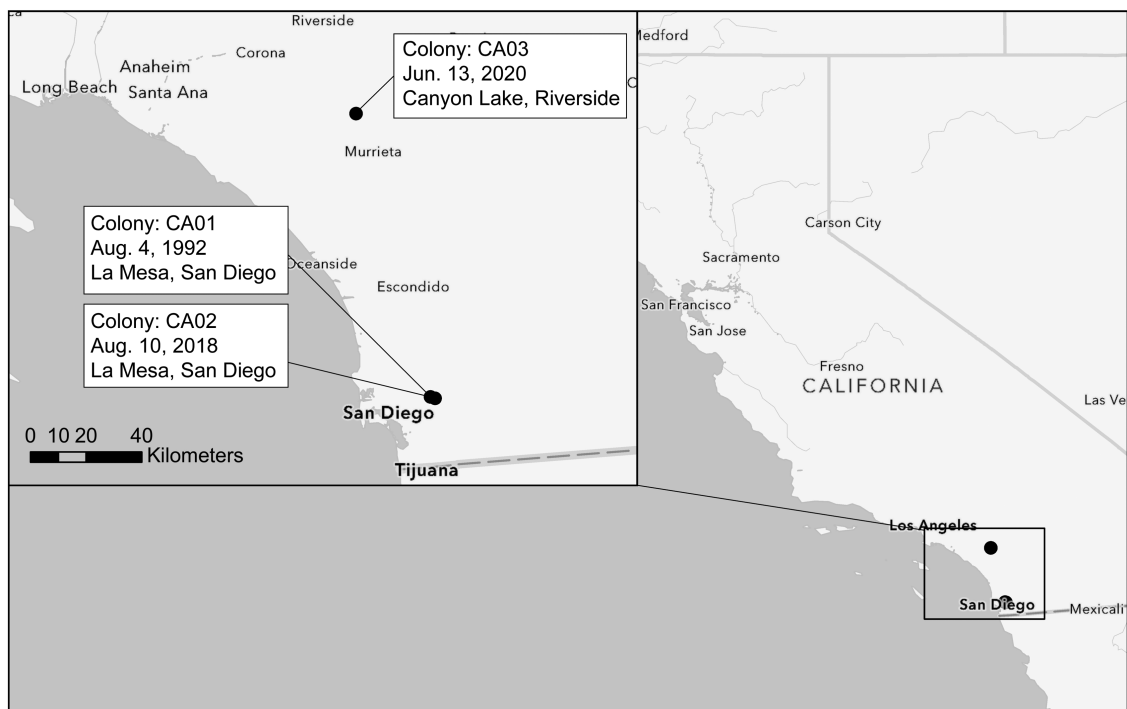


Fig. 1. Locations where *C. formosanus* samples were collected in California.

analyses. To compare sequence similarity, additional mtDNA sequences were obtained from GenBank (Supp Table 2 [online only]) and included for phylogenetic analysis. The reference sequences downloaded from GenBank and sequences obtained in this study were aligned using MUSCLE as implemented in MEGA 6 (Tamura et al. 2013) with default settings and trimmed to the appropriate size, 371 bp, 632 bp, and 373 bp fragment for COI, COII, and 16S, respectively. The maximum likelihood (ML) phylogenetic analysis was conducted with RAxML 8.0.0 (Stamatakis 2014), implementing the optimal substitution model and partitions estimated in PartitionFinder version 2.1.1 (Lanfear et al. 2016). To find the optimal ML tree, we performed 20 independent accurate ML searches from 20 parsimony starting trees generated under the default parsimony model in RAxML. Branch support for the RAxML tree was estimated with 100 rapid bootstrapping replicates. A minimum spanning mtDNA haplotype network was constructed using POPART (Leigh and Bryant 2015) to infer relationships among haplotypes.

Microsatellite Genotyping and Characterization

Fifteen individuals from colony CA01, eight individuals from CA02, and eight individuals from CA03 were scored using seven microsatellite loci (Cf4:1A2-4, Cf12-4, Cf4-10, Cf10-4, Copf01, Copf06, and Copf14) as described earlier in Vargo and Henderson (2000) and Liu et al. (2012). To genotype individual termites, we performed multiplex

PCR reactions with fluorescently labeled universal primers following the procedure described in Blackett et al. (2012). Two fluorescently labeled universal primers (Tail A and Tail B; Blackett et al. 2012) and modified locus-specific primers with a 5' universal primer sequence tail were used. The resulting PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) at the University of Arizona Genomic Analysis and Technology Core Facility (GATC). Microsatellite Analysis Software (available on Thermo Fisher Cloud) was used to visualize and score alleles. Allele frequencies were summarized using GenAEx 6.5 software (Peakall and Smouse 2006). Genetic differentiation, as expressed by Wright's F_{ST} (F_{ST}), Jost's estimate of differentiation (Dest), and Hedrick's standardized GST for small number of populations (G'_{ST}), was estimated using GenAEx 6.5 software (Peakall and Smouse 2006). Genetic clusters were further validated using a principal coordinate analysis (PCoA) based on genetic distance in Genalex v6.5 (Peakall and Smouse 2006). Termite colony CA01 was excluded from the PCoA and genetic differentiation analysis due to a large proportion of missing data, possibly due to DNA degradation of the 29-yr-old samples.

Results

MtDNA Sequence Comparison

The two colonies of La Mesa (CA01 and CA02) shared identical sequences using COI and COII, but they differed from the Canyon Lake colony (CA03) by 2-bp in each locus. CA02 and CA03 shared identical sequences at 16S. The 16S gene of the CA01 colony was not sequenced because PCR amplification failed. Phylogeny based on concatenated COI and COII sequences suggest that the La Mesa (San Diego County) and the Canyon Lake (Riverside County) colonies were from two different maternal lineages (Fig. 2).

Most *C. formosanus* sequences available in GenBank are partial COII genes, and the available COI and 16S sequences are relatively limited. Seventy-four COII sequences were downloaded and compared with the sequences obtained from this study. Most U.S. samples belong to two haplotypes, Hap01 and Hap02, except one sample from Texas (Fig. 3). CA03 belongs to Hap02, and this haplotype was widely distributed across the United States; it was found in Mississippi, Hawaii, Louisiana, Florida, and Georgia (Fig. 3). CA01 and CA02 belong to Hap01, predominantly found in Asia but also occurred in Mississippi and Hawaii (Fig. 3).

Allele Frequency and Genetic Differentiation

All the individuals from CA02 and CA03 were successfully genotyped at seven loci, while some of the samples from CA01 failed to be genotyped. The information of the number of individuals successfully genotyped per locus was listed in Table 1. The microsatellite alleles frequencies across all studied loci of both La Mesa colonies (CA01 and CA02) are relatively similar (Table 1). Some differences between the Canyon Lake colony and the La Mesa colonies were observed. There were several allele differences, e.g., alleles 190 and 202 at Copf06, were only observed in CA03 but not in CA01 and CA02; allele 181 at Cf4:1A2-4 was only observed in CA03 but not in CA01 and CA02; allele 355 at Copf01 was only observed in CA02 but not in CA03 (Table 1). Genetic differentiation analyses supported this difference. Significant genetic differentiation between colonies CA02 and CA03 colonies was detected ($F_{ST} = 0.24$; Dest = 0.30; $G'_{ST} = 0.55$; $P < 0.01$). PCoA of the microsatellite showed that the first and second principal coordinates accounted for 68.83% of the genetic variation (50.63 and 18.2%, respectively). The genetic differentiation between CA02 and CA03 was also found in PCoA

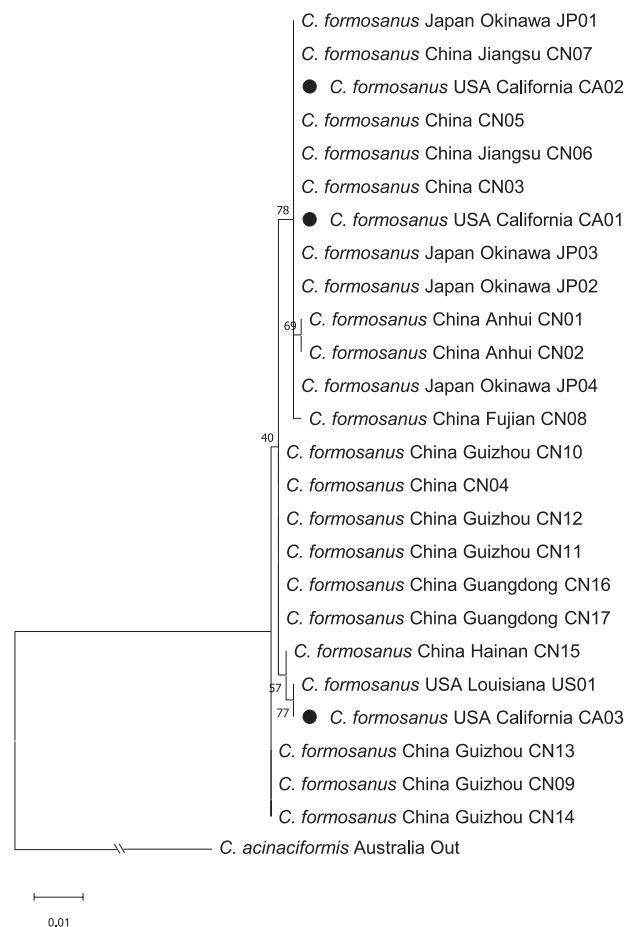


Fig. 2. Maximum likelihood phylogeny of *C. formosanus* based on concatenated COI and COII sequences (1003 bp). Numbers at nodes indicate bootstrap support values (100 replicates). Black circles denote the samples obtained from the present study.

analysis. Individuals of CA02 and CA03 were separated by the first coordinate of PCoA, which accounted for 50.63% of the total genetic variation (Fig. 4).

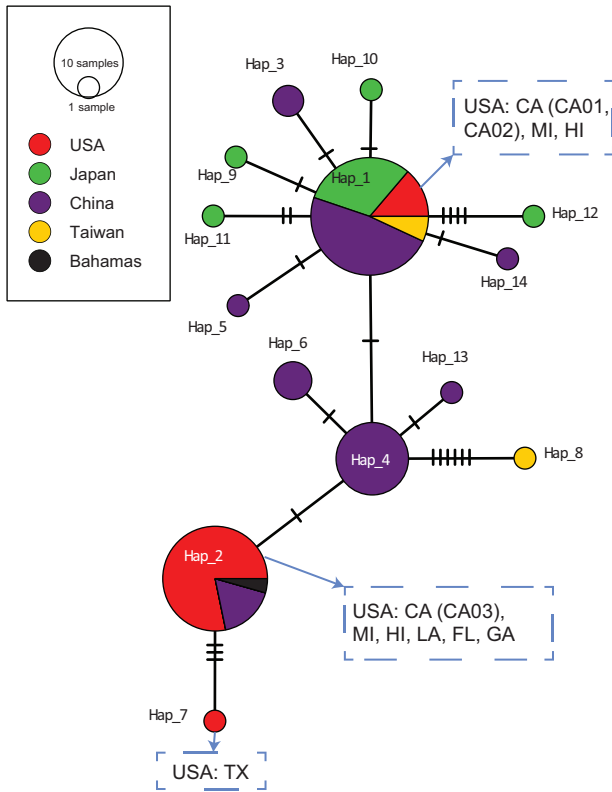


Fig. 3. Minimum spanning network for the COII gene (632 bp). Circle sizes are proportional to the number of sequences per haplotype. Colors represent countries where the haplotype was found.

Discussion

This is the first genetic analysis of the *C. formosanus* colonies that were discovered in California. The La Mesa and the Canyon Lake colonies are represented by different mitochondrial haplotypes and belong to two distinct genetic groups based on microsatellite. The most parsimonious explanation for the observed data is these *C. formosanus* infestations were the outcomes of two different introductions. *Coptotermes formosanus* was first introduced to La Mesa, San Diego county, most likely in the 1980s. Despite eradication efforts using baits, the residual population persisted and was rediscovered in 2018. On the other hand, the colony found in Canyon Lake, Riverside County, was not related to the La Mesa colonies, suggesting that it was from a separate introduction. At this stage, we cannot determine how the infestation of *C. formosanus* started in Canyon Lake. However, La Mesa’s infestation was traced to a family who brought back wood and potted plants with them when they moved from Hawaii. Since *C. formosanus* is common in Hawaii, one possibility is that one or multiple colonies could have been transported to La Mesa through these items. However, the rediscovered 2018 colony is likely descended from the original 1998 colony (Rust et al. 1998).

Formosan subterranean termites are typically distributed between the latitude of 26° and 35°N and 26° and 35°S (Su and Tamashiro 1987). The infestation of *C. formosanus* in southern California corresponded well with its putative distribution range (La Mesa: 32.7629, -117.0068; Canyon Lake: 33.69845, -117.27530). The recent discovery of *C. formosanus* in Petah Tikva, Israel (32.0979, 034.8971), with relatively similar latitude to that of southern California, shared similar Mediterranean climate conditions (Scheffrahn et al. 2020). However, the Mediterranean climate is atypical of the conditions of all other endemic or introduced localities of this species (Scheffrahn et al. 2020). *Coptotermes formosanus* is active in temperature ranges

Table 1. Allele frequencies and sample size (N) for each microsatellite across three *C. formosanus* colonies collected in California

Locus	N/Allele	CA01	CA02	CA03
Cf4:1A2-4	N	2	8	8
	181	0.000	0.000	0.438
	184	0.750	0.500	0.375
	187	0.250	0.500	0.188
Cf12-4	N	4	8	8
	179	0.000	0.000	0.125
	188	1.000	1.000	0.875
Cf4-10	N	4	8	8
	235	0.375	0.500	0.625
	241	0.625	0.500	0.375
Cf10-4	N	9	8	8
	164	0.667	0.750	0.563
	167	0.333	0.250	0.438
Copf01	N	0	8	8
	343	NA	0.000	0.938
	355	NA	0.125	0.000
	357	NA	0.875	0.063
Copf06	N	2	8	8
	190	0.000	0.000	0.500
	196	0.250	0.063	0.000
	198	0.750	0.938	0.313
Copf14	N	4	8	8
	225	1.000	1.000	0.625
	243	0.000	0.000	0.375

NA: Not available.

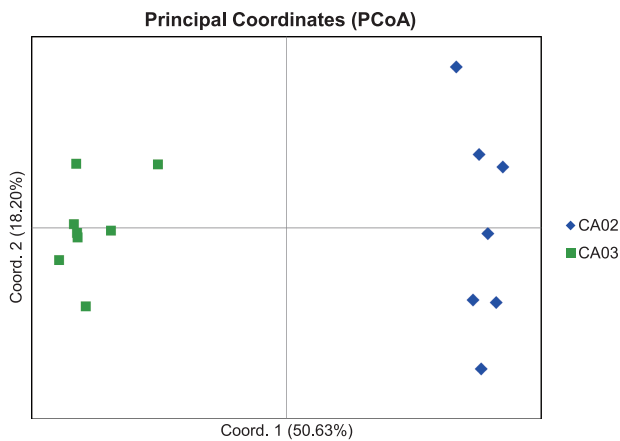


Fig. 4. Principal coordinate analysis of *C. formosanus* individuals. The first and second axis explained 50.63% and 18.20% of the total genetic similarities, respectively.

of 9.3–38.1°C (Cao and Su 2016), but its mortality could increase quickly at a temperature of 30–35°C and low relative humidity (55–65%; Wiltz 2012). With enough irrigation, plenty of shade trees, and milder summer temperature (~30°C), it is possible for the Formosan subterranean termite to survive, as evident from the >29-yr-old infestations in La Mesa.

Previous studies attempted to infer invasion routes of *C. formosanus* in the United States, but the lack of mitochondrial genetic variation at the population level has obscured the precise inference of the source population and the routes of introduction (Austin et al. 2006, Husseneder et al. 2012, Blumenfeld et al. 2021). Even in the native populations of *C. formosanus*, there are only nine COII haplotypes (Fang et al. 2008). MtDNA may be insufficient to resolve the relationship among invasive populations. The colonies that shared the same mtDNA haplotype may not necessarily share the same invasion sources. Although microsatellite data could reveal the sources and routes of invasions in detail, we could not pinpoint the source of the Californian populations due to lack of samples from possible origins, such as Asia, Hawaii, and the continental United States. More comprehensive sample collections incorporating more microsatellite or SNPs data will be able to illuminate the possible sources of Californian populations.

The extent of *C. formosanus* infestation in Canyon Lake remains undetermined. Although located in an arid environment, the surrounding landscapes with plants and trees are heavily irrigated, which may explain the survival and support the potential expansion and dispersal of this termite into the neighborhood. Rust et al. (1998) reported sporadic alate flights occurred from May to September in La Mesa, especially in the early evening and days when the daytime temperature was >31°C. According to the owner of the infested house in Canyon Lake, indoor swarms had been observed in late May/early June over the last 8 yr, suggesting that *C. formosanus* may have already reached other properties in the neighborhood. More detailed surveys along the surrounding properties and landscape may reveal new infestation and help to delimit the current distribution of this termite in Canyon Lake and elsewhere.

Supplementary Data

Supplementary data are available at the *Journal of Economic Entomology* online.

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