

Phylogenetic Analysis of Subterranean Termites (*Coptotermes* spp., Isoptera: Rhinotermitidae) Indicates the Origins of Hawaiian and North American Invasions: Potential Implications for Invasion Biology

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Abstract. Subterranean termites in the genus *Coptotermes* Holmgren are structural pests that have become globally distributed beyond their native range in Southeast Asia. Because of their destructive nature, it is useful to understand the pathways of their spread. Additionally, phylogenetic analysis of evolutionary relationships may lead to increased accuracy of insecticide-based management on the basis that related species are likely to share similar physiology. Cytochrome oxidase II nucleotide sequences were used to construct phylogenies of subterranean termites using both maximum parsimony and maximum likelihood models. The data set included subterranean termites (Rhinotermitidae), including *C. formosanus* Shiraki, and used drywood termites (Kalotermitidae) as putative outgroups. Both methods supported the main results, that Hawaiian infestations likely originated in Asia and that some infestations in North America either came through Hawaii or originated independently from the same ancestral region as the Hawaiian infestations. *Coptotermes formosanus*, the most significant pest, appears to be paraphyletic with respect to several other species in the genus, and may represent two cryptic species. Other infestations in North America appear to have originated separately in Asia. A phylogeographic hypothesis based on non-molecular information was also supported by these data.

Key words: invasive species, invasion pattern, structural pests, *Coptotermes formosanus* Shiraki

Introduction

Formosan subterranean termites are destructive structural pests adept at colonizing new locations (Woodrow et al. 2001, Kirton 2005; Messenger et al. 2005). In 1997, costs incurred as a result of termite infestation were estimated at over one billion dollars in the United States alone (Osbrink et al. 2001). In Hawaii, both Rhinotermitidae (subterranean) and Kalotermitidae (drywood) termites cause significant structural harm, but the Formosan subterranean termite *Coptotermes formosanus* Shiraki is the most important pest in the state. This rhinotermitid damages crops and trees in addition to causing over US\$100 million annually in structural costs (Tamashiro et al. 1996). Kirton (2005) reviewed the importance of constructing accurate termite phylogenies in order to tailor effective management strategies, including recognizing special susceptibility (or resistance) based on genetic bottlenecks or single invasion events and delineating the boundaries of particular species groups in complex ecosystems.

Termites have cryptic foraging and breeding habits (Campora and Grace 2001, Ross 2001). The subterranean nature of rhinotermitids obfuscates the study of complex social structures including variable plasticity among castes and the possibility of satellite reproductive pairs (Vargo et al. 2003), further complicating taxonomic analysis. Although the extent of the damage caused by termites is well known, the phylogeny of the global genus *Coptotermes* – which includes some of the most economically damaging termites – remains unclear. The genus contains an estimated 71 species, while the family Rhinotermitidae includes 15 genera (Kambhampati and Eggleton 2000). Over a third (33) of the described *Coptotermes* species have been reported from China (Gao et al. 1990, Wang and Grace 1999).

Recent research has focused on morphology-based phylogenies for the order (Donovan et al. 2000), microsatellite or mitochondrial DNA-based analyses of the colonial structure of populations (Vargo et al. 2003), biogeographic analysis (Thompson et al. 2000; Jenkins et al. 2001. Ohkuma et al. 2004), and distinguishing different species based on cuticular hydrocarbons (Page et al. 2002; Copren et al. 2005. Haverty et al. 2005). With the exception of a substantial amount of work on the rhinotermitid genus *Reticulitermes* (Thompson et al. 2000; Jenkins et al. 2001. Page et al. 2002. Ye et al. 2004; Copren et al. 2005), and Australian *Coptotermes* (Lo et al. 2006), little molecular research has aimed to determine evolutionary relationships among termites at the genus or species level. Donovan et al. (2000) emphasize the need for additional data, including that from behavioral, nucleotide, and biological sources, to construct a more accurate phylogenetic hypothesis for these complex insects.

A morphology-based study of termites from the families Kalotermitidae, Rhinotermitidae, and Termitidae in Puerto Rico and the U.S. Virgin Islands suggested an over-water dispersal route from western North America and South American sources (Scheffrahn et al. 2003), providing a plausible hypothesis for how termites arrived in the West Indies from the American continent. Lo et al. (2006) showed some Australian termites to be more closely related to Asian termites than to other species native to Australia, again suggesting the possibility of over-water movement for a short distance.

The most widely accepted hypothesis for the origin of *C. formosanus* was presented by Kistner (1985), with his description of a termitophilous staphylinid beetle, *Sinophilus xiai*. This was the first termitophilous beetle described from continental China and was the first termitophile to be found in *C. formosanus* nests; as a result, *C. formosanus* is thought to have originated in continental China. Recent molecular work has suggested that the order Isoptera ought to be sunk within cockroaches (Inward et al. 2007), and Lo et al. (2000) also suggest that wood-eating cockroaches in the genus *Cryptocercus* serve as an evolutionary intermediate lineage between the basally-branching cockroaches and the more derived termites; it is interesting to note that Park et al. (2004) have hypothesized the origin of *Cryptocercus* in east or northeast China. The historic dispersal of *C. formosanus* from continental China to its current range appears to have followed trade routes prior to the 20th century and military movement during and shortly after the second World War – a path *C. vastator* appears to be following at present (Woodrow et al. 2001), although this latter species was recently synonymized with *Coptotermes gestroi* (Wasmann) (Yeap et al. 2007). Because *Coptotermes* species are such damaging invaders it is important to understand the pathways by which they have, and may in the future, disperse across the globe. Such data will be of use in identifying and monitoring current and future invasion routes.

The goal of this project was to determine the dispersal pattern of *C. formosanus* based on cytochrome oxidase II sequence data. Termites in the genus *Coptotermes* are widespread pests, and samples for our analysis were chosen from invaded regions of the United States and likely source populations in Asia in an effort to identify likely invasion pathways into North America. Such an analysis might support or refute the hypothesis that *C. formosanus*

originated in continental China (Kistner 1985). Additionally, by comparing relationships within *Coptotermes* to other genera within Rhinotermitidae and Kalotermitidae we hoped to better understand the evolutionary relationships among the most destructive species and their less economically important sister taxa.

Methods

Genome and species selection. Cytochrome oxidase II sequences were selected because the gene has been informative for species-level analysis in other termite studies (e.g., Miura et al. 2000; Jenkins et al. 2001; Austin et al. 2004; Copren et al. 2005). In order to construct the tree, all available sequence data with location data specified was downloaded from GenBank for the following taxa: *C. formosanus* (multiple populations for analysis of origins and invasion pathways) (n = 21), *C. acinaciformis* (n = 2), *C. curvignathis* (n = 1), *C. gestroi* (n = 2), *C. kalshoveni* (n = 1), and *C. lacteus* (n = 2) (Table 1). The kalotermitids, serving as the putative outgroup, were represented by *Incisitermes immigrans*, *Cryptotermes brevis*, and *Cr. cynocephalus*. It is important to note we relied upon the species identifications given in GenBank and that any future reassessments of the accuracy of these identification may modify the results of the present study.

GenBank was scanned for available *Coptotermes* and Kalotermitidae COII nucleotide sequences. All available sequences were manually aligned; the aligned sequence length was consistent across all samples and was a maximum of 449 base pairs. Accession numbers and detailed collection location data are listed in Table 1.

Phylogeny reconstruction. PAUP* 4.0b10 (Swofford 2003) was used to conduct both maximum parsimony and maximum likelihood analyses, and TreeView v1.6.6. (Page 1996) was used to visualize the trees. We analyzed the nucleotide data under a maximum parsimony model (characters with equal weights, heuristic search, 1000 replicates). Bootstrapping to assess branch support (1000 replications) was also conducted in PAUP for the maximum parsimony model. We used ModelTest v3.5 (Posada and Crandall 1998) to select the model of nucleotide substitution that best fit the data; a maximum likelihood (ML) model was selected, and run using PAUP* (heuristic search, 1000 replicates); a single most parsimonious tree was produced.

Results

There were 409 parsimony informative characters, and the maximum parsimony and maximum likelihood trees were identical (Figures 1, 2). ModelTest recommended the general-time reversible (GTR) model with proportion of invariable sites and Gamma distribution estimated independently. The parsimony bootstrap values (Figure 1) strongly support the branches of the tree indicating close relationship between the *C. formosanus* on the continental US, Hawaii, and Japan; between *C. acinaciformis*, *C. curvignathis*, and *C. formosanus* from China-China-US (Florida)-US (Georgia); between *C. kalshoveni* and *C. gestroi*; and between these three clades. The four kalotermitid outgroup taxa are grouped together separately from the rhinotermitid taxa, with 100% bootstrap support in the parsimony model. The kalotermitids are split into two groups, one containing the two *Cr. cynocephalus* taxa (with 100% bootstrap support) and another with *I. immigrans* and *Cr. brevis* (61% bootstrap support). For the parsimony phylogeny, there were 409 parsimony informative characters and 18 most parsimonious trees all with 747 steps. The consistency index was 0.922, and the rescaled consistency index was 0.890, which was the same for all the most parsimonious trees. For the maximum likelihood phylogeny the ML score was $-\ln 2693.3184$.

Both the maximum parsimony and maximum likelihood trees highly support a clade

Table 1. Nucleotide GenBank accession numbers, species, and detailed collection location information.Co. = *Coptotermes*; Cr. = *Cryptotermes*; In. = *Incisitermes*

Accession no.	Abbreviation	Species	Collection location
<i>Coptotermes formosanus</i> Shiraki (Rhinotermitidae) taxa			
AY536404	CformosCHINA	<i>Co. formosanus</i>	Zhongsan University (China)
AY536403	CformosCHINA2	<i>Co. formosanus</i>	Zhongsan University (China)
AY168204	CformosFL	<i>Co. formosanus</i>	Golden Beach, FL (USA)
AY536405	CformosGA	<i>Co. formosanus</i>	Georgia (USA)
AY683220	CformosGA2	<i>Co. formosanus</i>	Cairo, GA (USA)
AY683219	CformosGA3	<i>Co. formosanus</i>	Savannah, GA (USA)
AY683218	CformosGA4	<i>Co. formosanus</i>	Savannah, GA (USA)
AY683216	CformosGA5	<i>Co. formosanus</i>	Paulding Co., Dallas, GA (USA)
AY683215	CformosGA6	<i>Co. formosanus</i>	Paulding Co., Dallas, GA (USA)
AY683214	CformosGA7	<i>Co. formosanus</i>	Dekalb Co., Tucker, GA (USA)
AY683213	CformosGA8	<i>Co. formosanus</i>	Gwinnett Co., Lawrenceville, GA (USA)
AY683212	CformosGA9	<i>Co. formosanus</i>	Gwinnett Co., Suwannee, GA (USA)
AY027489	CformosGA10	<i>Co. formosanus</i>	Athens, Georgia (USA)
AY683221	CformosGA11	<i>Co. formosanus</i>	Cairo, GA (USA)
AY536406	CformosHI	<i>Co. formosanus</i>	Hawaii (USA)
AY453588	CformosHI2	<i>Co. formosanus</i>	Oahu, Hawaii (USA)
AB109529	CformosJPN	<i>Co. formosanus</i>	Iriomote Island (Japan)
AY536407	CformosLA	<i>Co. formosanus</i>	New Orleans, LA (USA)
AY683217	CformosLA2	<i>Co. formosanus</i>	Chatham Street, New Orleans, LA (USA)
AF107488	CformosLA3	<i>Co. formosanus</i>	New Orleans, LA (USA)
AF525317	CformosTX	<i>Co. formosanus</i>	Galveston, TX (USA)
Other <i>Coptotermes</i> taxa (Rhinotermitidae)			
AF262610	Cacina UNK	<i>Co. acinaciformis</i>	Unspecified
AY536401	CacinaAUS	<i>Co. acinaciformis</i>	Griffin, Canberra, New South Wales (Australia)
AY536402	CcurvigMAL	<i>Co. curvignathus</i>	Penang Island (Malaysia)
AY295078	Cgestroi UNK	<i>Co. gestroi</i>	Unspecified
AY536408	CgestroiMAL	<i>Co. gestroi</i>	Penang Island (Malaysia)
AY536409	CkalshovMAL	<i>Co. kalshoveni</i>	Penang Island (Malaysia)
AY536410	Clacteus AUS	<i>Co. lacteus</i>	Canberra, New South Wales (Australia)
AF220600	Clacteus UNK	<i>Co. lacteus</i>	Unspecified
Kalotermitidae taxa			
AAG17066	CryptobreviAUS	<i>Cr. brevis</i>	Australia (exact locale unspecified)
AF189084	CryptocynoAUS	<i>Cr. cynocephalus</i>	Mossman, Queensland, (Australia)
AF189083	CryptocynoAUS2	<i>Cr. cynocephalus</i>	Mt Webb, Queensland, (Australia)
AB109542	IimmigASIA	<i>In. immigrans</i>	Asia (Japan or Thailand)

consisting of *C. formosanus* taxa from China, Malaysia, Australia, Florida, and Georgia plus both *C. acinaciformis* and *C. curvignathus*; *C. gestroi* taxa are in a separate clade with *C. kalshoveni*. Both of these clades had 100% bootstrap support in the parsimony method. Also, in both models *C. formosanus* populations from Hawaii and Japan appeared in a separate clade with 63% support in the parsimony tree, though this only represented a two-base pair difference from the North American clade. The clade containing the remainder of the *C. formosanus* populations from the continental United States and another Hawaiian sample

was supported with a 57% bootstrap value. Thus, *C. formosanus* is rendered paraphyletic with respect to all the other *Coptotermes* species used in this analysis.

Discussion

Both the maximum parsimony- and maximum likelihood-based trees offer some support for the theory that *C. formosanus* may have moved from Asia to the continental United States through Hawaii (Kistner 1985), though no definitive answer was provided by this study. In trees from both methods *C. acinaciformis* and *C. curvignathis* were sister taxa to the *C. formosanus* China-China-Florida-Georgia polytomy, which was a sister clade to the rest of the *Coptotermes* taxa. The data set had low sequence variability for the *C. formosanus* populations but the models were consistent in estimating the relationships within the genus; though divergence was low, as might be expected due to bottlenecks in a recently invading species, some resolution for the phylogeography analysis was offered. Future research using nuclear and mitochondrial markers may provide further information to support or refute this invasion hypothesis.

Phylogeographic analysis of the nucleotide data suggested high levels of diversity in the Hawaiian samples and also suggests that Hawaiian *C. formosanus* probably originated in Asia, possibly from more than one location. This is based on some Hawaiian samples grouping in with the larger *C. formosanus* polytomy from the southeastern United States, and another in a clade with a sample from Japan. This suggests multiple invasions of *C. formosanus* into Hawaii, perhaps associated with military movements of equipment and personnel at the end of World War II. Further, this invasion pattern within the country suggests multiple invasions to the continental United States from different locations, at least one of which may have come from Hawaii. The most remarkable result of this analysis is the clade appearing in both models containing *C. acinaciformis* (from Australia and an unknown sampling location), *C. curvignathis* (from Malaysia), and *C. formosanus* (from China and the southern United States). Such a result suggests multiple, independent invasions of *C. formosanus* directly from Asia into North America. More importantly, it reveals a complexity in mitochondrial DNA relationships between *Coptotermes* taxa in the same geographic region. It is possible that the shared mitochondrial DNA sequence (no variation across some individuals from all three species) is the result of long-term lineage sorting, or recent introgression or hybridization, all of which can confound studies based solely on molecular data (Cameron et al. 2006, Rubinoff et al. 2006, Will et al. 2005).

Coptotermes formosanus, the most significant pest in the genus, is apparently two distinct lineages that are each more closely related to other species in the genus, than to each other. Such a result has significant implications for efforts to contain and control the pest, since the two lineages may represent cryptic species that behave very differently. Further, the paraphyly, and possible cryptic species phenomenon suggests a more complex evolutionary history for the genus, which should be explored further. It may be that two species currently identified as *C. formosanus*, have retained some superficial ancestral traits leading to taxonomic confusion. Thus, while this study demonstrates the utility of a rapidly evolving molecular marker like COII for retracing invasion pathways, it also reveals the complexity of evolutionary relationships even at and below the species level. Future research on termite systematics must consider the possibility of introgression of mitochondrial markers and use multiple sources of data, such as nuclear DNA sequence and morphology, to understand broader evolutionary patterns in the group. The multiple invasions suggested by this study reveal that monitoring one route to North America, such as Hawaii, is unlikely to prevent future invasions since past invasion pathways not only came from Asia via Hawaii but also apparently directly from Asia into the eastern United States.

Figure 1. Maximum parsimony phylogeny with collection location (where known) indicated. Bootstrap values calculated using 1000 replicates.

Collection location key: UNK: unspecified; AUS: Australia; MAL: Penang Island, Malaysia; JPN: Irimote Island, Japan; all others are state abbreviations from the United States. China, Asia not abbreviated.

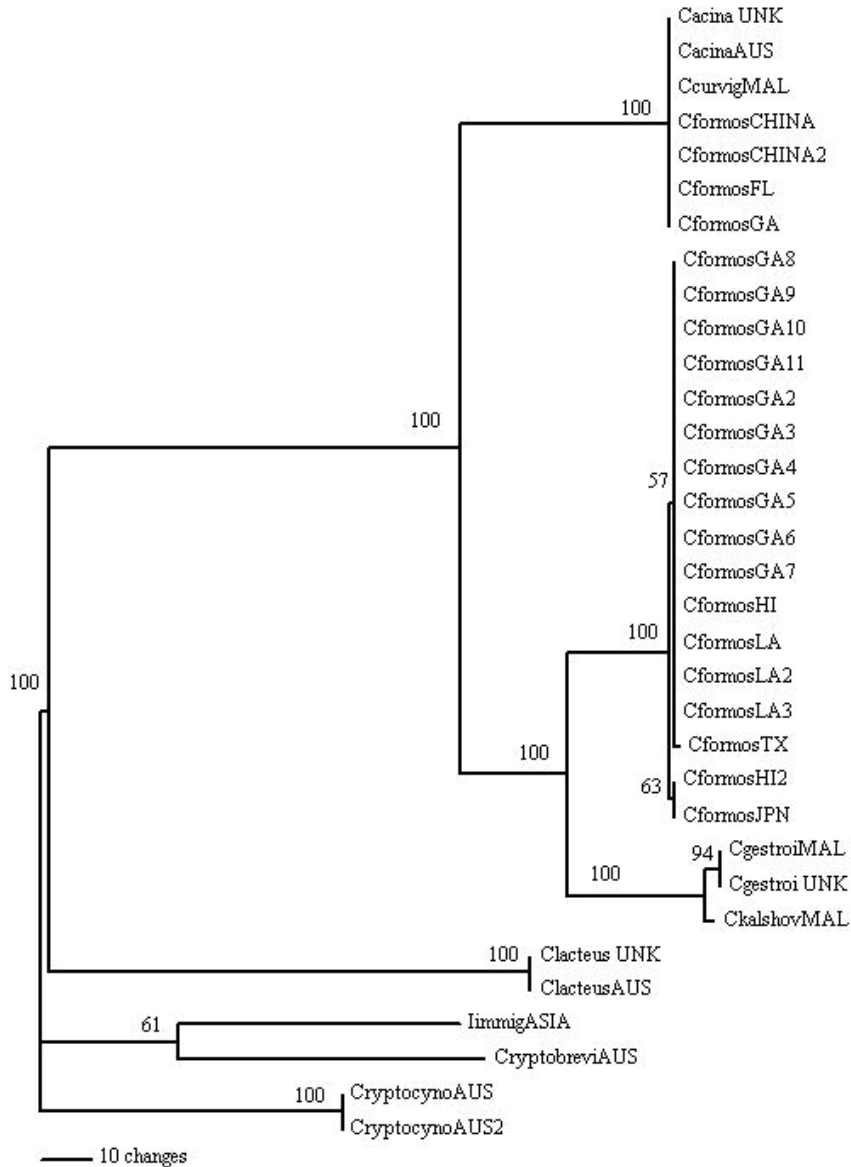
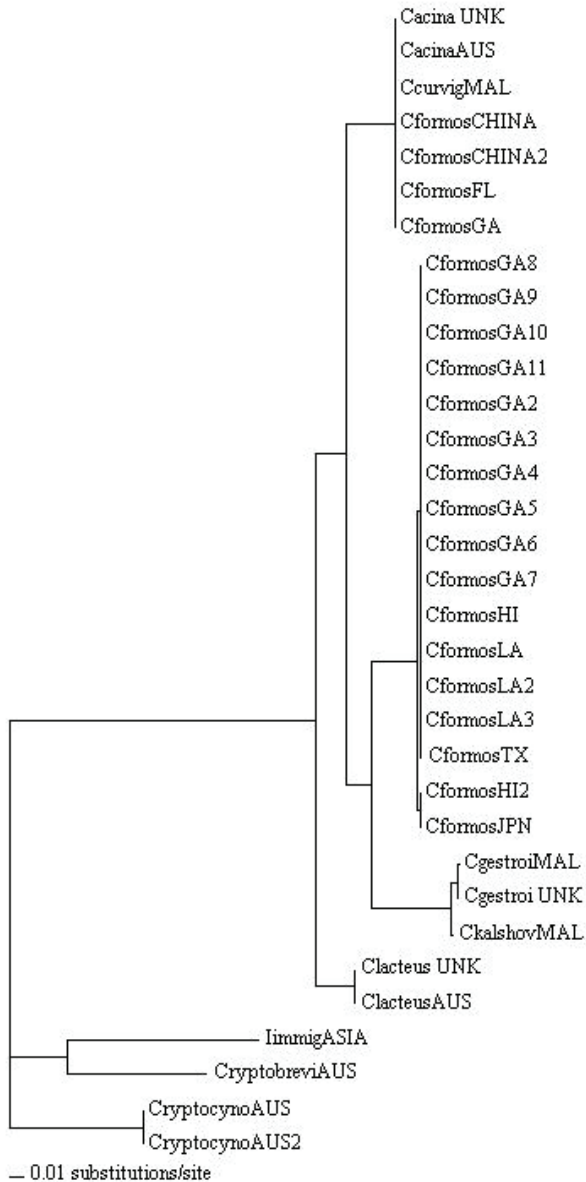


Figure 2. Maximum likelihood phylogram with collection location (where known) indicated.

Collection location key: UNK: unspecified; AUS: Australia; MAL: Penang Island, Malaysia; JPN: Irimote Island, Japan; all others are state abbreviations from the United States. China, Asia not abbreviated.



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