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TAXONOMIC QUESTIONS ON MALAYSIAN TERMITES (ISOPTERA: TERMITIDAE) ANSWERED WITH MORPHOLOGY AND DNA BIOTECHNOLOGY

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Abstract Accurate species identification underlies all termite field studies and population genetic research. With more than 170 species of termites in Peninsular Malaysia, it has been challenging to elucidate proper taxonomic records among the species studied, particularly within the family Termitidae. This lack of taxonomic clarity hampers many studies on termites in this area of the world. We initiated a multidisciplinary research project designed to study the taxonomy of species within the family Termitidae. Individual termites were first identified to species and or genus by morphometric characters. Taxonomic fidelity from morphometric characters was then determined by distance matrix and character-state phylogenetic analyses of COII sequence. We discuss this work in light of its implications for termite systematics and population genetics.
Key Words Termite phylogeny, COII, Termitidae, Macrotermitinae, Termitinae, Nasutitermitinae

INTRODUCTION

Termites of the order Isoptera are classified into seven families, Termitidae being the largest family, 14 subfamilies, 280 genera and over 2600 species (Krishna, 1970; Pearce and Waite, 1994; Kambhampati and Eggleton, 2000; Eggleton, 2001, Ohkuma et al., 2004). These insects are serious economic threats to agriculture and urban structures in most subtropical and tropical countries including Malaysia (Lee et al., 1999; Lee et al., 2003; Su and Scheffrahn, 2000; Chirasak et al., 2002). In Malaysia alone, it is estimated that there could be 180 species representing 48 genera (Tho, 1992). The cost of termite control in Malaysia accounted for 50% of the control industry's business, which in 2000 amounted to US $10 million (Lee, 2002). Although termites are important economic pests in Malaysia, there has been little in depth study of Malaysian termite biology and taxonomy especially those in the urban environment (Lee, 2002).

Phylogenetic studies using morphometric (Donovan et al., 2000; Noirot, 1995) and molecular (Aanen et al., 2002; Kambhampati and Eggleton, 2000; Kambhampati et al., 1996; Lo et al., 2000; Miura et al., 1998; Thompson et al., 2000) characters analyzed under phenetic and cladistic (Eggleton; 2001; Sazlanski et al., 2004) assumptions have recently made inroads in our knowledge of termite taxonomy generally. But there has not been a study to date, dedicated to understanding the taxonomy of Malaysian termites specifically.

We report on an initial multidisciplinary collaborative study the purpose of which is to use field observations, morphometric characters and mitochondrial DNA (mtDNA) cytochrome oxidase II sequence characters to determine the taxonomy of Malaysian termites in the family Termitidae (Isoptera). To this end termites were collected in Malaysia that were morphologically identified to three presumptive subfamilies, Macrotermitinae, Termitinae, and Nasutitermitinae. Morphological identifications were then verified, identified or confirmed by phylogenetic analyses of COII gene sequence.

MATERIALS AND METHODS

Termites. Termites were collected from Penang National Park and Universiti Sains Malaysia campus located in Penang Island, Malaysia (Table 1) and preserved in 95-100% ethanol. Collections were initially identified to species using morphometric characters (Tho, 1992; Thapa, 1981) (Table 2) and then verified using individual-specific mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) gene sequence.

Table 1. Termite sample data

Subfamily	Species[1, 2]	GenBank #	Source
Macrotermitinae			
	Macrotermes carbonarius	AB109525	Ohkuma et al. (2004)
	Macrotermes carbonarius (M1)	AY940131	C. Y Lee
	Macrotermes malaccensis	AB109528	Ohkuma et al. (2004)
	Macrotermes malaccensis (M2)	AY940132	C. Y. Lee
	Macrotermes malaccensis (M5)	AY940134	C. Y. Lee
	Macrotermes malaccensis (M6)	AY940135	C. Y. Lee
	Macrotermes gilvus	AB109256	Ohkuma et al. (2004)
	Macrotermes gilvus (M3)	AY940133	C. Y. Lee
	Microtermes obesi	AB109523	Ohkuma et al. (2004)
	Microtermes pakistanicus (M17)	AY940138	C. Y. Lee
	Microtermes pakistanicus (M18)	AY940139	C. Y. Lee
Termitinae			
	Dicupiditermes nemorosus	AB011410	Miura et al. (1998)
	Dicuspiditermes nemorosus (M14)	AY940136	C. Y. Lee
	Microcerotermes crassus	AB109503	Ohkuma et al. (2004)
	Microcerotermes crassus (M16)	AY940137	C. Y. Lee
Nasutitermitinae			
	Hospitalitermes ataramensis	AB109499	Ohkuma et al. (2004)
	Hospitalitermes bicolor (M31)	AY940142	C. Y. Lee
	Nasutitermes perparvus	AB109490	Ohkuma et al. (2004)
	Nasutitermes parvonasutus	AB37337	Miura et al. (2004)
	Nasutitermes spp A (M29)	AY940140	C. Y. Lee
	Nasutitermes walkeri	AB037332	Miura et al. (2004)
	Nasutitermes spp (M30)	AY940141	C. Y. Lee

[1]Species identification numbers for samples collected from Penang Island, Malaysia are in parenthesis (M1). All other samples were downloaded from GenBank.
[2]*Zootermopsis angusticollis*, GenBank # M839681 (Liu and Beckenbach 1992), was the outgroup taxon with which trees were rooted (Figures 1 and 2).

DNA Extraction. Individual worker termites were dissected to provide either the head segment or the joined head and thorax for molecular analyses. DNA was then extracted from an individual termite using the E.Z.N.A. Mollusc DNA kit (Omega Bio-Tek, Inc., Doraville, GA)

Amplification and Sequencing. The polymerase chain reaction (PCR) was used to amplify a ~ 800-bp fragment of the COII gene from each termite.

Primers TL2J3037 (5'-ATGGCAGATTAGTGCAATGG-3') and TKN3785 (5'TTTAA GAGACCAGTACTTG-3') (Liu and Beckenbach, 1992; Simon et al., 1994; Jenkins et al., 1999) were used to amplify the fragment and prime 36 forward and reverse sequencing reactions.

Table 2. Morphological measurements for each termite collection[1].

I.D.#	Species	Measurements (mm)[2,3]						Distinct Characters[4]
		HW	HL	WB	AS	LN	LM	
M1	*Macrotermes carbonarius* Hagen	3.945 (Ms)	4.195 (Ms)	2.010 (Ms)	-	-	-	a
		2.780 (ms)	3.010 (ms)	1.280 (ms)	-	-	-	
M2	*Macrotermes malaccensis* (Haviland)	3.480 (Ms)	3.780 (Ms)	1.740 (Ms)	-	-	-	b
M3	*Macrotermes gilvus* Hagen	1.655 (ms)	1.900 (ms)	0.805 (ms)	-	-	-	c
M5	*Macrotermes malaccensis* (Haviland)	3.240 (Ms)	3.200 (Ms)	1.610 (Ms)	-	-	-	b
		1.970 (ms)	2.350 (ms)	0.870 (ms)	-	-	-	
M6	*Macrotermes malaccensis* (Haviland)	3.900 (Ms)	4.020 (Ms)	1.800 (Ms)	-	-	-	b
M14	*Dicuspiditermes nemorosus* (Haviland)	1.174	2.138	0.901	14	-	-	l
M16	*Microcerotermes crassus* Snyder	0.911	1.529	0.456	13	-	-	m
M17	*Microtermes pakistanicus* Ahmad	0.804	0.835	0.495	-	-	-	n
M18	*Microtermes pakistanicus* Ahmad	0.804	0.835	0.495	-	-	-	n
M29	*Nasutitermes* sp. *A*	-	-	-	13	1.220	1.921	s
M30	*Nasutitermes* sp.	-	-	-	13	0.869	1.331	t
M31	*Hospitalitermes bicolor* (Haviland)	-	-	-	14	1.051	1.666	u

[1] Morphological measurements based on Tho (1992).
[2] (Ms) = Major soldier; (ms) = Minor soldier
[3] Uppercase dual letters represent: HW = head width; HL = head length; WB = width at base of mandibles; AS = antennae segments; LN = length of head to tip of nasus; LM = length of head to base of mandibles.
[4] Distinct characters represented by the lower case letters are: a = capsule length > 4.2 mm (major); > 3.0 mm (minor); b = capsule length > 4.5 mm (major); > 2.5 mm (minor); c = capsule length < 4.0 mm (major); < 2.5 mm (minor); l = antero-lateral corners of head extended as tubercle-like projections; antennae with 14 segments; m = head rectangular and parallel-sized, labrum wider than long, lateral margin parallet, mandibles long and finely serrated, two thirds the length of the head; n = inner margins of mandibles entire, postmentum broad, large and arched, subglobose head and bearsscattered isolated setae; s = round head which is large in relation to the body, orange head with a ferruginous orange nasus, and antennae have 12 or 13 segments; t = head round, not very produced behind and not constricted behind the antenna carinae, nasus directed forwards and not held at an angle to the head; u = head and abdominal tergites which the lighter coloured thoracic nota and abdominal sternites.

PCR was performed in a standard 25-µl reaction with 10-20 ng of total genomic DNA, 1 pmol of each primer, 2.0 mM $MgCl_2$, 1X buffer, 0.16 mM dNTP, 1X BSA, and 0.05 U/µl *Taq* DNA polymerase. Amplification was accomplished in a Perkin-Elmer Gene Amp PCR system 9600 or 9700 (Applied Biosystems, Foster City, CA). The procedure included a pre-cycle denaturation at 94º C for 2 min., a post-cycle extension at 70º C for 5 min., and 35 cycles of a standard three-step PCR (94º C for 1 min, 50º C for 1 min., and 70º C for 2 min.). Fragments were treated with exonuclease I (10U/µl) and shrimp alkaline phosphatase (1 U/µl) according to Jenkins et al. (2001) to remove single stranded DNA (ssDNA) fragments and cleave phosphate groups respectively. All PCR products were further purified according to protocol with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Samples of the amplified DNA fragment from individual termites were then sent to the Sequencing and Synthesis Facility (SSF) at Integrated Biotechnology Laboratories (Athens, GA) for direct sequencing in both directions.

Data Analyses. Individual electropherograms were edited and contigs formed using Sequencer 3.1.1 software (Gene Codes Corp., Ann Arbor, MI). Sequences that were exactly alike were compiled into a consensus sequence prior to alignment. Phylogenetic assumptions were tested according to Avise (1994) (Figures. 1 and 2). CLUSTALW (v. 1.7) (http://bioportal.bic.nus.edu.sg) (Thompson et al., 1994) was used to align 724 COII nucleotides for PHYLIP and NEXUS formats. Aligned sequences were then imported into PHYLIP (v. 3.6) (Felsenstein, 1993). A bootstrapped (1000 pseudoreplicates) neighbor-joining (Saitou and Nei, 1987) (Figure 1) analysis was generated. Genetic distances were calculated with DNAdist (PHYLIP v. 3.6) according to the Kimura 2-parameter model (Kimura, 1980) with a transition/ transversion ratio of 2. An unweighted parsimony analysis with gaps treated as missing data was also done in PAUP* (v. 4.0b4a) by David Swafford (distributed by Sinauer Associates, Inc., Fitchburg, MA) using the heuristic search option (Figure 2). Rooted trees were generated in TREEVIEW (Page, 1996). *Zootermopsis angusticollis*, GenBank M839681, (Liu and Beckenbach 1992) was used as the outgroup taxon with which to root trees (Figure 1 and 2). Assessment of node support was accomplished by 1000 nonparametric bootstrap pseudoreplicates (Hillis et al., 1996) for neighbor-joining (Figure. 1) and parsimony analyses respectively. Bootstrap node support 70% is referred to as strong (Hillis and Bull, 1993) (Figures. 1 and 2). All GenBank accession numbers are in Table 1.

RESULTS AND DISCUSSION

Phylogenetic Analyses

Distance matrix and character-state phylogenetic analyses of 724-bases of COII gene fragment sequence were used to assess taxonomic relationships among presumptive Termitidae subfamilies. The PAUP* heuristic parsimony algorithm (Swofford, 1999) determined that, of the total characters, 441 were constant, 65 variable but parsimony-uninformative, and 218 parsimony-informative. Phenogram (Figure 1) and cladogram (Figure 2) tree topologies confirmed the subfamily-specific species status of our dataset. These tree topologies supported the hypothesis of linearity between genetic divergence and ancestor lineage (Graur and Li, 1999). Node support, assessed by bootstrapping (Felsenstein, 1985), was strong for all terminal branches in both trees. Terminal branches leading to a single species are found in every tree and always get full bootstrap support (J. Felsenstein, personal communication), as was observed in our analyses. The terminal nodes of 100% are, therefore, not significant (J. Felsenstein, personal communication). The significance is in the terminal branch species verification, identification or confirmation.

This study was able to verify with mtDNA sequence the species status (Table 1, Figures. 1 and 2) of seven collections identified to species with morphological characters, M1, M3, M14, M16, M17, M18, M31, (Table 2). Two collections that could not be unequivocally classified to species by morphology alone, M5 and M6, were identified by their DNA sequence (Figures 1 and 2). Two additional collections could not be identified to species, M29 and M30, by either morphological or DNA characters. DNA data, however, did confirm that the two were different species in the same genus (Figures 1 and 2). Thus, this study demonstrated the value of using DNA-specific data for verification, identification and confirmation of termite taxonomy, which traditional nonmolecular observations could not accomplish alone (Avise, 1994).

The tree topologies (Figures 1 and 2), even though generated by two separate assumptions, produced similar results. This indicated that the relationship between those assumptions is linear. Thus, by using two methods of phylogenetic analyses we not only determine the relationship between extant termite species based on overall similarities, but gained insight into the branching relationships among individuals.

Morphological and DNA Characters

Morphological and molecular character analyses of the dataset partitioned termites into three Termitidae subfamilies, Macrotermitinae, Termitinae, and Nasutitermitinae. The Nasutitermitinae included only the non-mandible nasute species (Nna) (Ohkuma et al., 2004). The Termitinae included termites from the *Amitermes*-group (Ami), soldiers with biting or slashing mandibles (Ohkuma et al., 2004), and termites from the *Termites*-group (Ter), soldiers with snapping mandibles (Ohkuma et al, 2004). Four species showed full bootstrap support for their terminal branches, *Microtermes gilvus, Macrotermes malaccensis, Dicupiditermes nemorosus,* and *Microcerotermes crassus.*

M2 was identified from morphological characters (Table 2) as *Macrotermes malaccensis.* M5 and M6 had distinct morphological characters in common with M2 (Table 2) while other morphological characters were more phenotypically polymorphic. M6 major soldier (Ms) head width (HW) was larger than either M2 or M5 (Table 2). M2 major soldier head width was larger than M5 head width. Head length (HL) was also largest in

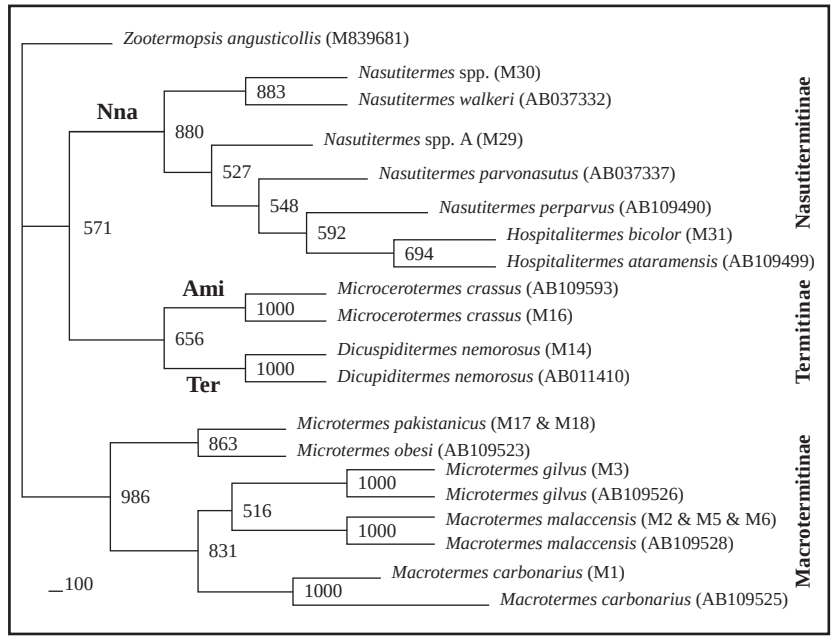

Figure 1. Neighbor-joining phenogram generated with PHYLIP 3.6. Node support resulted from 1000 bootstrap or nonparametric pseudoreplicates. Termitidae subfamilies, Macrotermitinae, Termitinae and Nasutitermitinae, identified in bold. Non-mandibulate nasutes, soldiers with biting or slashing mandibles (Amitermes group), and soldiers with snapping mandibles (Termes group), identified in bold as Nna, Ami, and Ter, respectively. Collections from Penang Island, Malaysia are identified in parenthesis after the species name. GenBank number in parenthesis after the species name indicates the sequence was downloaded from GenBank.

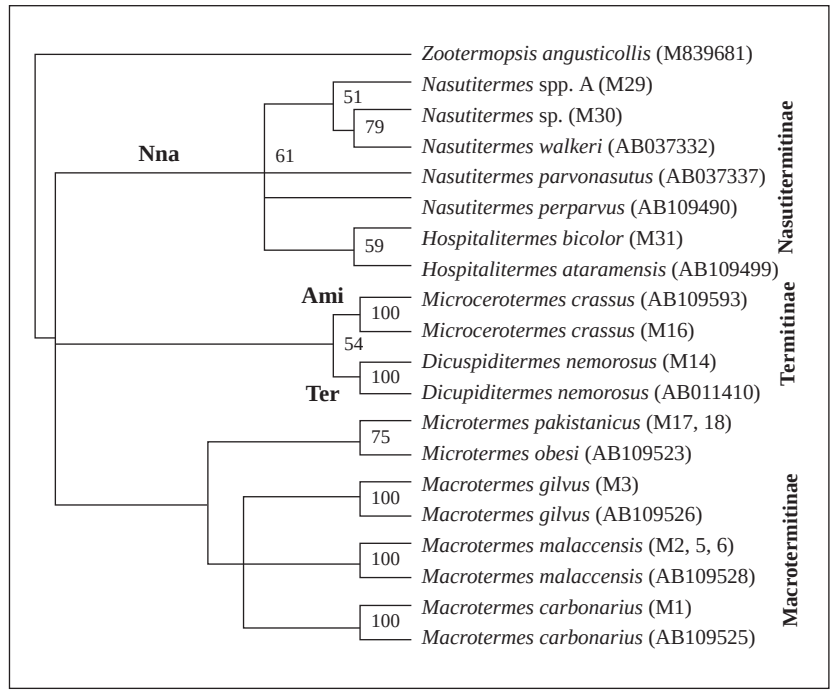

Figure 2. Parsimony cladogram generated by PAUP*4.0b10 analysis. Node support resulted from 1000 nonparametric pseudoreplicates. Termitidae subfamilies, Macrotermitinae, Termitinae and Nasutitermitinae, identified in bold. Non-mandibulate nasutes, soldiers with biting or slashing mandibles (Amitermes group), and soldiers with snapping mandibles (Termes group), identified in bold as Nna, Ami, and Ter, respectively. Collections from Penang Island, Malaysia are identified in parenthesis after the species name. GenBank number in parenthesis after the species name indicates the sequence was downloaded from GenBank.

M6 (4.020 mm) with M2 head length larger than M5 head length (Table 2). Width at the base of the mandibles (WB) was wider in M6 than either M2 or M5. Yet all three collections formed a single 770-bp COII contig of consensus sequence (M2 & M5 & M6). Phenetic (Figure 1) and cladistic (Figure 2) phylogenetic analyses of 724 nucleotides of this consensus sequence resulted in a terminal *Macrotermes malaccensis* species branch. Based, therefore, on common distinct characters (Table 2), DNA consensus sequence, and phylogenetic analyses (Figures 1 and 2), M5 and M6 were classified as *Macrotermes malaccensis*.

M1, M3, M14, M16, M17, M18, and M31 were identified by morphological characters as *Macrotemes carbonarius*, *Microtermes gilvus*, *Dicuspiditermes nemorosus*, *Microcerotermes crassus*, *Microtermes pakistanicus*, *Microtermes pakistanicus*, and *Hospittalitermes bicolor* respectively (Table 1). COII sequence confirmed the morphological identification through both phenetic (Figure 1) and cladistic (Figure. 2) phylogenetic analyses. M17 and M18 had identical sequence and, therefore, formed a consensus *Microtermes pakistanicus* sequence (M17 & M18) and a terminal species branch (Figures 1 and 2). *Macrotermes carbonarius, M crassus*, and *D. nemorosus* also formed terminal species branches with species-specific GenBank sequence (Miura et al., 1998; Ohkuma et al., 2004).

M29 and M30 were not identified to species from morphological characters. Although antennae segments (AS) were the same for M29 and M30, other morphological characters, including distinct characters (Table 2), were different for each sample. Phylogeny analyses of 724 DNA nucleotides revealed a strongly significant neighbor-joining node (88%) (Figure 1) and a meaningful parsimony node (51%) (Figure 2) (J. Felsenstein, personal communication) separating the two. The morphological and molecular characters, therefore, indicated that these two samples were not the same species, nor were they members of any species group in this study. Morphological phenotypic characters and the DNA database of species-specific COII sequence were insufficient to identify these two samples to species.

Gene Flow Observations

Collections M2, M5 and M6 and collections M17 and M18 each formed a consensus species sequence of *M. malaccensis* and *M pakistanicus* respectively (Table 1). Based on the close proximity of the field collection sites, a more thorough population genetics study using nuclear DNA markers as well as mtDNA markers would likely provid*e* insight into species-specific gene flow and population structure.

CONCLUSION

This study demonstrated the value of a collaborative, multidisciplinary approach (Forschler and Jenkins, 2000) to studying the termite taxonomy of Malaysia. The field collections and observations coupled with the morphological and DNA gene sequence character analyses provided insights into termite biology and taxonomy. We plan to continue our collaborative, multidisciplinary study of Malaysian termite taxonomy as we add more morphometric, population genetics and eventually behavior datasets to this initial project.

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