GENETIC VARIATION, DIVERSITY AND MOLECULAR PHYLOGENETIC OF HIGHER GROUP TERMITE
Macrotermes carbonarious Hagen (BLATTODEA: TERMITIDAE)

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ABSTRACT

Limited studies pertaining to evolutionary history and intraspecific diversity of *Macrotermes* genus have been carried out by researchers. Such studies are vital as from phylogeny we can understand the origin of species in a particular region. Thus, in this study, we investigated the phylogenetic relationships and genetic diversity among the population of *Macrotermes carbonarius* around USM campus. Seventeen *Macrotermes carbonarius* colonies were used to conduct a molecular genotyping study and the mitochondrial (COII) gene was used as marker. The data obtained were analyzed using T-Coffee, Clustal X, Mega 7.0 and DnaSP software programs. Our results show one main monophyletic clade that consists of two groups: HP1 and HP2. The samples from Stadium Hoki were classified in HP2, differing from the other populations studies by one singleton base. Our HP2 shows some similarity with some sequence from Pasoh Forest Reserved Malaysia, suggesting a state diversity connection. The disparity index suggests that all the samples are homogeneous populations, supported by the low value of mean pairwise distance between the samples. This study will serve as a platform for scientific community to enhance the knowledge on the phylogeny and genetic diversity of *Macrotermes* genus in South East Asia region.

Key words: *Macrotermes carbonarius,* phylogeny, genetic diversity, haplotype, mitochondrial COII gene

INTRODUCTION

Termites are terrestrial social insects, which belong to order Blattodea, consists of five families of lower termites and 1 family of higher termites (Noirot, 1992; Lee *et al*., 2005). Termites are the most elegant engineers of nature and are well known for their super architectural ability, cryptobiotic nature of life and enormous economic importance. They are an amazing group of social insects with highly evolved organization, cast system and division of labour (Sharma *et al*., 2013). Termites are arthropods that degrade complex substances like cellulose and hemicelluloses found in plant materials (Brune, 1998). Termites are also the most destructive insect pest of wood in the world. In the States, an approximate 5 billion USD cost of damage were done by termites every year, more than the damage caused by fire and wind storm combined. Termites were found around the world especially in the tropical and sub-tropical regions. Because of their vast economic importance, it has become vital for researcher to study them to unravel their origin and taxonomic position (Sharma *et al*., 2013).

Macrotermes carbonarius Hagen is a fungus growing termites from subfamily Macrtermitinae that are widely distributed throughout the Old-World tropics. These termites belong to higher group termites that utilize the symbiotic relationship with the fungus (genus of *Termitomyces*) to produce "food" for the termites. The fungus gardens are provided with plant substrates continuously, as the older parts that have been well decomposed by the fungus will then be consumed by the termites (Aanen & Eggleton, 2005). *M. carbonarius* is the only open foraging *Macrotermes* species, which forages above ground with extensive trails and the only black species. The species originated from Africa (Coaton *et al*., 1978; Aanen *et al*., 2002; Aanen & Eggleton, 2005; Aanen & Boomsma,

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2005) but are also found in South East Asia and parts of Indochina. They are also known to construct an extensive system of underground passages to have access to their foraging sites (Darlington, 1988). As mentioned earlier *M. carbonarius* mound contain fungus garden which made up of *Termitomyces,* a white rot fungus that are not digestible for all organism.

Singla *et al*. (2016) investigated the phylogenetic relationship among Indian termite species using COII gene sequence were able to identify the unidentified species and further confirmed using DNA barcoding approach. Their phylogeny shows different species of termites and different genetic relationship with each other in different geographical regions. Another study on the molecular phylogeny of Asian termites of Termitidea and Rhinotermitidea also had been done using mitochondrial COII sequences, which gives an important implication for the termite evolution and classification. Their results show monophyly in Termitidea, Macrotermitinae and Apicotermitinae but not in Rhinotermitidea, Termitinae and Nasutitermitinae in the terms of snapping mandibles characteristic (Singla *et al*., 2016; Lee *et al*., 2005). Unfortunately, their inferred phylogenetic relationships are still premature to draw definitive conclusion since there is still a great majority of termite genera that remained untouched (Ohkuma *et al*., 2004). The objectives for our study are to investigate the phylogenetic relationships, genetic variation and diversity among the population of *Macrotermes carbonarius* around Universiti Sains Malaysia Main Campus, Penang, Malaysia.

MATERIALS AND METHODS

Termite collection

Macrotermes carbonarius termites were garnered from 17 sites around USM Minden campus, (Figure 1). After collecting the termites from the site, they were stored temporarily in a sealed petri dish and labelled properly for identification. The locations of the site were mapped using GPS 72H (Garmin, USA) coordinates. The collected termites were brought back to the lab, then kept in the universal vial with 98% of alcohol and separated according to the captured location.

DNA isolation and PCR amplification

The DNA extracted from the heads of *M. carbonarius* collected from the field using (HiYieldPlus Genomic DNA mini kit, Taiwan, Real Biotech Cooperation) adopted from Seri Masran and Ab Majid (2017) protocol. The specimens were washed with sterile distilled water for 3 times, then homogenized in the cell lysis buffer with Proteinase K at 4°C and incubated for an hour under 60°C. After that, the lysates were subjected to protein precipitation and ethanol wash, followed by elution step using 50 µL buffer. The quantity and quality of DNA were measured using a NanoDrop 2000c (Thermoscientific, USA) at the wavelength of 260/280 nm and fragments of DNA were observed using 1% agarose gel electrophoresis. All the DNA extracts were stored in a -20°C freezer. The extracted DNA samples were amplified by polymerase chain reaction. The PCR reaction mix consists of 15 µL of dilute distilled water, 2.5 µL of each forward and
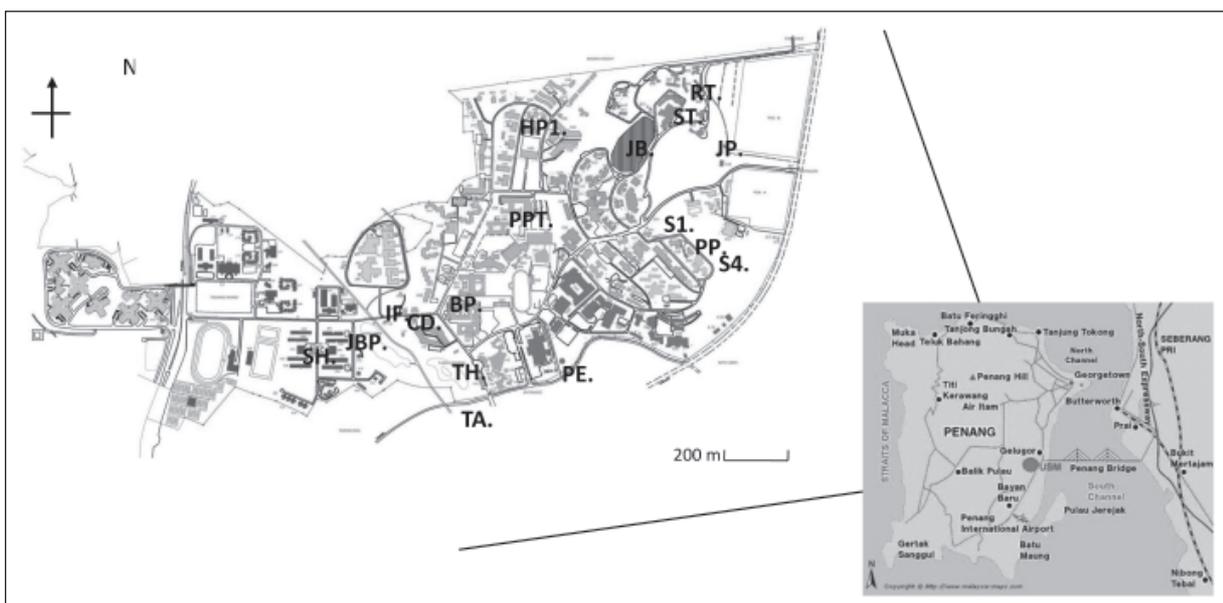
Fig. 1. Map of University Sains Malaysia Main Campus, Penang, Malaysia. The abbreviations that are marked on the map indicates the sites where the *M. carbonarius* populations were being collected. The abbreviation details are in Table 1.

reverse primer, 25 μL of master mix and 5 μL of DNA template. The tubes were placed in a thermal cycler and the conditions of amplification were as follows: an initial denaturation of 94°C for 5 min, ensued by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min and extension at 65°C for 3 min. The final extension was set at 72°C for 10 min before storage at 4°C. The primers used for amplification were ~407 bp in size, with forward primer '5-TTC TTC CAC GAC CAC GCA TT-3' and reverse primer '5-ACG TCT GCT GCT GTT ACG AT-3'. The PCR products were subjected to 1% agarose gel electrophoresis in order to evaluate the quality of DNA extracted. Five microliters of the amplified products were then purified via MEGA quick-spin™ Total Fragment DNA purification kit, Korea, iNtRON Biotechnology prior to sequencing at Apical Scientific Sdn. Bhd.

Genetic diversity and phylogenetic tree construction

Nucleotide sequence multiple alignments and quality checking were performed using T-Coffee (Notredame *et al*., 2000) and Clustal X 2.1 (Larkin *et al*., 2007). The aligned sequences were then deposited in the GenBank with the assigned accession numbers provided. Molecular Evolutionary Genetics Analysis (MEGA) 7.0 (Kumar *et al*., 2016) was used to examine the phylogenetic relationship tree among the *M. carbonarius* population. Neighbor-joining (Saitou & Nei, 1987)

and Maximum likelihood analyses were also done by using MEGA. The genetic diversity and nucleotide diversity were estimated using DNaSP 5.10 (Librado & Rozas, 2009).

RESULTS

The seventeen sequenced *Macrotermes carbonarius* amplicons revealed an average size of 380 bp. The gene sequences were then subjected to multiple alignments and gap elimination via T-Coffee Molecular Software and Clustal X 2.1 with the inclusion of outgroup species *M. gilvus* (AB109526.1) and *Coptotermes gestroi* (EU805757.1). A consensus 322 bp long partial coding sequence of mito-chondrial COII gene was left behind. A FASTA file that entails all the trimmed sequences was generated and submitted to NCBI GenBank database for accession number. The assigned accession numbers for the sequences range from KY273116 to KY273132 (Table 1).

Concomitantly, the sequences were also sent for NCBI Blast in order to validate the identity of the termite species sampled. There were three Blast Hits that attained a resemblance of 100% with the query sequences – two of which the *M. carbonarius* were sampled from Penang Island (**AY536412.1, AY940131**) and one from Pasoh Forest Reserve, Malaysia (**AB051878.1**). Thus, it was corroborated that our samples were precisely identified as *M.*

Table 1. Collection site, isolated code, group, and accession number of each *M. carbonarius* in this study

Isolated Code	Collection Site	State	Collection Date	Isolation Source	Lat_Lon	Group	Accession Number
JP	Jalan Pemimpin, USM campus	Penang	20-Oct-16	Mound	5.360N 100.307E	Hp01	KY273116 (MC1)
RT	Rumah Tetamu, USM campus	Penang	20-Oct-16	Mound	5.362N 100.307E	Hp01	KY272117 (MC2)
ST	Saga Tree near Rumah Tetamu, USM campus	Penang	20-Oct-16	Mound	5.361N 100.306E	Hp01	KY271118 (MC3)
TH	Tasik Harapan, USM campus	Penang	21-Oct-16	Mound	5.354N 100.300E	Hp01	KY270119 (MC4)
TA	Tasik Aman, USM campus	Penang	21-Oct-16	Mound	5.353N 100.300E	Hp01	KY269120 (MC5)
PE	Perpustakaan, USM campus	Penang	24-Oct-16	Mound	5.354N 100.303E	Hp01	KY268121 (MC6)
PP	Pusat Pengajian Bahasa, Literasi, USM campus	Penang	24-Oct-16	Mound	5.357N 100.307E	Hp01	KY267122 (MC7)
S1	Kolam Renang, USM campus	Penang	27-Oct-16	Mound	5.358N 100.306E	Hp01	KY266123 (MC8)
S2	Kolam Renang, USM campus	Penang	27-Oct-16	Mound	5.357N 100.307E	Hp01	KY265124 (MC9)
JB	Jalan Bendahari, USM campus	Penang	3-Nov-16	Mound	5.360N 100.305E	Hp01	KY264125 (MC10)
BP	Desasiswa Bakti Permai, USM campus	Penang	29-Oct-16	Mound	5.356N 100.300E	Hp01	KY263126 (MC11)
HP1	Hadanah Pusat Islam, USM campus	Penang	29-Oct-16	Mound	5.361N 100.302E	Hp01	KY262127 (MC12)
PPT	Pusat Pengajian Teknologi, USM campus	Penang	29-Oct-16	Mound	5.358N 100.302E	Hp01	KY261128 (MC13)
JPB	Jabatan Pembangunan, USM campus	Penang	3-Nov-16	Mound	5.355N 100.297E	Hp01	KY260129 (MC14)
SH	Stadium Hoki, USM campus	Penang	3-Nov-16	Mound	5.355N 100.296E	Hp02	KY259130 (MC15)
IF	Informm, USM campus	Penang	3-Nov-16	Mound	5.356N 100.298E	Hp01	KY258131 (MC16)
CD	CDR, USM campus	Penang	3-Nov-16	Mound	5.356N 100.298E	Hp01	KY257132 (MC17)

carbonarius. The average frequency distributions of nucleotides in the 1st, 2nd and 3rd codon positions are as followed: T= 37.7%, 16.8% and 15.9%; C=21.1%, 22.2% and 25.7%; A= 29.8%, 57.5% and 38.9%; and G=11.4%, 3.5% and 19.5% respectively. In order to statistically determine the homogeneity of substitution patterns between the 17 sequences, a Disparity Index test (Monte Carlo Test) (Kumar & Gadagkar, 2001) with 500 replicates was deployed to gauge the *P*-value, which in this scenario showed no significant bias (p= 1.00). Thus, all the seventeen sequences in our study have the same pattern of nucleotide substitution.

The phylogenetic relationship of *M. carbonarius* populations in USM Minden campus was analysed via two main tree construction approaches: distance matrix and character-based method. A Neighbour-Joining (Figure 2) and a Maximum Likelihood tree (Figure 3) was constructed based on two different evolutionary models, Tamura-Nei (Tamura & Nei, 1993) and Hasegawa-Kishino-Yano (Hasegawa *et al.*, 1985) model respectively. MEGA 7.0 software determined that, among the total 322 bp, 321 were conserved sites and one variable but parsimony-

uninformative (singleton sites). Due to the lack of parsim-info site, Maximum Parsimony method cannot be harnessed to generate a phylogenetic tree.

Both the trees displayed monophyly of *M. carbonarius* with respect to outgroup species, *M. gilvus* and *C. gestroi*, underpinned by 100% bootstrap value (Figure 2 and Figure 3). A single monophyletic clade which encompasses the 17 *M. carbonarius* samples was observed, although "SH" taxon had a short protruding branch from the other 16 samples. Besides, a major monophyletic clade which encompasses *M. carbonarius* and *M. gilvus* was also observed, separating the Termitidae family from Rhinotermitidae family. Based on DnaSP v.5.10.01analysis of the haplotype diversity, *M. carbonarius* populations in USM Minden campus can be classified into two major haplotypes – Hp01 and Hp02. Hp02 consists of "SH" taxon, which can only be found around the Stadium Hoki, while Hp01 comprises all the other 16 *M. carbonarius* populations. Hp02 varies from Hp01 only by one singleton site at position 275. This point mutation is not a missense mutation as the changes in base triplet from ATA to ACA, renders a different amino
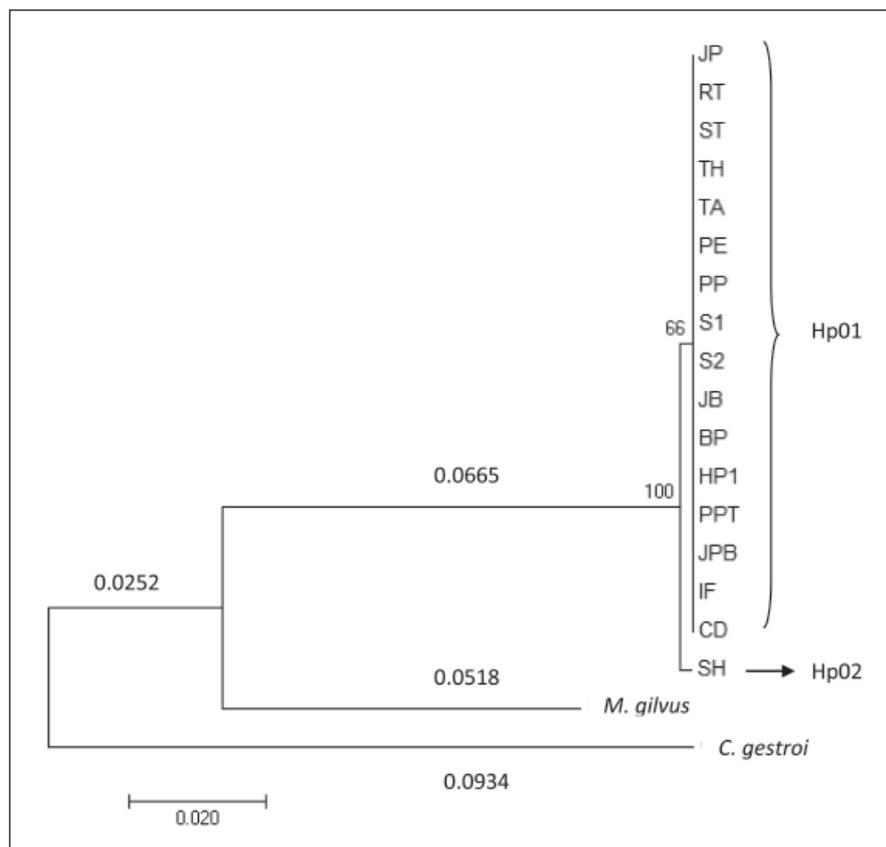

Fig. 2. Neighbor-Joining tree inferred from sequences of the mitochondrial COII gene of 17 *M. carbonarius* population in USM Minden Campus, with the inclusion of *M. gilvus* and *C. gestroi* as outgroup. Abbreviations are listed in Table 1. Numbers displayed on each internal node represents the bootstrap values (%) obtained after 1000 replications. The sum of branch length is 0.2401.
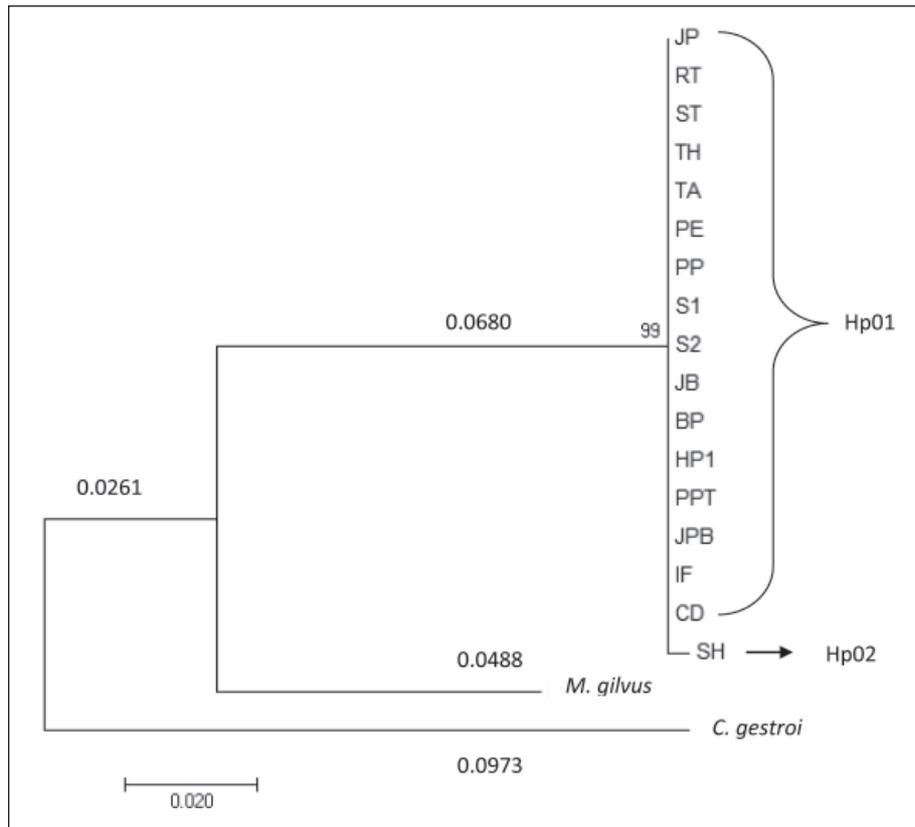
Fig. 3. Maximum Likelihood tree inferred from sequences of the mitochondrial COII gene of 17 *M. carbonarius* population in USM Minden Campus, with the inclusion of *M. gilvus* and *C. gestroi* as outgroup. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The log likelihood of this tree is -709. 1746. Abbreviations are listed in Table 1. Numbers displayed on each internal node represents the bootstrap values (%) obtained after 1000 replications. The sum of branch length is 0.2401.

acid (Methionine to Threonine) to be incorporated into the polypeptide chain. Further analysis can be carried out to study how this missense mutation affects the protein structure of cytochrome c oxidase II. The tree is also underpinned by an overall mean distance of 0.000348, implying that all the isolates are very close to each other and have no conspicuous variance between different populations in USM. Lastly, the pairwise distance with bootstrap method (1000 replicates) between "SH" population and other *M. carbonarius* populations indicates 0.00319, as compared to other 16 *M. carbonarius* population.

DISCUSSION

Mitochondrial DNA (mtDNA) is often harnessed by researches to infer phylogenetic relationship among closely related species because it evolves much faster than nuclear DNA (Moore, 1995). Such property of mtDNA facilitates the accumulation of significant differences in sequence variation within a population, even though they might have just diverged for a brief period of time. Among the mitochondrial DNA, cytochrome c oxidase I, II and 16s rRNA are the three most common genes used for studies in evolutionary history among taxa (Miura *et al.*, 2000). Nowadays, a 648 bp region of mitochondrial *COI* gene is widely acknowledged as the "DNA barcode" for identification of almost all animal groups because it has a highly conserved functional domain and a relatively fast evolution rate in animal (Hebert *et al.*, 2003).

Classification of termites rooted on morphological characteristic is comparatively less accurate and problematic due to their miniature size, morphological features that alter as a result of environmental factor and the ubiquity of biotypes that renders identification process difficult (Sobti *et al.*, 2009). In this study, mitochondrial *COII* gene was chosen as a genetic marker to study the phylogenetic relationship of *M. carbonarius* in USM Minden campus. Utilization of *COII* region of

mtDNA in the study of phylogeny of termites has been demonstrated previously (Lo *et al*., 2004; Ohkuma *et al*., 2004).

The discrepancy in the haplotype diversity can possibly be explained by the occurrence of polygyny within some of the *M. carbonarius* mounds, notably "SH population". Brandl *et al*. (2004) mentioned that *M. michaelseni* in Kenya displays a considerable variation in the establishment of polygyny across years, ranging from 5 to 50%. The phenomenon of polygyny can be attributed to several factors. During colony foundation, few female alates may work hand in hand, engendering pleometrosis. When they become reproductively active, each of them contribute to the generation of steriles. Thus, in a single mature polygynous colony, several lineages of steriles stay and cooperate together. Hacker *et al*. (2005) suggested that the connectedness of the primary reproductive determines the mean relatedness of the steriles generated. By using multilocus fingerprints and microsatellites to study the relatedness of colony-founding queen, they reported that the nestmate queens are in fact unrelated. Hence, we inferred that the major soldiers that we collected from 'SH population' are from a different matriline of sterile. Besides, the queen that produces the same matriline of sterile with the other 16 populations might be killed as ramification of conflict and overt aggression. Evidence shows that antennal mutilation of queens is ubiquitous in mature polygynous colonies (Darlington, 1988).

Brandl *et al*. (2005) highlighted that *M. michaelseni* have a low value of genetic differentiation (F_{ST}) across a 50km spatial scale based on a microsatellite loci study. This indicates that it is quite rare that two different haplotypes to exist within a small region. In fact, our *M. carbonarius* sequences exhibits 100% identity with a sample from Pasoh Forest Reserve, Malaysia, indicating that an inter-state connection is present. Besides, they also highlighted that dispersion by human transportation can be precluded for mound-builders like *M. michaelseni.* In a study conducted by Austin *et al*. (2008), 41 different haplotypes were detected from 106 samples of *Reticulitermes tibalis* based on 16S rRNA sequence. One haplotype was found to appear in 24% of the samples, while there are 23 haplotypes that only present once. They speculated that glaciation and sky islands can be attributed to the haplotype variation as underpinned by molecular clock.

In this present study, we did not highlight much on the branch length within each internal node as the utmost purpose of this research is to determine how diverged the *M. carbonarius* populations within USM Minden campus and not how it evolved with respect to other termite species. However, it is noteworthy that Rhinotermitidae in certain extent is paraphyletic with respect to Termitidae (Lo *et al*., 2004) and that both Termitidae family and Macrotermitinae subfamily were monophyletic (Ohkuma *et al*., 2004). The monophyly of Termitidae insinuates that the loss of gut flagellate symbionts happens only once (Ohkuma *et al*., 2004); and the monophyly of Macrotermitinae implied that the agricultural symbiosis between Macroteritinae and fungi Termitomyces occurs only once (Anen *et al*., 2002).

CONCLUSION

In conclusion, this study has provided an insight into the phylogenetic relationship of *M. carbonarius* populations in USM Minden campus. The *M. carbonarius* populations are quite conserved in terms of evolutionary history, with only one singleton base difference among the sequences tested. Different termite species and genera should be collected from the campus in order to have a more detailed understanding of the phylogenetic relationship among the termites.

REFERENCES

Aanen, D.K. & Boomsma, J.J. 2005. Evolutionary dynamics of the mutualistic symbiosis between fungus-growing termites and Termitomyces fungi. *Insect-Fungal Associations: Ecology and Evolution*, 191-210.

Aanen, D.K. & Eggleton, P. 2005. Fungus-growing termites originated in African rain forest. *Current Biology,* **15(9)**: 851-855.

Aanen, D.K., Eggleton, P., Rouland-Lefevre, C., Guldberg-Frøslev, T., Rosendahl, S. & Boomsma, J.J. 2002. The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences,* **99(23)**: 14887-14892.

Austin, J.W., Szalanski, A.L., McKern, J.A. & Gold, R.E. 2008. Molecular phylogeography of the subterranean termite *Reticulitermes tibialis* (Isoptera: Rhinotermitidae). *Journal of Agricultural and Urban Entomology,* **25(2)**: 63-79.

Brandl, R., Hacker, M., Bagine, R.K.N. & Kaib, M. 2004. Yearly variation in polygyny in the termite *Macrotermes michaelseni* (Sjöstedt). *Insectes Sociaux,* **51(3)**: 294-298.

Brandl, R., Hacker, M., Epplen, J.T. & Kaib, M. 2005. High gene flow between populations of *Macrotermes michaelseni* (Isoptera, Termitidae). *Insectes Sociaux,* **52(4)**: 344-349.

Brune, A. 1998. Termite guts: the world's smallest bioreactors. *Trends in Biotechnology,* **16(1)**: 16-21.

Coaton, W.G.H., Sheasby, J.L. & Ruelle, J.E. 1978. National survey of the Isoptera of southern Africa. Department of Agriculture and Fisheries, South Africa. pp. 48-49.

Darlington, J.P.E.C. 1988. Mutilation of the primary reproductives in termites of the genus Macrotermes. *International Journal of Tropical Insect Science,* **9(01)**: 81-83.

Hacker, M., Kaib, M., Bagine, R.K.N., Epplen, J.T. & Brandl, R. 2005. Unrelated queens coexist in colonies of the termite *Macrotermes michaelseni*. *Molecular Ecology,* **14(5)**: 1527-1532.

Hasegawa, M., Kishino, H. & Yano, T.A. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution,* **22(2)**: 160-174.

Hebert, P.D., Cywinska, A. & Ball, S.L. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Sciences,* **270(1512)**: 313-321.

Kumar, S. & Gadagkar, S.R. 2001. Disparity index: a simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. *Genetics,* **158(3)**: 1321-1327.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution,* **33(7)**: 1870-1874.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. & Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics,* **23(21)**: 2947-2948.

Lee, C.Y., Forschler, B.T., Jenkins, T.M. & Robinson, W.H. 2005. Taxonomic questions on Malaysian termites (Isoptera: Termitidae) answered with morphology and DNA biotechnology. In *Fifth International Conference on Urban Pests, Singapore,* 11-13 July 2005. pp. 205-211. International Conference on Urban Pests (ICUP).

Librado, P. & Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics,* **25(11)**: 1451-1452.

Lo, N., Kitade, O., Miura, T., Constantino, R. & Matsumoto, T. 2004. Molecular phylogeny of the Rhinotermitidae. *Insectes Sociaux,* **51(4)**: 365-371.

Miura, T., Roisin, Y. & Matsumoto, T. 2000. Molecular phylogeny and biogeography of the nasute termite genus Nasutitermes (Isoptera: Termitidae) in the Pacific tropics. *Molecular Phylogenetics and Evolution,* **17(1)**: 1-10.

Moore, W.S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution,* **49(4)**: 718-726.

Noirot, C. 1992. From wood-to humus-feeding: an important trend in termite evolution. In *First European Congress of Social Insects, Leuven (Belgium),* 19-22 August 1991. Leuven University Press.

Notredame, C., Higgins, D.G. & Heringa, J. 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology,* **302(1)**: 205-217.

Ohkuma, M., Yuzawa, H., Amornsak, W., Sornnuwat, Y., Takematsu, Y., Yamada, A., Vongkaluang, D., Sarnthoy, O., Kirtibutr, N., Noparatnaraporn, N., Kudo, T. & Inoue, T. 2004. Molecular phylogeny of Asian termites (Isoptera) of the families Termitidae and Rhinotermitidae based on mitochondrial COII sequences. *Molecular Phylogenetics and Evolution,* **31(2)**: 701-710.

Sabtu, F.S. & Ab Majid, A.H. 2017. Genetic variation and population structure of the arboreal bicolored ant *Tetraponera rufonigra* Jerdon from selected urban locations in eastern Penang Island, Malaysia. *Journal of Asia-Pacific Entomology,* **20**: 1350-1357.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4(4)**: 406-425.

Seri Masran, S.N.A. & Ab Majid, A.H. 2017. Genetic diversity and phylogenetic relationships of cytochrome C oxidase subunit 1 in *Cimex hemipterus* (Hemiptera: Cimicidae) populations in Malaysia. *Journal of Medical Entomology,* **54(4)**: 974-979.

Sharma, V.L., Singla, M. & Sobti, R.C. 2013. Phylogenetic position of Indian termites (Isoptera: Termitidae) with their respective genera inferred from DNA sequence analysis of the mitochondrial cytochrome oxidase gene subunit I compared to subunit II. *Molecular and Cellular Biochemistry,* **384(1-2)**: 39-45.

Singla, M., Goyal, N., Sharma, R., Singla, N., Chaudhuri, A., Sobti, R.C. & Sharma, V.L. 2016. Reconstructing phylogenetic relationship among Indian termite species inferred from COII gene sequences (Blattodea: Isoptera: Termitidae). *Journal of Entomology and Zoology Studies,* **4(4)**: 1-7.

Sobti, R.C., Kumari, M., Sharma, V.L., Sodhi, M., Mukesh, M. & Shouche, Y. 2009. Sequence analysis of a few species of termites (Order: Isoptera) on the basis of partial characterization of COII gene. *Molecular and Cellular Biochemistry,* **331(1-2)**: 145-151.

Tamura, K. & Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution,* **10(3)**: 512-526.