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SHORT COMMUNICATION

Phylogeny of Australian *Coptotermes* (Isoptera: Rhinotermitidae) species inferred from mitochondrial COII sequences

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Abstract

Six Australian species of *Coptotermes* are traditionally recognized, but recent cuticular hydrocarbon studies suggest that some of these may represent more than one species. An understanding of the phylogenetic diversity of Australian *Coptotermes*, particularly the pest species, is likely to be important for the improvement of termite management strategies. A study of phylogenetic relationships among species of this genus was performed, based on the mitochondrial cytochrome oxidase (COII) gene, comparing the data with recent data from Asian species. Representatives of the species *C. lacteus* (Froggatt), *C. frenchi* Hill and *C. michaelseni* Silvestri were each found to form closely related monophyletic groups, however representatives of *C. acinaciformis* (Froggatt) were not. For *C. acinaciformis*, representatives from northern mound-building populations were found to form a distinct group to southern, tree-nesting forms. Among southern *C. acinaciformis*, two Western Australian representatives were found to be divergent from other populations. The results suggest that *C. acinaciformis* probably represents a complex of species rather than one, as has been suggested previously. One unidentified *Coptotermes* sp. taxon from Melbourne was found to be divergent from other taxa. Notably, some Australian species were more closely related to Asian species than other Australian species.

Keywords: termite, subterranean, pest, mound

Introduction

Coptotermes (Isoptera: Rhinotermitidae) may be the most economically important genus of termites worldwide. *Coptotermes formosanus* Shiraki, an Asian species, has become established in a number of countries, including the USA, where its economic impact is in the order of hundreds of millions of dollars of damage annually (Su & Scheffrahn, 2000). In Australia, members of the genus cause much of the 780 million dollars of termite damage and associated repair and management costs annually (Archicentre, 2003). Based on morphological characters, six species of *Coptotermes* have been described in Australia: *C. acinaciformis* (Froggatt), *C. frenchi* Hill, *C. lacteus* (Froggatt), *C. michaelseni* Silvestri, *C. brunneus* Gay and *C. dreghorni* Hill (Hill, 1926, 1942; Gay & Calaby, 1970). *Coptotermes acinaciformis* has the widest

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distribution, and is considered the most serious pest species. In the north (Queensland, Northern Territory, northern Western Australia), it generally forms mounds, while in the south (New South Wales, Victoria, South Australia, southern Western Australia), it generally nests inside trees. In Western Australia, two subspecies are recognized, *C. a. acinaciformis* and *C. a. raffrayi*. The latter dominates the south-western corner of the state. *Coptotermes frenchi*, also a pest, occurs in much of mainland Australia, particularly in the south and east, but it is not known from the Northern Territory, western Queensland or northern Western Australia. It commonly nests in trees, but is also known to build low mounds. *Coptotermes lacteus*, not generally considered of economic importance, usually forms mounds. It is found from the extreme south-east corner of Queensland down through eastern New South Wales and into Victoria. One *Coptotermes* sp. with morphological characteristics similar to *C. lacteus* has been recorded causing significant damage to houses and in the absence of any mounds in the vicinity (R.H. Eldridge, unpublished observation). *Coptotermes michaelseni* and *C. brunneus* are found only in Western Australia, the former forming subterranean nests, and the latter forming mounds. *Coptotermes dreghorni* is restricted to the tablelands and coastal areas of north Queensland, and nests mainly in stumps or logs.

Studies of the cuticular hydrocarbon profiles and esterase patterns of *Coptotermes* species indicate that some morphology-based species may represent more than one species (Brown *et al*., 1990, 1994, 2004; Wang & Grace, 2000). Northern, mound-building *C. acinaciformis* have been shown to have notably different profiles to those in the south, and among southern populations at least three well-characterized hydrocarbon phenotypes are recognized. For *C. lacteus*, tree-nesting species (found along the south-east coast) were found to have distinct hydrocarbon profiles and distinct alate flight time differences to mound builders, and are likely to represent a different species (currently designated *Coptotermes* sp. P) (Brown *et al*., 2004). Within mound-builders, two hydrocarbon phenotypes exist: one of which exists across the distribution of *C. lacteus*, and another which may be specific to the north-west area of its distribution (type location: Armidale, designated *Coptotermes* sp. A.). For *C. frenchi*, two hydrocarbon phenotypes were identified, one of which is present in the south-eastern corner of the continent as well as in northern Queensland, and another (designated *C. labiosus* (Hill)), which is found in inland Victoria and New South Wales, as well as Western Australia.

An understanding of the phylogenetic diversity of *Coptotermes* is of interest from a basic scientific point of view, and is also of potential importance for the development of environmentally friendly management strategies. For example, if a species of pest termite in a given area actually comprises three or four different species, the individual species may respond in diverse ways to alternative management strategies; and some may actually be benign rather than pest species. In these cases, correct identification is important.

Recently, phylogenetic studies have been performed on the Rhinotermitidae at the among-genera level (Austin *et al*., 2004; Lo *et al*., 2004; Ohkuma *et al*., 2004), and some studies have focused on *Coptotermes* from Asia and the Americas, including the introduced *C. formosanus* (Szalanski *et al*., 2004; Ye *et al*., 2004). Here the first sequence-based study of a number of populations of Australian *Coptotermes* is described, focusing on the three most widespread species: *C. acinaciformis*, *C. frenchi* and *C. lacteus*.

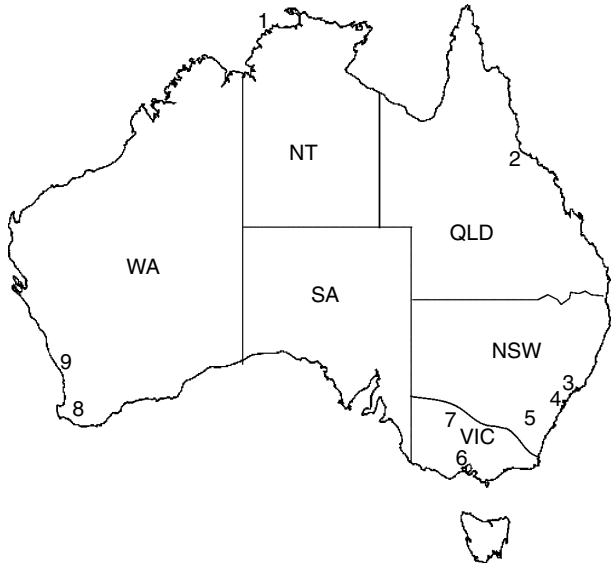

Fig. 1. Collection locations for Australian *Coptotermes* spp. 1, Darwin, NT; 2, Townsville, QLD; 3, Olney State Forest, NSW; 4, Sydney, NSW; 5, Canberra, ACT; 6, Melbourne, VIC; 7, Walpeup, VIC; 8, Manjimup, WA; 9, Perth, WA. Australian state abbreviations are as follows: VIC, Victoria; NSW, New South Wales; NT, Northern Territory; WA, Western Australia; ACT, Australian Capital Territory; QLD, Queensland.

Materials and methods

Termites

Figure 1 shows the collection locations of Australian termites examined in this study. *Coptotermes acinaciformis* were obtained from mounds in Darwin (NT, number 1 in fig. 1) and Townsville (QLD, 2), and either from tree nests or infested timber in Olney State Forest (NSW, 3), Sydney (NSW, 4), Canberra (ACT, 5), Walpeup (VIC, 7), Manjimup (WA, 8) or Perth (WA, 9). *Coptotermes lacteus* were obtained from mounds in Olney State Forest, Canberra and Melbourne (VIC, 6). *Coptotermes frenchi* were obtained from infested timber in Canberra and Melbourne, and *C. michaelseni* was obtained from infested timber in Perth. Termites were assigned to species based on soldier head morphological characteristics (Hill, 1942). In the case of *C. acinaciformis* from Western Australia, samples were not identified at the sub-species level. One unidentified species from Melbourne was also included.

DNA extraction, PCR and sequencing

One to three worker termites were used for each extraction. Following removal of the gut, DNA was extracted from termites using standard methods involving proteinase K and phenol/chloroform, as previously described (Lo *et al*., 2000). Polymerase chain reaction (PCR) of mitochondrial COII was performed using primers A-tLEU and B-tLYS

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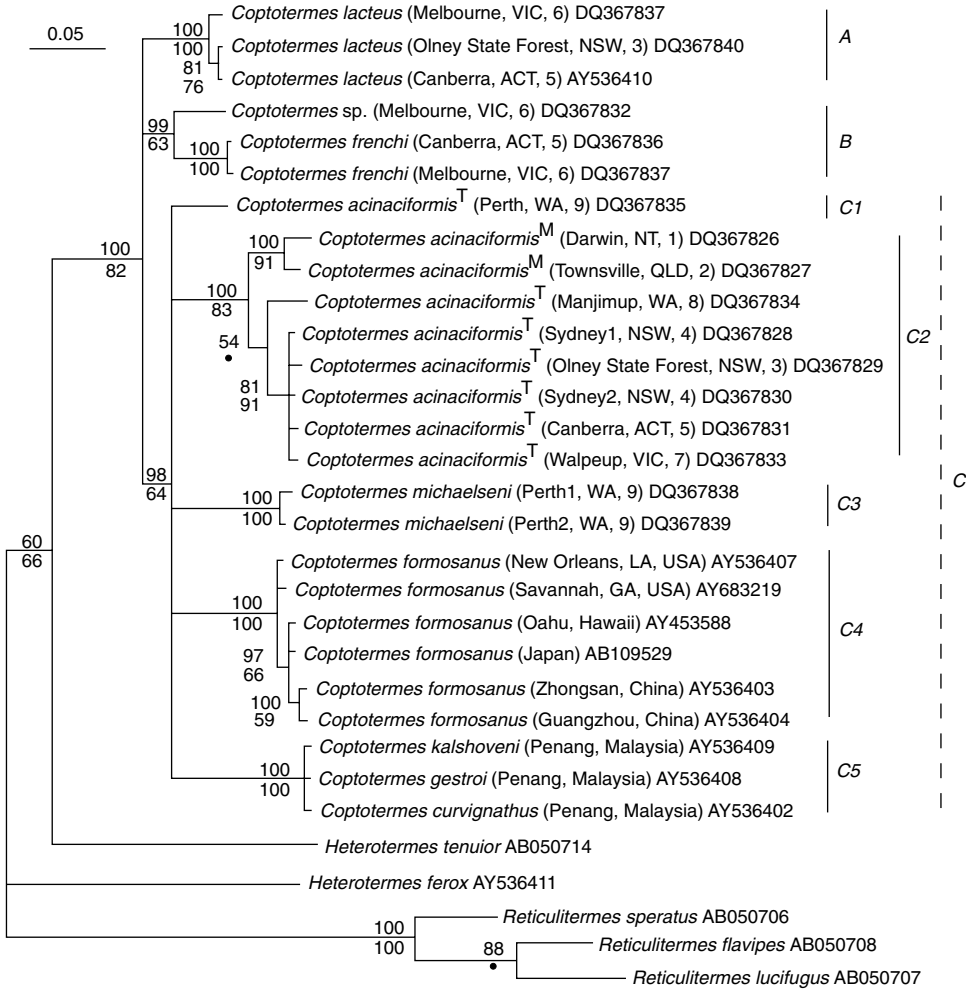

Fig. 2. Molecular phylogeny of *Coptotermes* spp. based on mitochondrial COII sequences. The topology shown was inferred using Bayesian Inference (BI). A maximum parsimony (MP) 50% majority-rule bootstrap analysis (1000 replicates) produced a tree that was identical to that shown, with the exception of two nodes with less than 50% MP bootstrap support that were collapsed to form polytomies (indicated by a bullet, also see text). Values above branches are posterior probability values from BI, while those below branches are bootstrap percentages from MP. The scale bar indicates substitutions/site. The letters A, B and C indicated the three basal *Coptotermes* lineages, and within C, five additional lineages/clades are indicated (C1–C5). Source of termite (including a number referring to fig. 1) and GenBank accession numbers are given next to each *Coptotermes* species name. For *C. acinaciformis* samples, both mound (M) and tree (T) nesting colonies were examined, and have been labelled as such.

(Simon *et al*., 1994). PCRs were performed in 50 µl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of forward and reverse oligonucleotide primer, 1 U of recombinant *Taq* polymerase (Takara, Japan) and 1 µl of DNA. Cycling conditions were: 1 cycle of 94°C for 1 min (initial denaturation); 30 cycles of 94°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1–2 min (extension); 1 cycle of 72°C for 2 min (final extension). For all PCRs, *Taq* polymerase was added following the initial denaturation step. Following purification by spin column chromatography (S-300HR, Pharmacia), both strands of each COII PCR product were determined by direct sequencing using ABI Prism BigDye terminator cycle sequencing kits (Perkin-Elmer). Sequencing reactions were analysed using an ABI 377 or 310 sequencing system (Perkin-Elmer). GenBank Accession numbers are shown next to each taxon in fig. 2.

Phylogenetic analyses

Coptotermes COII sequences were conceptually translated to protein sequences, and combined with other *Coptotermes* sequences available in GenBank. Previous studies (Lo *et al*., 2004; Ohkuma *et al*., 2004) indicated that the closest related genus to *Coptotermes* is *Heterotermes*, while the next most closely related genus is *Reticulitermes*. Sequences from these genera were thus obtained from GenBank, and alignment was performed in Clustal X. This protein alignment, which was not found to contain any gaps, was used to perform a manual alignment of nucleotide sequences (available on request). Trees were inferred using Bayesian inference (BI) and maximum parsimony (MP) bootstrap analysis using the programs MrBayes 3.0 (Huelsenbeck & Ronquist, 2001) and PAUP4.0b10 (Swofford, 2000) respectively. To verify that each of the datasets contained significantly more

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phylogenetic structure than random data, we measured the skew (g_1) in the distributions of tree lengths for each gene, based on 1000 random generated trees ('generate trees option' in PAUP*4.0b8) (Hillis & Huelsenbeck, 1992; Swofford, 2000). The significance of g_1-values was assessed using the critical values for four-state character data listed previously (Hillis & Huelsenbeck, 1992). To check for potential variations in base composition among the sequences in each dataset, the chi-squared test for stationarity in Tree-Puzzle 5.0 (Strimmer & Haeseler, 1996) was used. For BI, parameters for the selected model of substitution were estimated from the data using MrModeltest (Nylander, 2002). A total of 15,000 trees were obtained (ngen = 1,500,000, samplefreq = 100), and the first 5000 of these were considered as the 'burn in' and discarded. A 50% majority-rule consensus tree of the remaining 10,000 trees, including branch lengths (savebrlens = yes) was produced. Three different replicates of these analyses were performed. For MP, 50% majority-rule bootstrap trees were produced using the heuristic search option (1000 replicates, 10 random addition replicates per bootstrap replicate). Analyses were performed with third position codon transitions down-weighted by a factor of 4 with respect to all other substitutions (Simon *et al*., 1994). *Coptotermes* taxa were not constrained to be monophyletic in any analysis. Trees were rooted using sequences from *Reticulitermes* spp.

Results and Discussion

A 677 bp fragment of the mitochondrial COII gene in several Australian *Coptotermes* taxa was sequenced, and analysed along with other sequences available in GenBank for this genus and closely related genera. No gaps were found in the COII alignment, and significant phylogenetic signal was found: skew values were below the critical values for significance at the $P < 0.01$ level (data not shown) (Hillis & Huelsenbeck, 1992). The COII sequences did not significantly differ in base composition, based on the test performed in TreePuzzle 5.0. All COII electrophoretograms were unambiguous, and the sequences contained uninterrupted open reading frames, suggesting they were not nuclear pseudogenes (which are expected to accumulate nonsense mutations over time) (Bensasson *et al*., 2001).

Figure 2 shows the topology for *Coptotermes* taxa obtained using Bayesian inference, with support values (posterior probabilities; PP) shown above branches. The topology inferred from MP analysis was almost identical to that shown (bootstrap values (BV) are shown below branches). The only difference was the lack of >50% MP bootstrap support for the clade containing *C. acinaciformis* from Manjimup and other southern locations. The topologies from three separate BI runs were identical, with only minor differences in PP values.

Coptotermes taxa were found to a form monophyletic group in BI and MP analyses with high support (100% PP, 82% BV). At the base of the *Coptotermes* clade, three lineages (labelled A, B, C) were recovered with high support from BI and varying levels of support from MP. Clade A (100% PP and BV) consisted of each of the three *C. lacteus* representatives examined (Olney, Canberra, Melbourne). Clade B (99% PP, 63% BV) consisted of two *C. frenchi* taxa plus one *Coptotermes* sp. from Melbourne. The larger clade C (98% PP, 64% BV) comprised *C. acinaciformis*, *C. michaelseni*, plus the Asian taxa *C. formosanus*, *C. gestroi*, *C. kalshoveni* and *C. curvignathus*. Notably, clade C revealed that some Australian taxa are more closely related to Asian taxa than to other Australian taxa. Mound-building Australian termites (*C. lacteus* and northern Australian *C. acinaciformis*) were found to be unrelated to each other, suggesting that the mound building phenomenon has evolved multiple times in the genus.

Within clade C, a polytomy consisting of five lineages was recovered (C1–C5). Clade C2 contained almost all of the *C. acinaciformis* examined. The two northern mound-building *C. acinaciformis* from Townsville and Darwin were found to be closely related, and divergent from two other lineages, one of which was represented by *C. acinaciformis* from Manjimup (southern WA), the other comprising taxa from Sydney, Olney, Canberra and Walpeup (central VIC). Interestingly, *C. acinaciformis* from Perth (C1 in fig. 2) was found to be divergent from other representatives of this species. *Coptotermes michaelseni* (clade C3) was also found to represent a divergent lineage within clade C. As expected on the basis of previous studies (Ye *et al*., 2004), *C. formosanus* from Asia and America (clade C4) were found to be closely related. Finally, a clade (C5) containing the species *C. kalshoveni*, *C. gestroi* and *C. curvignathus* was recovered (Ye *et al*., 2004). Since these species contain identical sequences, it is unlikely that they each represent different species. Alternatively, they may have been misidentified. Resolution within clade C was not high enough to enable us to infer whether the Asian clades (C4, C5) are more closely related to each other than they are to the Australian lineages (C1, C2, C3).

The relationships inferred from COII sequences are largely in agreement with results previously obtained using cuticular hydrocarbons, and, in many cases, geographically diverse representatives from a species clustered together. For example, a number of *C. acinaciformis* from areas separated by over 1000 km (Olney, Sydney, Canberra, Walpeup) were found to be highly genetically similar, in agreement with cuticular hydrocarbon studies of *C. acinaciformis* material from similar areas. The finding of two relatively divergent *C. acinaciformis* taxa (from WA) is similar to the finding of diverse hydrocarbon profiles in some taxa of this species from various locations (Brown *et al*., 2004). Although not directly comparable due to the fact that samples were collected in different areas, both hydrocarbon and molecular studies suggest that *C. acinaciformis* is genetically diverse, and may contain several species. Mound-building *C. lacteus* from the three geographically diverse sites (Olney, Canberra, Melbourne) were found to be closely related on the basis of COII, also in agreement with cuticular hydrocarbon studies of taxa in these areas. Unfortunately we were not able to include samples of the tree-nesting form of this species to test the possibility that it is genetically divergent from the mound-building forms. The two *C. frenchi* samples examined, from Canberra and Melbourne, were closely related, as were the two *C. michaelseni* samples from Perth. One exception to the pattern of the COII data being in agreement with cuticular hydrocarbon data concerns *C. lacteus* and *C. michaelseni*. These two species were found to have highly similar hydrocarbon profiles (Brown *et al*., 1994), however they were not closely related on the basis of COII.

Future molecular-based studies of Australian *Coptotermes* would ideally include all those taxa suggested by cuticular hydrocarbon profiles to be genetically distinct (Brown *et al*., 2004), as well as material from regions that have not yet been

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sampled. Based on the results thus far obtained, it is likely that further sampling will reveal several genetically distinct lineages. In order to further understand relationships among various lineages, additional genes need to be sequenced, including some nuclear loci. Of particular interest is the grouping of some Australian taxa (such as *C. acinaciformis*) with Asian taxa, rather than with other Australian taxa such as *C. lacteus* and *C. frenchi*.

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