Genetic Relationship Between *Coptotermes heimi* and *Coptotermes gestroi* (Isoptera: Rhinotermitidae)

by

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ABSTRACT

Coptotermes heimi (Wasmann) is a destructive subterranean termite species commonly found on the Indian subcontinent. Using partial sequences of the mitochondrial genes rRNA small subunit 12S, rRNA large subunit 16S and cytochrome oxidase subunit 2 (COII), together with morphometric measurements, we determined the relationship between *C. heimi* and *Coptotermes gestroi* (Wasmann). Gene sequences obtained from 13 populations of *C. heimi* from Pakistan and one population of *C. gestroi* from Malaysia, along with other sequences of *C. heimi*, *C. gestroi*, *Coptotermes formosanus* Shiraki and other *Coptotermes* spp. from GenBank, were used in the analysis. Seventeen morphometric measurements of *C. gestroi* and *C. heimi* revealed numerous overlaps. The morphological analysis using Principle Component Analysis (PCA) and Discriminant Function Analysis (DFA) failed to differentiate *C. heimi* from *C. gestroi*. Individuals from both species were clustered together in the scatter plots. Phylogenetic analysis of 12S, 16S, and COII gene sequences suggested that both species are likely conspecific. Based on the molecular phylogenetics and morphometric data, we propose that *C. heimi* is a junior synonym of *C. gestroi*.

Key Words: *Coptotermes heimi*, *Coptotermes gestroi*, mitochondrial 12S rRNA, 16S rRNA, COII rRNA, synonymity

INTRODUCTION

Coptotermes heimi is a destructive species of subterranean termite limitedly distributed in Pakistan and India (Roonwal 1959, Roonwal & Chhotani 1962, Gay 1967, Chaudhary & Ahmad 1972) to Bhutan, and has become

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established in the arid Arabian peninsula (Roonwal & Chhotani 1989). It was originally described as *Arrhinotermes heimi* (Wasmann 1902), but was later renamed *C. heimi* by Holmgren (1911). Another species, *Coptotermes parvulus* (Holmgren 1913), was found to be a junior synonym of *C. heimi* (Snyder 1949). *Coptotermes heimi* often attacks both dead and living trees and shrubs (Gay 1969). It has been reported to damage living shisham (Indian rosewood), morus (mulberry), and poplar trees in Pakistan. In India, *C. heimi* is a common polyphagous species that attacks over 35 different species of plants (Roonwal 1970). Besides being a serious pest of forest trees, this species also infests buildings and wooden structures, especially in Sind and the Northwest Frontier Province of Pakistan (Akhtar 1983).

Coptotermes gestroi (Wasmann 1896) is a highly destructive subterranean termite species (Lee 2007, Yeap *et al*. 2007). The distribution of *C. gestroi* extends from Southeast Asia (Assam of India, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Singapore, and Indonesian Archipelago) through the Philippines, Taiwan, and Hawaii, to the New World, including Florida, the West Indies, Mexico, and Turks and Caicos Islands in the Caribbean (Light 1929, Wasmann 1986, Scheffrahn *et al*. 1990, Su *et al*. 1997, Scheffrahn & Su 2000, Kirton & Brown 2003, Yeap *et al*. 2007, Li *et al*. 2009). It is the primary subterranean termite species in urban areas in Southeast Asia, and it causes serious damage to timber and wooden structures (Yudin 2002, Acda 2004, Kirton 2005, Yeap *et al*. 2009). On the Indian subcontinent, *C. gestroi* has only been reported in Northern India (Assam); it never has been recorded in Pakistan.

Both *C. heimi* and *C. gestroi* are very similar based on morphological characteristics (Roonwal & Chhotani 1962). Kirton & Brown (2003) suggested that *C. heimi* might be a junior synonym of *C. gestroi*. They stated that inconsistencies in the pest status of different species of *Coptotermes* in different geographical regions likely were due to persistent misidentifications in the taxonomic literature, and the use of different names for the same species likely was the result of unrecognized synonymies. The recognition of a single pest species of *Coptotermes* originating from Southeast Asia will enable the reorganization and pooling of scientific information from different countries, which in turn will facilitate the development of effective management strategies for the common pest species (Kirton & Brown 2003, Kirton 2005, Yeap *et al*. 2007).

Molecular phylogenetic analyses using mitochondrial genes can reveal the relationship among populations and can differentiate species (Miura *et al*. 2000, Jenkins *et al*. 2007, Li *et al*. 2009, Yeap *et al*. 2009). Using the combination of molecular phylogenetic analyses and analysis of morphological characteristics, recent studies have revealed synonymy among several termite species, such as *Reticulitermes flavipes* (Kollar) and *Reticulitermes santonensis* (Feytaud) (Austin *et al*. 2005); *Nasutitermes corniger* (Motsuchulsky) and *Nasutitermes costalis* (Holmgren) (Scheffrahn *et al*. 2005); *C. gestroi* and *Coptotermes vastator* Light (Yeap *et al*. 2007); and *Coptotermes formosanus* Shiraki, *Coptotermes dimorphus* (Xia & He), and *Coptotermes cochlearus* (Xia & He) (Yeap *et al*. 2009). In this study, we investigated the genetic relationship between *C. heimi* and *C. gestroi* using morphometrics and three mitochondrial genes (12S, 16S, and COII). We concluded that *C. heimi* is a junior synonym of *C. gestroi*.

MATERIALS AND METHODS

Termite samples

Thirteen populations of *C. heimi* and one population of *C. gestroi* were collected from Pakistan and Malaysia, respectively (Table 1). Samples were preserved in 70% ethanol for morphological studies and in 100% for molecular studies. Other data on *C. heimi*, *C. gestroi*, *C. formosanus*, *Globitermes sulphureus* (Haviland) and *Reticulitermes flaviceps* (Oshima) and other *Coptotermes* spp. from the GenBank, were also used for phylogenetic studies (see Table 1).

Morphological measurements

Twenty soldiers from 13 populations of *C. heimi* were measured using a stereomicroscope (model SZ2-LGB, Olympus, Tokyo, Japan) connected to a computer-assisted imaging camera with 0.01 mm precision. The following seventeen morphological characteristics were measured: total length, length without head, length of head at base of mandibles, head (length to fontanelle), maximum width of head, width of head at base of mandibles, segment I of antennae (length), segment I of antennae (width), segment II of antennae (length), segment II of antennae (width), labrum length, maximum width of labrum, minimum gula width, maximum gula width, gula length, pronotum length, and pronotum width. These data along with earlier morphological

Table 1: Information on termite samples used in this study.

Sample code	Species	Collection sites	GenBank accession no.		
			12S	16S	COII
Samples from this study					
CH001PK	*C. heimi*	Pakistan, Lahore, Cheecha Watni	GQ859246	GQ859252	GU931692
CH002PK	*C. heimi*	Pakistan, Lahore, Model Town	GQ859247	GQ859253	GU931693
CH003PK	*C. heimi*	Pakistan, Lahore, Johar Town	GQ859248	GQ859254	GU931694
CH004PK	*C. heimi*	Pakistan, Lahore, DHA	GQ859249	GQ859255	GU931695
CH005PK	*C. heimi*	Pakistan, Lahore, Flate	GQ859250	GQ859256	GU931696
CH006PK	*C. heimi*	Pakistan, Lahore, LCWU	GQ859251	GQ859257	GU931697
CH007PK	*C. heimi*	Pakistan, Jhang	GU812408	GU812415	GU812423
CH008PK	*C. heimi*	Pakistan, Jhang	GU812409	GU812416	GU812424
CH009PK	*C. heimi*	Pakistan, Jhang	GU812410	GU812417	GU812425
CH010PK	*C. heimi*	Pakistan, Kasur	GU812411	GU812418	GU812426
CH011PK	*C. heimi*	Pakistan, Kasur	GU812412	GU812419	GU812427
CH012PK	*C. heimi*	Pakistan, Attock	GU812413	GU812420	GU812428
CH013PK	*C. heimi*	Pakistan, Lahore	GU812414	GU812421	GU812429
CG088MY	*C. gestroi*	Malaysia, Kedah	GU177845	GU177846	GU812422
Yeap *et al.* (2007)					
CG001MY	*C. gestroi*	Malaysia, Penang, USM	EF379982	EF379963	EF379945
CG004MY	*C. gestroi*	Malaysia, Kuala Lumpur, Bangsar	EF379987	EF379969	EF379951
CG005MY	*C. gestroi*	Malaysia, Johor	EF379988	EF379970	EF379952
CG001SG	*C. gestroi*	Singapore, Serenity Terrace	EF379983	EF379964	EF379946
CG002SG	*C. gestroi*	Singapore, Serangoon Avenue 3	EF379985	EF379967	EF379949
CG001TH	*C. gestroi*	Thailand, Bangkok 1	EF379977	EF379965	EF379947
CG002TH	*C. gestroi*	Thailand, Bangkok 2	EF379986	EF379968	EF379950
CG001IN	*C. gestroi*	Indonesia, Cibinong	EF379981	EF379962	EF379944
CG002IN	*C. gestroi*	Indonesia, Bogor.	EF379984	EF379966	EF379948
CV001PH	*C. gestroi*	Philippines, Laguna Philippines, Los Banos	EF379989	EF379972	EF379954
CV002PH	*C. gestroi*	Philippines, Laguna Philippines, Los Banos	EF379991	EF379973	EF379955
CV001HW	*C. gestroi*	USA, Hawaii, Oahu	EF379990	EF379971	EF379953
CF001JP	*C. formosanus*	Japan, Wakayama	EF379978	EF379959	EF379941
CF002JP	*C. formosanus*	Japan, Wakayama	EF379979	EF379960	EF379942
CF003JP	*C. formosanus*	Japan, Okayama	EF379980	EF379961	EF379943
CF001HW	*C. formosanus*	USA, Hawaii, Oahu	EF379976	EF379958	EF379940
GS001MY	*G. sulphurues*	Malaysia, Penang, USM	EF379993	EF379975	EF379957

Table 1: Information on termite samples used in this study (continued).

Sample code	Species	Collection sites	GenBank accession no.		
			12S	16S	COII
Yeap *et al.* (2009)					
CG001TW	*C. gestroi*	Taiwan, Tainan1	FJ235115	FJ376672	FJ384649
CG002TW	*C. gestroi*	Taiwan, Chang Jung Christian University	FJ235116	FJ376673	FJ384650
CF004JP	*C. formosanus*	Japan, Kagoshima A	FJ235103	FJ376661	FJ384640
CF005JP	*C. formosanus*	Japan, Kagoshima B	FJ235104	FJ376662	FJ384641
CF006JP	*C. formosanus*	Japan, Kagoshima C	FJ235105	FJ376663	FJ384642
CF007JP	*C. formosanus*	Japan, Kagoshima 1	FJ235106	FJ376664	FJ384643
CF008JP	*C. formosanus*	Japan, Kagoshima 3	FJ235107	FJ376665	FJ384644
CF009JP	*C. formosanus*	Japan, Kagoshima 5	FJ235108	FJ376666	FJ384645
CF001TW	*C. formosanus*	Taiwan, Taichung 1	FJ235096	FJ376659	FJ384647
CF002TW	*C. formosanus*	Taiwan, Taichung 2	FJ235097	FJ376660	FJ384648
CF001CN	*C. formosanus*	China, Guangzhou, Guangdong	FJ235098	FJ376668	FJ384636
CF002CN	*C. formosanus*	China, Guangzhou, Sun Yat-sen University	FJ235099	FJ376669	FJ384637
CF003CN	*C. formosanus*	China, Guangzhou, AVON Co.	FJ235100	FJ376670	FJ384638
CF004CN	*C. formosanus*	China,Guangzhou, Guandong Entomological Inst.	FJ235101	FJ376671	FJ384639
CK001MY	*C. kalshoveni*	Malaysia, Penang, USM	FJ235118	FJ376684	
CK002MY	*C. kalshoveni*	Malaysia, Penang, Pantai Keracut	FJ235119	FJ376685	FJ384652
CA001AU	*C. acinaciformis*	Australia, Darwin, NT	FJ235092	FJ376675	FJ384630
CA002AU	*C. acinaciformis*	Australia, Griffith, NSW	FJ235093	FJ376676	FJ384631
CFR001AU	*C. frenchi*	Australia, Canberra, ACT	FJ235094	FJ376677	FJ384632
CL001AU	*C. lacteus*	Australia, Canberra, ACT	FJ235095	FJ376678	FJ384633
Scheffrahn *et al.* (2004)					
	C. heimi	India		AY558908	
Sobti *et al.* (unpublished)					
	C. heimi	India	EU553816	EU553817	
	C. heimi	India	EU553818		
Li *et al.* (2008)					
TW223	*R. flaviceps*	Taiwan, Taitung County, Lanyu Township	EU627778	EU627780	EU627782

data collected for *C. gestroi* (Yeap *et al*. 2007) were subjected to analysis of variance (ANOVA), and means were separated by Tukey HSD (Table 2) using STATISTIX Version 7.0.

Next, the original values for the 17 characters were transformed to standardize the data set according to the formula: Mtrans = M x 100/SL by Schindler & Schmidt (2006), where M is the original measurement, Mtrans represents transformed measurement, while SL is the standard length (in this study, we used the total length of the soldier head). The transformed data set was subjected to Principal Components Analysis. Components are considered useful for explaining the data if the eigenvalues are greater than one (Kaiser 1960). To improve the interpretation of the data, varimax rotation was conducted on the original principal components.

Discriminant Function Analysis (DFA), a linear ordination technique, was also performed to discriminate among groups. The coefficients of the linear combination obtained through maximizing the ratio of the between- to within-groups variance define canonical vectors (Owen & Chmielewski 1985). In this study, Wilks' lambda was used in a stepwise method to calculate the percentage overlap between each group. All calculations were performed using SPSS software v18 (SPSS Inc.). Voucher specimens were kept at the Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

DNA extraction, amplification, and sequencing

Total genomic DNA of a single worker termite from each of the 13 populations of *C. heimi* and one population of *C. gestroi* was extracted. The preserved specimen was washed with distilled water and laid flat to dry on a piece of filter paper. The intact termite was frozen with liquid nitrogen and ground in a 1.5 ml tube. After grinding, the DNA was extracted using the CTB Tissue Extraction Kit (Intron, Seongnam-Si, Gyeonggi-do, Korea). Amplification of 12S, 16S, and COII mitochondrial genes was carried out using polymerase chain reaction (PCR) with the primers 12SF (5'-TACTATGTTACGACTTAT-3') and 12SR (5'- AAACTAGGATTAGATACCC-3') for 12S (Simon *et al*. 1994, Kambhampati & Smith 1995, Yeap *et al*. 2007), LRJ-13007 (5'-TTACGCTGTTATCCCTAA-3') and LRN- 13398 (5'-CGCCTGTTTATCAAAAACAT-3') for 16S (Simon *et al*. 1994, Kambhampati & Smith

Table 2: Morphometric data (in millimeters) of the soldier termites of *C. heimi* and *C. gestroi*. Means followed by different letters within the same column are significantly different ($P < 0.05$; Tukey HSD).

Species	n	Length	Length without head	Length of head at base of mandibles	Head, length to fontanelle	Maximum width of head	Width of head at base of mandibles	Segment I of antennae, length	Segment I of antennae, width
C. gestroi									
CG001IN*	20	4.92 (4.59-5.29) h	3.01 (2.81-3.23) f	1.36 (1.30-1.44) de	1.30 (1.26-1.35) e	1.15 (1.10-1.18) ef	0.54 (0.46-0.58) ab	0.15 (0.13-0.18) bc	0.08 (0.07-0.09) de
CG002IN*	20	4.86 (4.15-5.54) h	2.92 (2.20-3.51) ef	1.32 (1.23-1.38) cde	1.21 (1.15-1.25) b-e	1.14 (1.11-1.18) def	0.58 (0.56-0.61) c	0.16 (0.13-0.18) bc	0.08 (0.08-0.10) e
CG001MY*	20	4.13 (3.60-5.11) a-d	2.31 (2.06-2.90) a-d	1.27 (1.12-1.44) b-e	1.27 (1.17-1.47) cde	1.15 (1.05-1.50) def	0.58 (0.50-0.75) c	0.16 (0.13-0.21) bc	0.08 (0.08-0.11) de
CG004MY*	20	3.73 (3.25-4.30) a	2.03 (1.76-2.47) a	1.24 (1.12-1.33) bcd	1.19 (1.16-1.28) bcd	1.02 (0.99-1.07) b	0.52 (0.50-0.54) c	0.15 (0.13-0.17) bc	0.08 (0.08-0.08) cde
CG001TH*	20	3.83 (3.51-4.16) ab	2.07 (1.68-2.33) ab	1.21 (1.13-1.26) abc	1.16 (1.12-1.22) bc	1.07(0.95-1.16) cde	0.52 (0.48-0.58) abc	0.15 (0.14-0.16) bc	0.08 (0.07-0.09) cde
CG002TH*	20	3.94 (3.66-4.27) abc	2.19 (1.96-2.45) abc	1.22 (1.15-1.30) bcd	1.15 (1.07-1.21) b	1.11 (1.06-1.19) def	0.53 (0.47-0.60) abc	0.15 (0.13-0.18) bc	0.08 (0.07-0.09) cde
C. heimi									
CH001PK	20	4.80 (4.60-4.95) gh	2.92 (2.65-3.19) ef	1.40 (1.15-1.49) e	1.29 (1.26-1.32) de	1.17 (1.13-1.21) f	0.57 (0.47-0.61) c	0.17 (0.16-0.18) c	0.09 (0.08-0.10) e
CH002PK	20	4.16 (4.05-4.30) a-d	2.47 (2.36-2.63) bcd	1.26 (1.13-1.33) b-e	1.21 (1.19-1.23) b-e	1.07 (1.04-1.10) cd	0.55 (0.52-0.61) abc	0.17 (0.15-0.19) bc	0.08 (0.07-0.08) b-e
CH003PK	20	4.34 (4.03-4.52) c-g	2.46 (2.05-2.73) bcd	1.16 (0.99-1.36) ab	1.22 (1.17-1.25) b-e	1.09 (1.06-1.15) c-f	0.54 (0.52-0.55) abc	0.16 (0.15-0.17) bc	0.08 (0.07-0.09) cde
CH004PK	20	4.24 (3.93-4.50) b-e	2.52 (2.15-2.81) cde	1.34 (1.16-1.43) cde	1.31 (1.30-1.32) e	1.15 (1.11-1.19) ef	0.55 (0.53-0.57) abc	0.16 (0.14-0.19) bc	0.08 (0.07-0.09) cde
CH005PK	20	4.46 (3.91-5.05) d-h	2.66 (2.13-3.06) def	1.29 (1.19-1.41) cde	1.23 (1.08-1.37) b-e	1.11 (1.04-1.18) def	0.55 (0.51-0.60) abc	0.16 (0.15-0.19) bc	0.08 (0.07-0.09) cde
CH006PK	20	4.72 (4.32-5.34) fgh	2.93 (2.28-3.71) ef	1.04 (0.94-1.26) a	1.03 (0.94-1.26) a	0.92 (0.87 -1.06) a	0.47 (0.45-0.52) a	0.13 (0.12-0.14) a	0.06 (0.06-0.07) a
CH007PK	20	4.35 (4.04-4.85) c-g	2.58 (2.24-3.16) c-f	1.24 (1.09-1.33) abc	1.21 (1.12-1.31) b-e	1.08 (1.03-1.18) cde	0.57 (0.53-0.62) c	0.15 (0.13-0.18) bc	0.08 (0.07-0.09) cde
CH008PK	20	4.35 (3.93-4.67) c-g	2.65 (2.27-3.04) def	1.27 (1.18-1.34) bcd	1.27 (1.20-1.31) cde	1.12 (1.07-1.16) def	0.58 (0.54-0.61) bc	0.15 (0.15-0.16) bc	0.08 (0.07-0.08) b-e
CH009PK	20	4.28 (4.04-4.64) b-f	2.55 (2.27-2.82) cde	1.25 (1.10-1.32) bcd	1.24 (1.20-1.32) b-e	1.10 (1.05-1.17) def	0.56 (0.52-0.60) c	0.15 (0.13-0.16) ab	0.07 (0.07-0.08) abc
CH010PK	20	4.65 (4.23-5.44) e-h	2.90 (2.57-3.59) ef	1.31 (1.22-1.35) cde	1.24 (1.15-1.32) b-e	1.15 (1.10-1.17) ef	0.59 (0.55-0.63) bc	0.16 (0.14-0.18) bc	0.08 (0.06-0.08) b-e
CH0011PK	20	4.57 (4.17-5.44) d-h	2.56 (2.27-2.81) cde	1.30 (1.22-1.35) b-e	1.24 (1.16-1.27) b-e	1.12 (1.06-1.17) def	0.58 (0.52-063) c	0.15 (0.13-0.18) bc	0.07 (0.06-0.08) bcd
CH012PK	20	4.57 (4.17-5.44) d-h	2.72 (2.13-3.38) def	1.26 (1.23-1.30) b-e	1.24 (1.12-1.31) b-e	1.11 (1.04-1.15) def	0.57 (0.54-0.60) c	0.15 (0.13-0.17) bc	0.08 (0.07-0.08) b-e
CH013PK	20	4.36 (3.93-4.63) c-g	2.58 (2.27-2.81) cde	1.22 (1.02-1.41) bcd	1.16 (0.97-1.37) b	1.14 (1.06-1.19) def	0.54 (0.46-0.60) abc	0.13 (0.12-0.14) a	0.07 (0.06-0.09) ab

*Based on Yeap *et al.* (2007)

Table 2: Morphometric data (in millimeters) of the soldier termites of *C. heimi* and *C. gestroi* (continued). Means followed by different letters within the same column are significantly different ($P < 0.05$; Tukey HSD).

Species	n	Segment II of antennae, length	Segment II of antennae, width	Labrum, length	Labrum, maximum width	Gula, minimum width	Gula, maximum width	Gula, length	Pronotum, length	Pronotum, width
C. gestroi										
CG001IN*	20	0.07 (0.06-0.08) ab	0.06 (0.06-0.07) bc	0.32 (0.29-0.37) abc	0.28 (0.27-0.30) bc	0.24 (0.22-0.25) bc	0.40 (0.40-0.41) de	0.95 (0.84-1.05) cd	0.40 (0.36-0.43) e-i	0.80 (0.75-0.84) e-h
CG002IN*	20	0.06 (0.05-0.07) a	0.06 (0.06-0.07) bc	0.37 (0.30-0.43) bc	0.28 (0.26-0.30) bc	0.22 (0.20-0.26) ab	0.38 (0.35-0.42) bc	1.04 (0.87-1.15) d	0.41 (0.38-0.43) e-i	0.79 (0.75-0.82) d-g
CG001MY*	20	0.07 (0.06-0.08) ab	0.06 (0.06-0.08) bc	0.34 (0.31-0.40) abc	0.29 (0.27-0.34) bc	0.23 (0.20-0.24) ab	0.39 (0.38-0.43) cd	0.86 (0.70-1.01) bc	0.39 (0.34-0.48) d-h	0.78 (0.67-1.06) c-f
CG004MY*	20	0.07 (0.05-0.08) ab	0.06 (0.06-0.06) abc	0.34 (0.32-0.36) abc	0.26 (0.24-0.28) a	0.21 (0.21-0.22) a	0.35 (0.33-0.36) a	0.79 (0.71-0.87) ab	0.32 (0.27-0.35) a	0.66 (0.64-0.68) a
CG001TH*	20	0.07 (0.06-0.07) ab	0.06 (0.06-0.07) abc	0.33 (0.26-0.37) abc	0.26 (0.23-0.28) ab	0.21 (0.19-0.23) a	0.35 (0.34-0.36) a	0.77 (0.67-0.93) a	0.34 (0.29-0.36) abc	0.72 (0.68-0.78) abc
CG002TH*	20	0.06 (0.05-0.08) ab	0.06 (0.06-0.07) bc	0.33 (0.29-0.38) abc	0.27 (0.26-0.28) abc	0.21 (0.18-0.24) a	0.36 (0.33-0.38) ab	0.79 (0.74-0.84) ab	0.36 (0.33-0.38) a-e	0.75 (0.70-0.80) b-e
C. heimi										
CH001PK	20	0.07 (0.06-0.08) ab	0.06 (0.06-0.07) bc	0.38 (0.34-0.41) c	0.29 (0.29-0.30) c	0.24 (0.23-0.25) bcd	0.38 (0.37-0.38) bc	0.82 (0.78-0.86) ab	0.38 (0.37-0.40) c-g	0.81 (0.78-0.82) e-h
CH002PK	20	0.06 (0.05-0.08) a	0.06 (0.05-0.07) bc	0.34 0.30-0.40) abc	0.27 (0.26-0.29) abc	0.22 (0.20-0.25) ab	0.36 (0.32-0.38) ab	0.80 (0.75-0.85) ab	0.32 (0.28-0.35) ab	0.72 (0.68-0.76) abc
CH003PK	20	0.07 (0.07-0.08) ab	0.06 (0.06-0.07) bc	0.35 (0.30-0.38) abc	0.28 (0.27-0.29) abc	0.21 (0.19-0.23) a	0.36 (0.35-0.37) ab	0.81 (0.77-0.85) ab	0.35 0.30-0.39) a-d	0.73 (0.69-0.75) bcd
CH004PK	20	0.07 (0.06-0.08) ab	0.06 (0.05-0.07) abc	0.34 (0.33-0.36) abc	0.28 (0.25-0.29) abc	0.21 (0.19-0.23) a	0.39 (0.37-0.40) cd	0.80 (0.74-0.90) ab	0.35 (0.31-0.40) a-d	0.76 (0.72-0.79) b-e
CH005PK	20	0.06 (0.06-0.08) ab	0.06 (0.06-0.07) abc	0.35 (0.26-0.43) abc	0.26 (0.23-0.29) ab	0.26 (0.25-0.27) cde	0.40 (0.39-0.42) cde	0.84 (0.74-0.92) ab	0.41 (0.35-0.44) ghi	0.86 (0.79-0.93) h
CH006PK	20	0.06 (0.05-0.06) a	0.05 (0.05-0.06) a	0.34 (0.25-0.44) abc	0.27 (0.25-0.29) ab	0.28 (0.26-0.29) e	0.41 (0.39-0.44) de	0.86(0.80-0.92) abc	0.36 (0.31-0.45) b-f	0.71 (0.66-0.81) ab
CH007PK	20	0.07 (0.06-0.08) ab	0.07 (0.06-0.08) c	0.36 (0.34-0.38) abc	0.26 (0.22-0.28) ab	0.25 (0.22-0.28) cde	0.41 (0.39-0.43) de	0.84 (0.77-0.91) ab	0.43 (0.39-0.48) hi	0.85 (0.78-0.89) gh
CH008PK	20	0.06 (0.05-0.07) a	0.07 (0.06-0.07) bc	0.36 (0.32-0.39) abc	0.27 (0.24-0.30) abc	0.27 (0.24-0.28) cde	0.42 (0.39-0.43) e	0.84 (0.80-0.90) ab	0.43 (0.40-0.48) hi	0.86 (0.84-0.91) h
CH009PK	20	0.07 (0.06-0.08) ab	0.06 (0.06-0.07) bc	0.32 (0.24-0.37) ab	0.26 (0.22-0.30) ab	0.27 (0.24-0.32) de	0.42(0.38-0.45) de	0.79 (0.73-0.89) ab	0.44 (0.41-0.46) hi	0.85 (0.82-0.87) fgh
CH010PK	20	0.07 (0.06-0.08) ab	0.06 (0.05-0.07) abc	0.36 (0.28-0.42) abc	0.27 (0.25-0.29) abc	0.28 (0.26-0.29) e	0.42 (0.40-0.44) e	0.86 (0.78-0.95) ab	0.44 (0.36-0.50) hi	0.84 (0.79-0.87) fgh
CH0011PK	20	0.07 (0.06-0.08) ab	0.06 (0.05-0.07) abc	0.35 (0.29-0.39) abc	0.27 (0.24-0.28) ab	0.28 (0.25-0.32) e	0.41 (0.38-0.45) de	0.82 (0.76-0.95) ab	0.44 (0.41-0.46) i	0.85 (0.82-0.87) fgh
CH012PK	20	0.06 (0.05-0.07) a	0.07 (0.06-0.08) c	0.31 (0.30-0.34) a	0.27 (0.23-0.29) ab	0.26 (0.22-0.28) cde	0.41 (0.39-0.43) de	0.85 (0.79-0.92) ab	0.44 (0.39-0.47) hi	0.85 (0.72-0.86) gh
CH013PK	20	0.07 (0.06-0.08) b	0.06 (0.05-0.07) ab	0.33 (0.29-0.36) abc	0.27 (0.23-0.29) ab	0.27 (0.26-0.29) e	0.40 (0.39-0.42) cde	0.83 (0.74-0.90) ab	0.41 (0.35-0.45) f-i	0.74 (0.71-0.82) b-e

1995, Yeap *et al*. 2007), C2F2 (5'- ATACCTCGACGWTATTCAGA-3') and TKN3785 (5' GTTTAAGAGACCAGTACTTG-3') for COII (Simon *et al*. 1994, Hayashi *et al*. 2003, Yeap *et al*. 2007). Two microliters of extracted DNA (> 50 ng/µl) from each sample was prepared for PCR using the PTC-200, Peltier Thermal Cycle (MJ Research, Watertown, MA, USA) with a profile consisting of a precycle denaturation at 94°C for 2 min, a postcycle extension at 72°C for 10 min, and 35 cycles of a standard three-step PCR (51.3, 53.1 and 58.2°C annealing). Amplified DNA from individual termites was purified using a SpinClean Gel Extraction kit (Intron, Seongnam-Si, Gyeonggi-do, Korea). Samples were sent to Macrogen Inc. (Seoul, South Korea) for direct sequencing in both directions, which was conducted under BigDyeTM terminator cycling conditions. The reacted products were then purified using ethanol precipitation and analyzed with a DNA analyzer (Automatic Sequencer 3730xl; Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

BioEdit v7.0.5 software (Hall 1999) was used to edit individual electropherograms and form contigs. Multiple consensus sequences were aligned using CLUSTAL W (Thompson *et al*. 1994). Multiple alignment parameters for gap opening and extension penalties were 10 and 0.2, respectively. The sequences for the outgroup species, *G. sulphureus* (Haviland) and *R. flaviceps* (Oshima), were obtained from GenBank from the work of Yeap *et al*. (2007) and Li *et al*. (2009), respectively. Published sequences of *Coptotermes* spp. from GenBank (www.ncbi.nlm.nih.gov) also were included in the alignments for phylogenetic comparisons (Table 1). The distance matrix option of PAUP* 4.0b10 (Swofford 2002) was used to calculate genetic distance according to the Kimura 2-parameter model of sequence evolution (Kimura 1980). Maximum parsimony analysis was performed with TBR branch swapping and 10 random taxon addition replicates under a heuristic search, saving no more than 100 equally parsimonious trees per replicate. To estimate branch support on the recovered topology, non-parametric bootstrap values were assessed with 1000 bootstrap pseudo-replicates (Felsenstein 1985). Before the maximum likelihood (ML) analysis, we ran Modeltest 3.7 to find the optimal model of DNA substitution (Posada & Crandall 1998). Phylogenetic reconstruction for ML was based on the best-fit model, which was selected by

the Akaike information criterion (Akaike 1974), as it is more advantageous than the hierarchical likelihood ratio test (Posada & Buckley 2004). Heuristic ML searches using TBR branch swapping were performed in PAUP 4.0b10 (Swofford 2002). ML nodal support was estimated using the non-parametric bootstrap (Felsenstein 1985) and was restricted to 1000 pseudo-replicates to limit computing time.

RESULTS AND DISCUSSION

Morphological characteristics

Morphologically, it is difficult to distinguish the soldiers of *C. heimi* and *C. gestroi*. Unlike *C. formosanus* (with two pairs of setae) (Scheffrahn *et al*. 1990, Su *et al*. 1997), both species have a pair of setae projecting dorsolaterally from the base of the fontanelle; a long tongue-shaped labrum extending beyond the middle of the mandibles; a hyaline tip with two long hairs at its base; a saddle-shaped pronotum with anterior lobes; and a head narrowed anteriorly. Our measurements of the 17 morphological characteristics of *C. heimi* soldiers all fell within the range of the morphometric data of *C. gestroi* soldiers (Yeap *et al*. 2007) (Table 2).

Roonwal & Chhotani (1962) stated that *C. gestroi* is close to *C. heimi*, as the range of the head length to the mandible base of both species overlapped (1.20–1.45 mm and 1.41–1.53 mm in *C. heimi* and *C. gestroi*, respectively). The measurement reported for *C. heimi* also fell within the range of the described characteristic for *C. gestroi* in Yeap *et al*. (2007) (Table 2). These overlapping data supported Kirton & Brown's (2003) report that *C. heimi* might be a junior synonym of *C. gestroi*. Roonwal & Chhotani (1962) also stated that the dorsum of the head capsule behind the fontanelle of *C. heimi* is crooked and slightly swollen, but it is straight and not swollen in *C. gestroi*. We found this characteristic to be highly variable in *C. heimi* in the 13 populations that we examined. Roonwal & Chhotani (1962) also reported great variation in the large samples that they examined and attributed this to intraspecific variation. Table 2 showed the variations in the characters occur within both *C. gestroi* and *C. heimi* species.

PCA revealed that the first principle component (PC 1) contributed over 89.5% of the morphological variation between *C. gestroi* and *C. heimi* (Table 3). PC I with all variables loaded strongly positive, representing the overall

Table 3: Eigenvector elements and percentage of total variance of principle components of morphometic data from *C. gestroi* and *C. heimi* soldiers.

Measurement	Description	PC1	PC2	PC3
Length	Total body length	0.996	-0.087	-0.007
LWH	Length without head	0.996	-0.087	-0.007
HF	Head, length to fontanelle	0.677	0.683	0.058
HM	Length of head at base of mandibles	0.664	0.652	-0.33
WH	Maximum width of head	0.694	0.651	0.114
WHM	Width of head at base of mandibles	0.586	0.556	0.05
GMAXW	Gula, maximum width	0.797	0.327	0.273
GMINW	Gula, minimum width	0.719	0.183	0.267
GL	Gula, length	0.753	0.216	0.276
LL	Labrum, length	0.591	0.323	0.031
LW	Labrum, width	0.714	0.431	0.121
PW	Pronotum, width	0.757	0.453	0.278
PL	Pronotum, length	0.686	0.282	0.346
AIL	Segment I of antennae, length	0.546	0.536	-0.132
AIIL	Segment II of antennae, length	0.349	0.361	0.052
AIW	Segment I of antennae, width	0.476	0.602	-0.007
AIIW	Segment II of antennae, width	0.523	0.562	0.124
% Variance		89.49	6.81	0.98

size. The second principle component (PC 2) accounted for 6.8% of the total variation and was correlated to almost all the characters except total length and length without head. The principle component scores were overlapped extensively in almost all the examined individuals (Fig. 1).

There were only eight variables (LWH, HF, WH, GMAXW, GMINW, GL, PW and AIL) that were included in the analysis of DFA. The first canonical variate (CV 1) and second canonical variate (CV 2) accounted for 53.4% and 17.9% of the total variance, respectively (Table 4). The plot of the first two canonical variates also failed to differentiate the two species (Fig. 2). Plots for both multidimensional analyses showed individuals of *C. gestroi* and *C.heimi* were clustered together, indicating that morphological characters of both species were highly overlapped. It is clearly shown that *C. gestroi* and *C. heimi* could not be distinguished by morphological characters.

Genetic analysis

Partial DNA sequences consisting of 420, 428 and 680 base pairs of 12S rRNA, 16S rRNA and COII rRNA were obtained from the 13 populations of *C. heimi* and one population of *C. gestroi* in this study. In addition to DNA sequences on *C. gestroi*, *C. formosanus* and other *Coptotermes* spp. (Yeap *et*

Table 4: Within-group correlation of variables to the canonical variates.

Measurement	CV1	CV2	CV3
LWH	0.476	-1.131	0.303
HF	-0.697	-0.111	0.254
WH	-2.258	-0.297	-2.132
GMAXW	1.34	-0.044	-0.04
GMINW	0.836	-0.263	-1.124
GL	-0.213	-0.706	0.919
PW	1.065	2.307	1.433
AIL	-0.484	0.264	0.572
% Variance	53.4	17.9	11.4

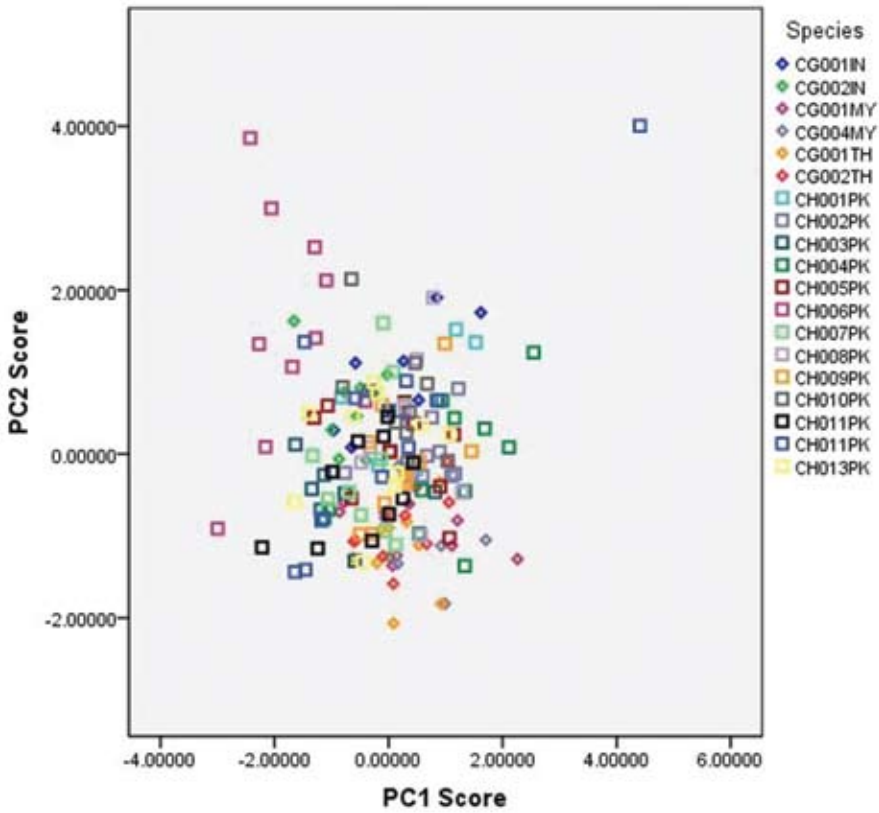

Fig. 1: Plot of the first two principal components for nineteen termite populations

al. 2007, 2009), *C. heimi* from India (Scheffrahn *et al*. 2004, Sobti *et al*. unpublished) were obtained from GenBank and included for phylogenetic analysis (Table 1). All of the three mitochondrial genes of all ingroup taxa were A + T rich (65.67% in 12S, 65.29% in 16S and 61.44% in COII genes) and had a base use with an excess of As. Of these gene fragments, COII was most variable (431 constant characters) and informative (120 informative sites). 16S had 376 constant characters with 52 informative sites. 12S was more conserved (368 constant characters) and less informative (38 informative sites). Based on chi-square tests for base frequency homogeneity among taxa, the base frequency distribution of the two gene fragments and their combined data set were homogenous ($P = 1.0$).

Among the 13 *C. gestroi* DNA sequences, the genetic diversity ranged from 0.00% to 0.56% in 12S, 0 - 0.50% in 16S, and 0- 1.43 % in COII genes.

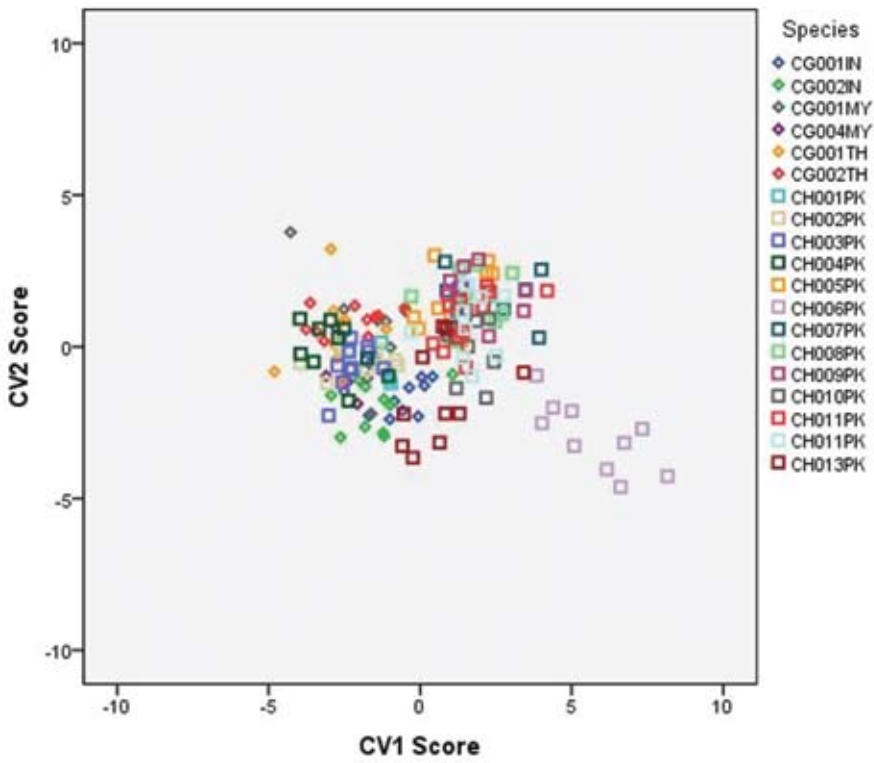

Fig. 2: Plot of the first two canonical variables for nineteen termite populations.

Among the 13 populations of *C. heimi* from Pakistan, there were four haplotypes in 12S with the genetic diversity ranged from 0.00% to 0.51%; two halpotypes in 16S, genetic diversity range from 0 to 0.51%, and three haplotypes in COII, genetic diversity range from 0 to 0.47 %. Across the three genes, the transition rate was 100% between *C. heimi* and *C. gestroi* in 16S genes, while in 12S and COII genes, transition and tranversion rate was in a ratio of 60:40. The genetic divergence between *C. heimi* and *C. gestroi* based on the Kimura 2-parameter varied up to 1.7% (7 bp) in the 12S gene, 1.4% (5 bp) in the 16S gene, and 2.63% (23bp) in the COII gene.

The genetic divergence between *C. gestroi* and *C. heimi* in the 12S and 16S genes is much lower when compared to the genetic divergence between *Nasutitermes corniger* (Motschulsky) and *Nasutitermes costalis* (Holmgren). Scheffrahn *et al*. (2005), who proposed the synonymity of the latter two

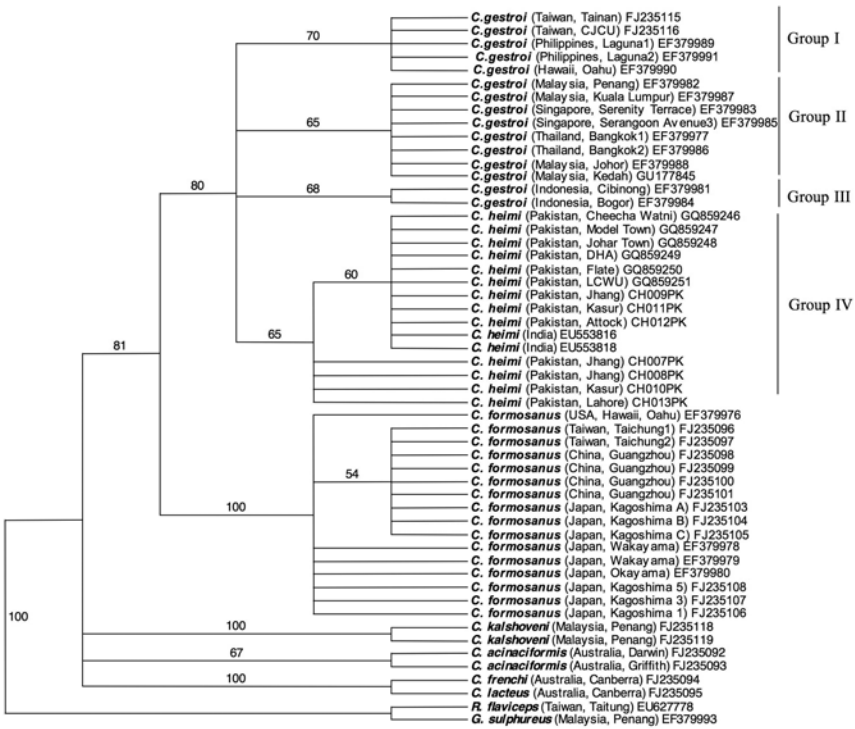

Fig. 3: A single most parsimonious tree obtained for the 12S gene by using a heueristic search option in PAUP4.0b10 (Swofford 2002). Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%.

species, had recorded genetic divergence of up to 1.8% with variability of 13 nucleotides in the 16S gene. We found a higher genetic divergence between *C. gestroi* and *C. heimi* in the COII gene (2.63%). As COII gene is considered as the fastest-evolving and the longest gene examined (Ye *et al*. 2004), the value of 2.63% is considered relatively low. Among other species which we included in the analysis, the least genetic divergence between two different species was 8.2%.

Coptotermes gestroi is a widespread species in the tropics and subtropics, the base substitutions between *C. heimi* and *C. gestroi* recorded in this study could be considered to be intraspecies variation. The slight divergence of *C. heimi* from *C. gestroi* may be due to natural selection (i.e., a mutation occurred as a result of the adaptation of *C. heimi* to different geographical,

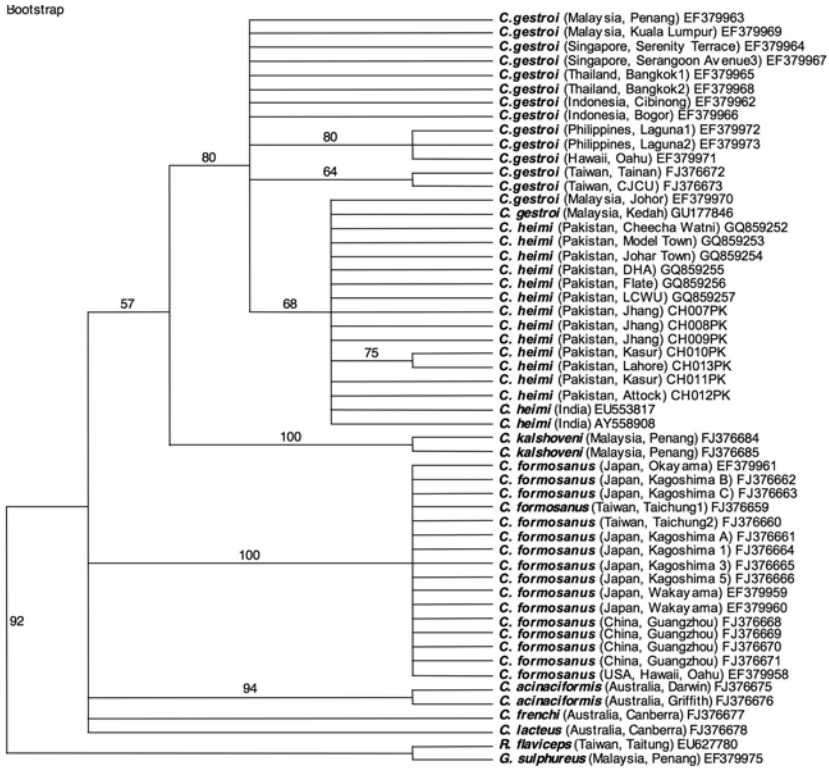

Fig. 4: A single most parsimonious tree obtained for the 16S gene by using a heueristic search option in PAUP4.0b10 (Swofford 2002). Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%.

climatic, and ecological niches) or possibly the limited gene flow in Pakistan and India (interbreeding among these isolated *C. heimi* populations, along with introduced and accumulated mutation).

Phylogenetic relationship inferred from 12S, 16S, COII, and combined gene analysis

Maximum parsimony analysis of the 12S, 16S, COII and the combined genes resulted in a total of 9, 16, 68, and 100 (initial MaxTrees setting = 100) equally most parsimonious trees, respectively (Fig. 3: length = 127, Consistency Index (CI) = 0.889, Retention Index (RI) = 0.674; Fig. 4: length = 168, CI = 0.912, RI = 0.684; Fig. 5: length = 223, CI = 0.877, RI = 0.763, Fig. 6: length = 237, CI = 0.902, RI = 0.859). The best model for the ML analysis of the 12S gene was "GTR+I+G" with the following parameter settings: base = (0.4533, 0.2244, 0.1189, 0.2034), Nst = 6, Rmat = (563043.9745,

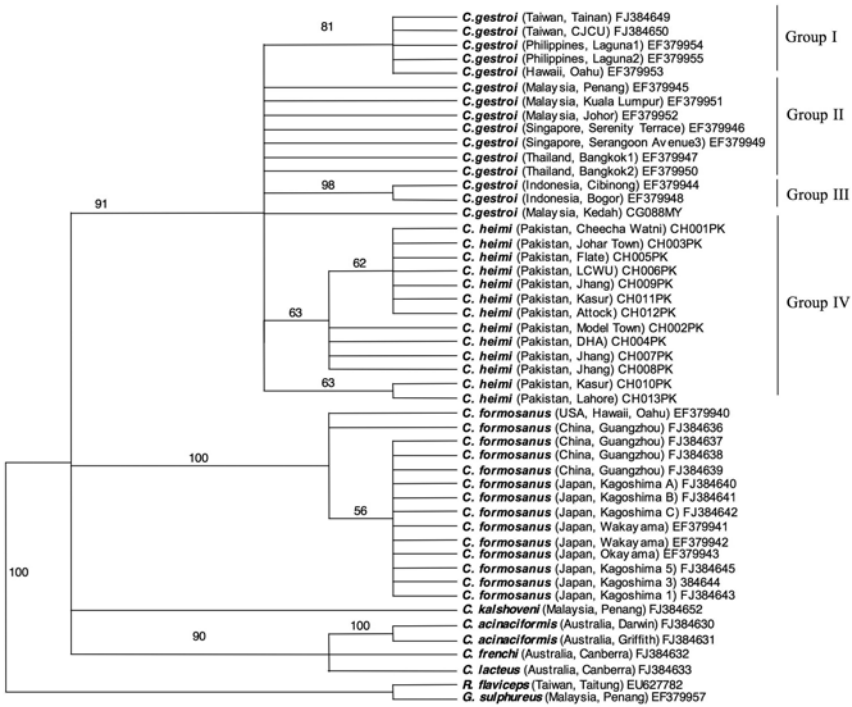

Fig. 5: A single most parsimonious tree obtained for the COII gene by using a heueristic search option in PAUP4.0b10 (Swofford 2002). Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%.

732350.2145, 266837.6836, 9673.2974, 1847327.7362), rates = gamma shape = 0.4975, and pinvar = 0.3272. For the 16S gene, the selected model was "HKY+I+G" with the following parameter settings: base = (0.4335, 0.2377, 0.1094, 0.2194), Nst = 2, TRatio = 3.7352, rates = gamma shape = 0.2937, and pinvar = 0.4627. For the COII gene, the selected model was "HKY+I+G" with the following parameter settings: base = (0.3894, 0.2514, 0.1341, 0.2250), Nst = 4, TRatio = 3.8645, rates = gamma shape = 0.3156, and pinvar = 0.4563. For the combined genes, the "GTR+I+G" model was selected with the following parameter settings: base = (0.4159, 0.2408, 0.1242, 0.2191), Nst = 6, Rmat = (2.7546, 15.9667, 2.6439, 0.6456, 38.9887), rates = gamma shape = 2.2751, and pinvar = 0.6395. A single tree was recovered for each of the genes (–In L 1276.8662 for 12S, –In L 1553.4895 for 16S, –In L 2695.3887 for COII, and –In L 28665.6983 for the combined genes,

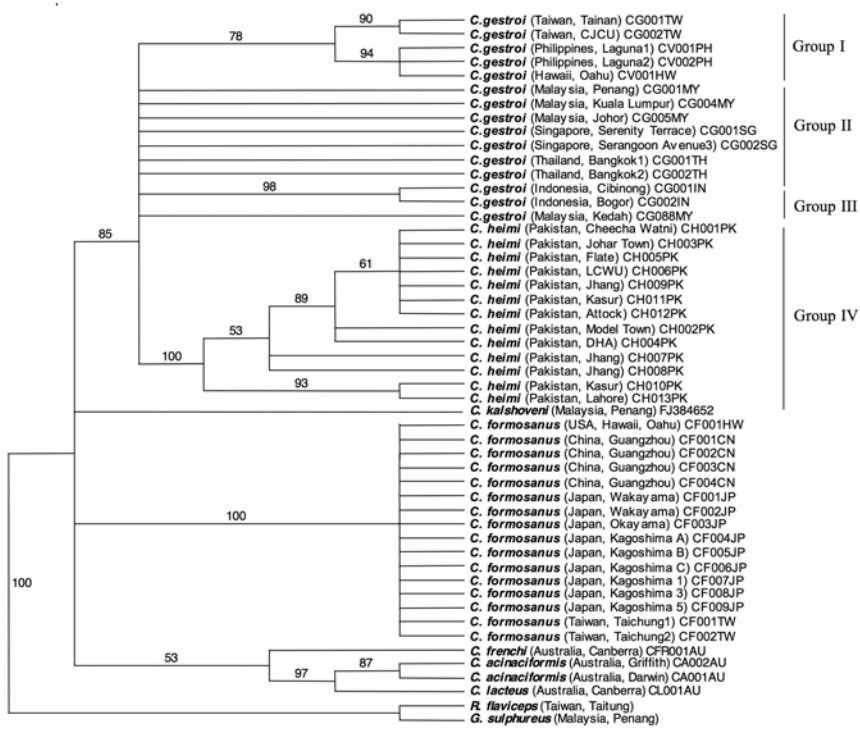

Fig. 6: The most parsimonious tree obtained for combined 12S, 16S and COII genes by using a heueristic search option in PAUP4.0b10 (Swofford 2002). Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%.

trees not shown). The most parsimonious trees with a topology similar to that generated by the ML analysis were chosen (Figs. 3–6).

A comparison of the three bootstrap trees revealed no cases in which a grouping with more than 50% bootstrap support in one tree conflicted with a grouping with more than 50% bootstrap support in another tree. Based on the overall low level of conflict between the three genes, the data were combined, and the most parsimonious tree was constructed.

For the parsimony tree of the 12S gene, populations of *C. gestroi* were partitioned into four groups (Fig. 3). Group I consisted of *C. gestroi* from Taiwan, Hawaii and the Philippines; group II contained *C. gestroi* from Malaysia, Singapore, and Thailand; group III had *C. gestroi* from Indonesia; and group IV consisted of *C. heimi* from Pakistan and India. These groupings, however, had moderate bootstrap values (< 70%). They were distinctly different from *C. formosanus* and other *Coptotermes* spp. which were included in the analysis. For the 16S gene, populations of *C. gestroi* did not partition into four groups as in 12S gene, yet, *C. gestroi* and *C. heimi* remained in the same clade. *Coptotermes gestroi* from Kedah and Johor, Malaysia grouped together with *C. heimi* with moderate bootstrap support of 68% (Fig. 4). For COII and combined genes, the relationship exhibited was similar to 12S gene (Figs. 5 & 6). *C. gestroi* and *C. heimi* formed a common clade with strong bootstrap support (91% and 85% in COII and combined genes, respectively).

Due to low intraspecies genetic variation, all *C. formosanus* populations from different regions fall into same clade without further subdivision into different geographical groups (Figs. 3–6). On the other hand, the combination of 12S, 16S and COII genes revealed the phylogenetic relationship of *C. gestroi* populations which agree with the report of Li *et al.* (2009) and Yeap *et al.* (2009). The additional group IV from *C. gestroi* clade in this study showed that *C. heimi* from Pakistan and India are belonged to another geographical group of *C. gestroi*. This result supported that *C. heimi* is a junior synonym of *C. gestroi*.

In summary, morphological analysis and molecular phylogenetic analyses using 12S, 16S, and COII mitochondrial genes suggested that *C. heimi* is a junior synonym of *C. gestroi.* We found that the morphological characters of *C. heimi* and *C. gestroi* overlapped greatly, that low genetic diversity existed between *C. heimi* and *C. gestroi* (1.4–2.63%). The nucleotide differences ob-

served could be intraspecies variations that are due to different geographical, climatic, and ecological niches.

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