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Genetic Evidence for Multiple Invasions of *Coptotermes formosanus* (Blattodea: Rhinotermitidae) in California

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Abstract

New infestations of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae), were discovered in southern California, namely in Rancho Santa Fe and La Mesa (San Diego County) and Highland Park (Los Angeles County) in 2021. We investigated whether these new infestations were related to the previous infestations in La Mesa (2018) and Canyon Lake, Riverside County (2020). We used two mitochondrial genes (COI and COII) and seven polymorphic microsatellite markers to infer the genetic relationship between southern California colonies and their breeding systems. The samples collected from seven localities belonged to five colonies (inter-colony distances ranged from ~160 m to 185 km, with an average of 97 km). Of these five colonies, two were simple families, and three were extended families. Structure analyses of microsatellite genotypes grouped the termite samples into three distinct genetic clusters, suggesting at least three independent introduction events in southern California.

Key words: Formosan subterranean termite, breeding system, microsatellite, genotype, invasive species

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is one of the most destructive urban pests in the world (Su 1990, Lowe et al. 2000, Evans et al. 2013, Cuthbert et al. 2021). It is also the only termite species listed in the world's 100 worst invasive organisms (GISD 2022). This species is endemic to East Asia (Kistner 1985, Evans et al. 2013, Chouvenc et al. 2016) but has been introduced and established in many tropical and subtropical regions (Jones et al. 2017, Scheffrahn et al. 2020, Blumenfeld et al. 2021), including the United States (Woodson et al. 2001, Su 2003, Evans et al. 2013). This species was first observed outside Asia in Oahu, Hawaii, in 1907 (Swezey 1914, Bess 1970) and on the U.S. mainland in Charleston, South Carolina, in 1957 (Chambers et al. 1988). Subsequently, it spread to the southeastern and south-central states and California (Woodson et al. 2001, Su 2003, Evans et al. 2013, Scheffrahn et al. 2020), likely through anthropogenic activities.

The damages due to *C. formosanus* amounted to more than $1 billion annually in the United States (Su 2002, Lax and Osbrink 2003, Pimentel et al. 2005). Only recently has *C. formosanus* become a concern to pest management professionals in California (Tseng et al. 2021). It was first discovered in California in La Mesa, San Diego County, in 1992 (Atkinson et al. 1993, Reierson et al. 1993). Although the colony in La Mesa was treated (Haagsma et al. 1995, Rust et al. 1998), the alates likely dispersed to adjacent areas. In August 2018, *C. formosanus* was rediscovered in La Mesa. In June 2020, another new infestation of the Formosan subterranean termite was discovered in a house in Canyon Lake, Riverside County (Tseng et al. 2021). Population genetic analyses showed that the Canyon Lake infestation was unrelated to the La Mesa population (Tseng et al. 2021).

In 2021, more infestations were found in Rancho Santa Fe, La Mesa (San Diego County), and Highland Park (Los Angeles County). This study investigates the relationship of the abovementioned infestations using mitochondrial DNA and microsatellite data to provide insight into the origin of those colonies. We also characterized the breeding system of the *C. formosanus* populations in Southern California using the same dataset.

Materials and Methods

Termite Samples

We collected fresh Formosan subterranean termite samples (workers, soldiers, and alates) from Rancho Santa Fe and La Mesa (San Diego County) and Highland Park (Los Angeles County) in June and July

2021 (Fig. 1). Samples in Rancho Santa Fe and Highland Park were collected from infested houses, while samples in La Mesa were collected from tree stumps near the infested home. Sampling sites LM2, LM3, and LM4 in La Mesa are located 36 m, 39 m, and 158 m apart, respectively, from the infested house, LM1, which was sampled in 2018. All samples were preserved in absolute ethanol before DNA extraction. We also included the samples previously collected from La Mesa and Canyon Lake in 2018 and 2020, respectively (Tseng et al. 2021; sites LM1 and CL1) for comparison.

DNA Extraction, mtDNA Sequencing, and Microsatellite Genotyping

DNA was extracted from each termite worker using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Three individuals collected from each sample site were used in mtDNA sequence comparisons. Portions of the cytochrome oxidase subunit I (COI) and cytochrome oxidase subunit II (COII) were amplified following the procedures described in Tseng et al. (2021). Bidirectional sequencing of PCR amplicons was performed by Retrogen Inc. (San Diego, CA, USA). Sequence data were assembled by using Sequencher 4.9 (GeneCodes), and multiple sequence alignments were generated using MUSCLE with default setting as implemented in MEGA 6 (Tamura et al. 2013). Haplotype data were obtained through DNA SP 6 software (Rozas et al. 2017), and haplotypes were named following Tseng et al. (2021). Sixteen workers from each sample site were genotyped at seven microsatellite loci (*Cf4:1A2-4*, *Cf12-4*, *Cf4-10*, *Cf10-4*, *Copf01*, *Copf06*, and *Copf14*) using published primer sequences (Vargo and Henderson 2000, Liu et al. 2012). Among the seven microsatellite loci used in this study, four (*Cf4:1A2-4*, *Cf4-10*, *Cf10-4*, and *Cf12-4*) are trinucleotide repeat loci, and three (*Copf01*, *Copf06*, and *Copf14*) are dinucleotide repeat loci. PCR procedures followed that described in Tseng et al. (2021). The resulting PCR amplicons were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) at the University of Arizona, Genomic Analysis and Technology Core Facility (GATC). Allele sizes were visualized and scored using the GeneMarker program (version 3.0.1, SoftGenetics LLC) and checked trice by eye. We genotyped twice for rare alleles (alleles that appeared in less than three individuals) to confirm the observed genotypes. Microsatellite and mtDNA sequence data for samples collected in sites LM1 and CL1 were obtained from a previously published study (Tseng et al. 2021) and reanalyzed.

Colony Affiliation and Breeding System

Coptotermes formosanus was collected from multiple sites in La Mesa in 2018 and 2021 (Fig. 1). To determine the colony affiliation of samples from these different sites, groups of termites from LM1, LM2, LM3, and LM4 were tested for significant genetic differentiation using permutation tests with standard Bonferroni corrections as implemented in FSTAT 2.9.4 (Goudet 1995). Samples with significant genetic differentiation were considered from different colonies (Vargo 2003; Husseneder et al. 2005, 2007). We tested whether the colony belonged to a simple family colony (i.e., colony headed by a single pair of reproductives) or an extended family colony (i.e., colony headed by multiple reproductives) based on the frequencies and classes of worker genotypes (Vargo 2003; Vargo et al. 2003, 2006; Husseneder et al. 2005). A colony was considered a simple

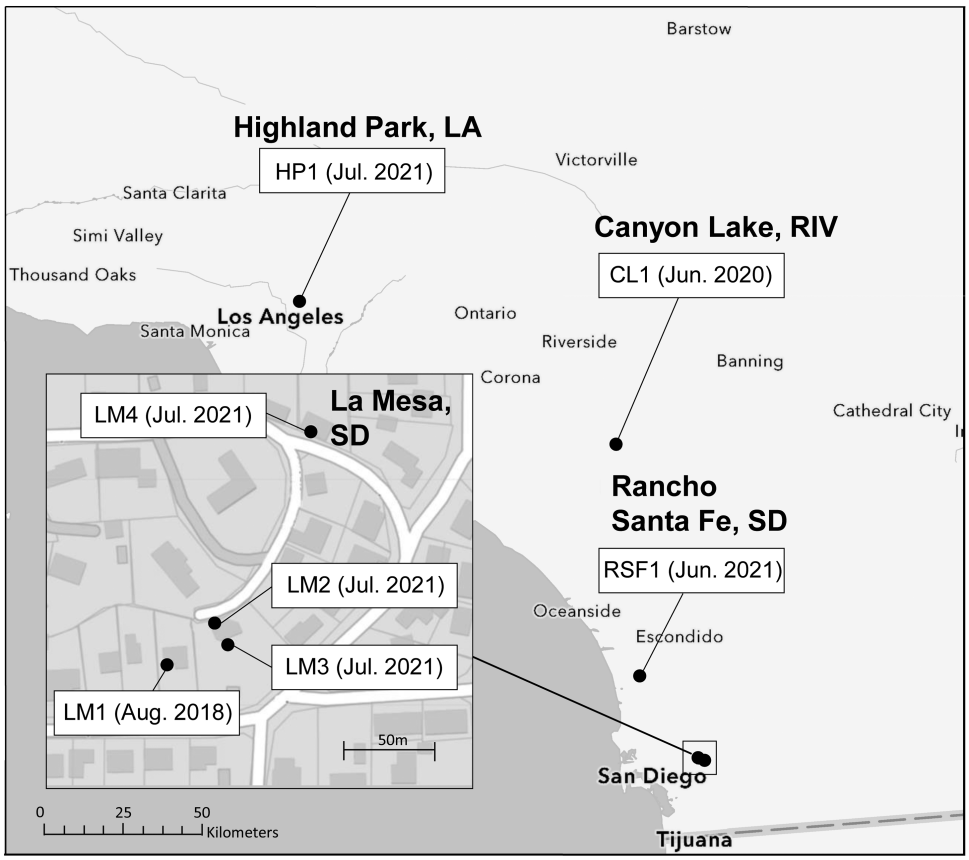

Fig. 1. Map of southern California, USA showing sampling sites for *Coptotermes formosanus* samples in this study.

Table 1. Colony affiliation, mtDNA haplotype, and breeding structure in five colonies of *Coptotermes formosanus* in southern California

Colony ID	Sampling site	mtDNA haplotype	Breeding structure
LM1+2+3	LM1, LM2, LM3	Hap01	Simple-family
LM4	LM4	Hap01	Extended-family
CL1	CL1	Hap02	Simple-family
RSF1	RSF1	Hap02	Extended-family
HP1	HP1	Hap02	Extended-family

family if the genotypes of the workers were consistent with those expected for the offspring of monogamous pairs of reproductives, and the observed genotype frequencies did not significantly differ from the expected Mendelian ratios determined by G-test. A colony was considered an extended family when the colony had no more than four alleles at a locus and contained genotypes inconsistent with a monogamous pair of parents (e.g., comprises five genotypes, three classes of homozygotes, or the observed frequencies of the genotypes deviated significantly from those expected in a simple family).

Population Structure

We investigated the genetic relationships among samples from different sites using two approaches. First, the genetic clustering of individuals within and among studied localities was visualized using a principal coordinate analysis (PCoA) based on genetic distance in Genalex 6.5 software (Peakall and Smouse 2006). The clustering of termite samples was also assessed by Bayesian model-based clustering software STRUCTURE 2.3.4 (Pritchard et al. 2000). We evaluated the potential number of populations (i.e., genetic clusters, K) from one to seven, with 10 replicates each. All simulations were performed for each K with 1,000,000 MCMC iterations (the first 100,000 iterations were discarded as burn-in). The most likely K was evaluated using Structure Selector (Li and Liu 2018) by calculating the median of medians (MedMedK) method and a maximum of medians (MaxMedK) with a threshold value of 0.8, which accounts for uneven sampling and hierarchical population structure (Puechmaille 2016). The sampling site information was used as a correcting factor. The STRUCTURE result was visualized using the CLUMPAK server (Kopelman et al. 2015).

Results

The concatenated sequences of both COI and COII genes with 1,003 characters revealed two mtDNA haplotypes (Hap01 and Hap02) that differ by 4-bp for the samples collected in southern California (Table 1). Seven microsatellite loci were successfully genotyped and were polymorphic, with allele numbers ranging between 2 and 5 (mean ± SD = 3.14 ± 1.21; Table 2). The number of alleles per microsatellite marker for each colony ranged between 1 and 4 (Table 2). The La Mesa samples (LM2–4) collected in 2021 shared the same haplotype as the La Mesa samples (LM1) collected in 2018. Genetic differentiation analysis based on microsatellite genotypes indicates that these samples belonged to two different colonies, where samples from sites LM1, LM2, and LM3 belong to colony LM1+2+3, and samples from site LM4 belong to colony LM4 (Fig. 1, Table 1). The *C. formosanus* samples collected from Canyon Lake, Rancho Santa Fe, and Highland Park shared an identical mtDNA haplotype, Hap02 (Table 1). However, the population structure analysis suggests that these samples belong to two different genetic clusters based on microsatellite genotype data (Fig. 2). PCoA plot reveals that *C. formosanus* samples are grouped into three distinct genetic clusters, also supported by the STRUCTURE analysis using MedMedK and MaxMedK criteria (Fig. 2A and B, also see Supp Fig. S1 [online only] for STRUCTURE results from K = 2 to 7). Genetic cluster I contains samples collected from La Mesa, cluster II includes samples collected from Canyon Lake, and cluster III contains samples collected from Rancho Santa Fe and Highland Park (Fig. 2A and C). Differentiation among the three genetic clusters was supported by one to four private alleles with private allele frequencies ranging between 0.016 and 0.375. Genotype profiles of *C. formosanus* workers collected from southern California were listed in Supp Table S1 (online only). Of the five colonies collected from southern California, two were simple families (LM1+2+3 and CL1), and three were extended families (LM4, RSF1, and HP1; Table 1).

Discussion

In this study, we performed population genetic analyses to infer the number of invasions of *C. formosanus* in southern California and their breeding systems. Our analysis clearly shows that the recent infestations consist of three genetically distinct populations. These clusters are represented by the following groups: 1) La Mesa, 2) Canyon Lake, and 3) Rancho Santa Fe and Highland Park (Fig. 2). We suggest that termites from each genetic cluster are descended from independent invasions into southern California. Genetic variability detected in this study is similar to that of invasive populations in Charleston, SC, and Rutherford County, NC, as measured by the average number of alleles per microsatellite loci (Vargo et al. 2006).

The 1992 La Mesa infestation (Atkinson et al. 1993) was treated until the program was discontinued in 1997 due to the lack of funding (Rust et al. 1998). When infestations were rediscovered in 2018, the closest location of infestation was only 100 m from the original site. Genetic data supported that the 2018's colony was closely related to the 1992's colony (Tseng et al. 2021). The 2021 samples were collected near the 2018 locations [36 m (LM2), 39 m (LM3), and 158 m (LM4)]. Our results indicated that the 2018 infestation (LM1) belonged to a simple family colony (LM1+2+3, Table 1). Although this colony was treated, it was not eliminated and persisted in the adjacent areas (LM2 and LM3). On the other hand, the termites in locality LM4 belonged to another extended family colony (LM4, Table 1) but were closely related to colony LM1+2+3. The finding of the new colony indicates that the Formosan subterranean termites have already spread beyond the house, with an observation of alates swarming in the yard of the infested house in 2018. An area-wide *C. formosanus* control program in La Mesa is urgently warranted, judging from the well-established and multiple infested sites.

Population genetic analyses indicated that Canyon Lake infestation was unrelated to La Mesa infestations (Tseng et al. 2021). The infested home was fumigated and treated with Termidor HE, and termites were not found 6 mo after the treatment. Additional investigation in this study suggests that the colony in Canyon Lake is a simple family. There is a need to have periodic inspections for termite activity in this area. The infestation in Rancho Santa Fe and Highland Park is 153 km

Table 2. Number of alleles per microsatellite marker for each colony collected. (LM: La Mesa; CL: Canyon Lake; RSF: Rancho Santa Fe; HP: Highland Park)

	LM1+2+3	LM4	CL1	RSF1	HP1	Overall
Cf4:1A2-4	2	2	3	2	3	3
Cf12-4	1	1	2	4	3	5
Cf4-10	2	2	2	1	1	2
Copf01	1	1	2	4	3	4
Copf14	1	1	2	1	1	2
Cf10-4	2	2	2	2	2	2
Copf06	2	3	3	1	1	4

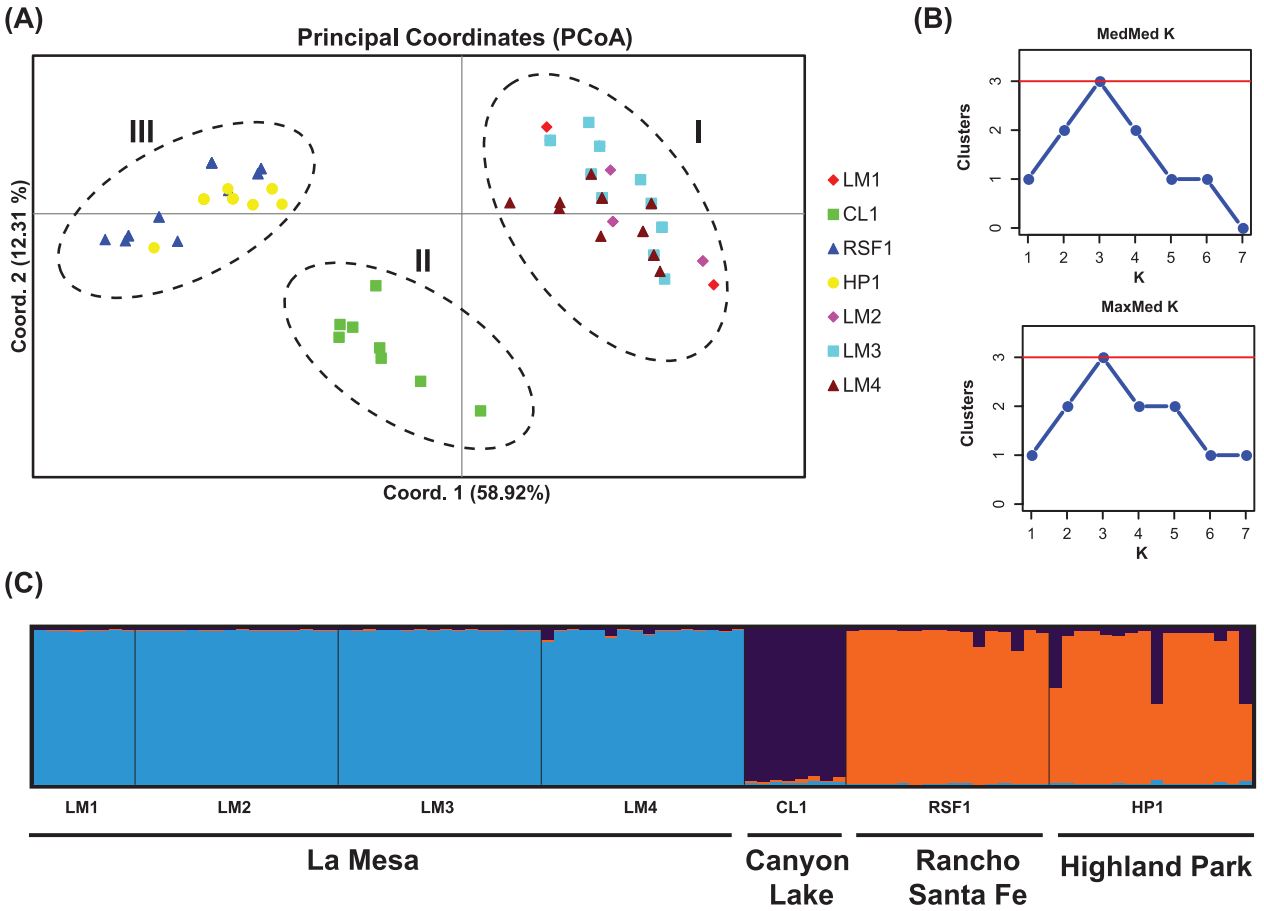

Fig. 2. Population structure of *Coptotermes formosanus* in southern California. (A) Principal coordinate analysis of *C. formosanus* individuals. The termite samples were divided into three genetic groups by the first and the second PCoA axes. The first and second axis explained 58.63% and 12.31% of the total genetic similarities, respectively. (B) Estimated number of genetic clusters (K) using the median of medians (MedMedK) and maximum of medians (MaxMedK) criteria in STRUCTURE. (C) Genetic clustering of *C. formosanus* in southern California inferred by STRUCTURE analysis based on the best K determined by MedMedK and MaxMedK. Also, see Supp Fig. S1 (online only) for STRUCTURE results from K = 2 to 7. A color characterizes each genetic cluster, and each individual is represented by a vertical bar according to its probability of belonging to each genetic cluster. The locations where samples were collected appear below the figure.

apart. Genetic analysis revealed that the two infestations were genetically similar (Fig. 2). Migration of termites between the two localities via natural dispersal is unlikely because alates of this species appear to have limited flight ranges (less than 1 km; Messenger and Mullins 2005). The most parsimonious explanation for this observation is that anthropogenic activities introduced two infestations from a common source (e.g., infested lumbers, potted plants, etc.). Rancho Santa Fe and Highland Park colonies are both extended families. Although there is no available data on the breeding structure concerning colony age for *C. formosanus*, extended family colonies are expected to be older than simple families because the secondary reproductives occur later in the colony development, after one or both founding primary reproductives die (Myles 1999). The lifespan of primary reproductives of *C. formosanus* in the field is not known, but primary reproductives of *C. formosanus* can survive up to 9 yr in the laboratory (Huang 1987, Osbrink et al. 2016). The two colonies in Rancho Santa Fe and Highland Park were likely mature colonies judging from thousands of alates that swarmed at both locations. Mature colonies (colony age > 5 yr old) could produce nymphs, the sexual line of individuals that become alates (Chouvenc and Su 2014). Undoubtedly, the infestations of the *C. formosanus* in Rancho Santa Fe and Highland Park started many years ago and remained undetected until 2021.

The sources of these infestations remain unknown but could likely be originated from Hawaii, southcentral, or/and southeastern regions of the United States where *C. formosanus* is abundant. Although *C. formosanus* naturally disperses through nuptial flights, the primary method of range expansion in its invasive areas is through the transport of infested materials, primarily recycled railway ties/sleepers, and potted plants and furniture (Atkinson et al. 1993, Jenkins et al. 2002, Messenger et al. 2002, Lee et al. 2009, Evans et al. 2013). Once introduced, the heavily irrigated urban landscape and shorter winter period in Southern California likely helped the colonies establish and thrive. The spread of *C. formosanus* will likely continue in southern California if no immediate action is taken. A statewide survey of Formosan subterranean termite infestation, especially in southern California, is urgently warranted, besides evaluating the common modes of spread and increasing public awareness to delimit further anthropogenic spread of this destructive species.

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Supplementary Data

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

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