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Estimating molecular phylogeny of some Indian termites combining partial COI sequences

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Abstract
To study the phylogenetic relationships of Indian termites (Insecta: Isoptera), mitochondrial DNA sequences of 334 bp of Cytochrome Oxidase subunit - I gene of nine species were subjected to MEGA 5.2. Phylogenetic trees were constructed using Neighbor-Joining and Maximum Likelihood methods. The results revealed the phylogenetic status of Indian termites with other species from different geographical locations. The results were also confirmed with additional molecular parameter, nucleotide composition and pairwise genetic distance.

Keywords: Cytochrome oxidase I, Termite, phylogeny.

1. Introduction
The evolutionary origin of termites remains unresolved for more than half a century [1-3]. The clear picture of termite ancestry is crucial for understanding as how these insects evolved because they lack haplodiploid genetic system associated with eusocial evolution in bees, ants, wasps and thrips [4, 5]. Several studies took into account to study the phylogeny of termites using different molecular markers. Among the 13 protein-coding genes within the mt genome, cytochrome c oxidase I has gained particular popularity for estimating relationships among closely allied taxa. Although COI may be matched by other mitochondrial genes in resolving such cases of recent divergence, this gene is more likely to provide deeper phylogenetic insights than alternatives such as cytochrome b [6] because changes in its amino acid sequence occur more slowly than those in this, or any other, mitochondrial gene. In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species [7, 8]. It is used as a molecular marker for evolutionary study because its rate of nucleotide evolution allows it to resolve evolutionary histories at the family, genus and species levels [9].

2. Materials and Methods
2.1 Material for molecular analysis
Termites for the present study were collected randomly from various regions of India (Table 1). Collection was Voucher specimens were preserved in rectified alcohol and maintained in the Department of Zoology, Panjab University, Chandigarh (UT), India. Soldier specimens of all the species collected (packed in scintillation vials in rectified alcohol) were got identified from Zoological Survey of India, Kolkata and Forest Research Institute, Dehradun using keys or descriptions of Chhotani [10]. The details of collection site, date of collection, source and name of the collector were mentioned on each vial. The period of study, including collection, experimental designs, data analysis was from 2010 till 2013.

2.2 DNA extraction, PCR and Sequencing
Genomic DNA was extracted from worker termites by using standard phenol: chloroform extraction method [11]. Sequencing represented 650 nucleotide base pairs per taxon in the species under investigation using primers, Forward (UEA-9-mod)- 5' GTAAATTTAACATTTTTTCCYCAACA 3' [12] and Reverse (C2-N-3389) - 5' TCATAACTTCAGTATCATTG 3' [13] by protocol of Williams *et al*. (1990) [14] followed with some modifications. These primers amplify a 3' portion of the mtDNA COI gene, tRNA-Leu and a 5' section of the COII gene. The amplified products were got sequenced directly from Chromous Biotech. Pvt. Ltd., Bangalore (India). To facilitate genetic comparisons of *COI* gene with existing GenBank DNA sequences, region of *COII* gene was excluded and the remaining portion of *COI* i.e. 334 bp was used for further analysis.

Table 1: Collection data of the different termite species belonging to three genera (Family: Termitidae)

S. No.	Identified Species	Collection Site	Family	Subfamily	Source
1	*Microcerotermes beesoni* (Snyder)	South Goa	Termitidae	Termitinae	Cashew nut tree
2	*Microtermes obesi* (Holmgren)	Hallu majra and Outskirts of Sukhna Lake, Chandigarh	Termitidae	Macrotermitinae	Damp wood
3	*Microtermes unicolor* (Snyder)	Village Kothe Radha, Jagraon, Ludhiana Distt. (Punjab)	Termitidae	Macrotermitinae	Log of wood
4	*Microtermes mycophagus* (Desneux)	Badehar, Hamirpur Distt. (H.P.)	Termitidae	Macrotermitinae	Tree trunk
5	*Odontotermes horni* (Wasmann)	Sec.11, Chandigarh	Termitidae	Macrotermitinae	Damp wood, tree
6	*Odontotermes obesus* (Rambur)	Malak Road, Jagraon (Punjab)	Termitidae	Macrotermitinae	ound
7	*Odontotermes gurdaspurensis* (Holmgren and Holmgren)	Mandi (Himachal Pradesh)	Termitidae	Macrotermitinae	Mound
8	*Odontotermes brunneus* (Hagen)	Pipli, Haryana	Termitidae	Macrotermitinae	Wood
9	*Odontotermes bhagwati* (Chatterjee and Thakur)	Sec. 10, Chandigarh	Termitidae	Macrotermitinae	Tree

2.3 Phylogenetic analysis

The sequences were first edited manually for discarding the ambiguous and skipped bases and files were converted into FASTA format by excluding COII region. The edited sequences were analyzed by using BLAST search [15] from NCBI. Around 17 sequences were retrieved from NCBI Gen Bank and aligned with our target sequences during phylogenetic analysis. Multiple sequence alignments were prepared using Clustal omega [16] with default setting and evolutionary distances were computed using the Kimura 2-Parameter (K2P) method [17]. Tree were constructed by Neighbor-Joining (NJ) method based on the Kimura 2-Parameter distance with the uniform rate substitution among sequences and Maximum likelihood (ML) method using MEGA 5.2. Phylogenies were assessed by a 1000 bootstrap replication [18] to test the reliability of inferred trees and all codon positions were included to verify the robustness of the internal nodes.

3. Results and Discussion

3.1 Nucleotide Analysis

The nucleotide composition was biased towards adenine and guanine. The A+T content varied from 62.98 to 64.35% and stretches of as were more common. (Table 2). It was in consistence with the data on other insect mitochondrial genes [19, 20].

Table 2: A+T/G+C (%) in nine species of termites based on COI gene

S. No.	Identified Species	A+T (%)	G+C (%)
1.	*Microcerotermes beesoni*	64.12	35.88
2.	*Microtermes obesi*	62.99	37.01
3.	*Microtermes unicolor*	64.35	35.65
4.	*Microtermes mycophagus*	62.98	37.02
5.	*Odontotermes horni*	64.01	35.99
6.	*Odontotermes obesus*	63.63	36.37
7.	*Odontotermes gurdaspurensis*	64.00	36.00
8.	*Odontotermes brunneus*	63.23	36.77
9.	*Odontotermes bhagwatii*	63.95	36.05

This high A+T content is a general feature of the *COI* mitochondrial DNA region in arthropods [21-23]. The figure 1 shows the graph depicting the clear picture of nucleotide composition in different termite species.

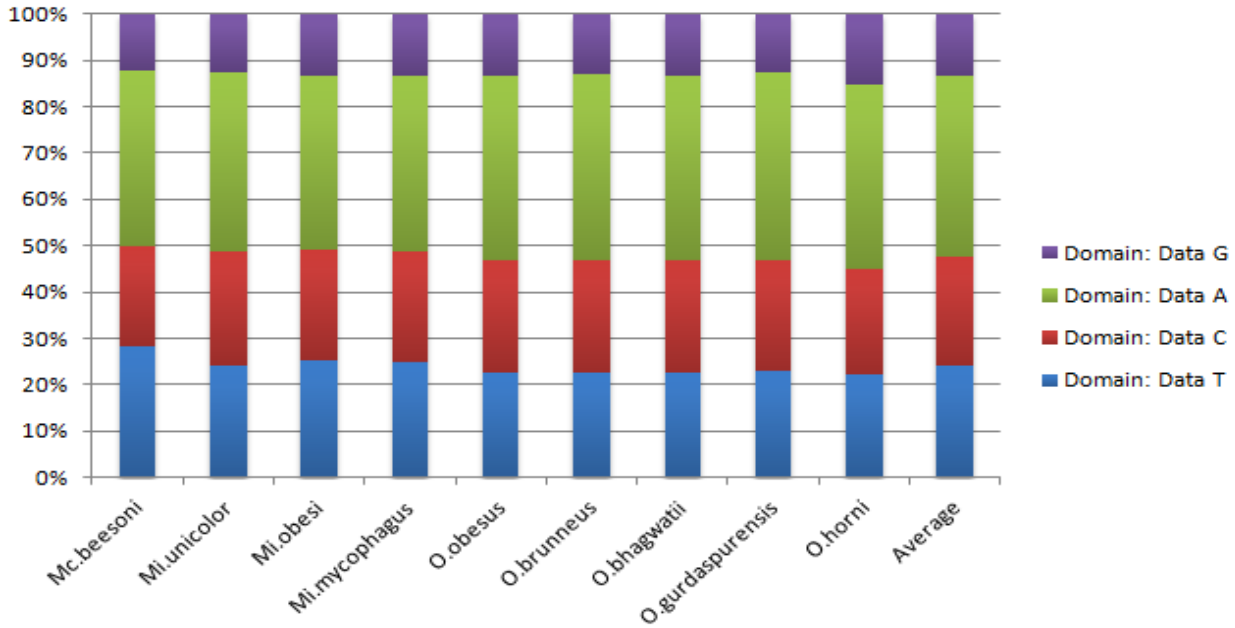

Fig 1: Graph indicating the nucleotide composition in different species of termites under study

Similar observations were made by Wahlberg *et al.* (2003) [24] while sequencing the 140 individuals of genus *Phyciodes* belonging to family Nymphalidae of order Lepidoptera. The A+T content of *COI* gene in genus *Bactrocera* (Diptera: Tephritidae) was slightly lower (63-68%) than that found in *Ceratitis capitata* (70%) [25].

The A+T content for the fragment of genes *COI, 16S* and *12S* in nasuta subgroup of *Drosophila* was 65.30, 77.90 and 75.20 respectively [26]. The genetic structures and arrangements of the mitogene *COI* sequence of the *Coptotermes formosanus* populations collected from the three isolated islands in the Ryukyu Archipelago of Japan were revealed A+T bias [27].

The present results are in accordance with the above studies, it can therefore be concluded that mitochondrial *COI* gene fragment shows A-T rich bias in most of the groups of insects. The phylogenetic analysis based on mitochondrial *COI* gene sequences, thus mirrored the homology between the different termite species and the degree of difference among them gives information about their relationship.

3.2 Pairwise Genetic Distance

The sequences of the partial fragment of *COI* gene from the species under study were used to calculate Pairwise genetic distance values (Kimura 2 parameter) based on *COI* gene using MEGA 5.2 (Table. 3).

Table 3: Pairwise genetic distances (Kimura 2-parameter) based on COI gene fragment in species under study

S. No.	Identified Species	1	2	3	4	5	6	7	8	9
1.	*Odontotermes obesus*									
2.	*Odontotermes brunneus*	0.022								
3.	*Odontotermes bhagwatii*	0.006	0.028							
4.	*Odontotermes gurdaspurensis*	0.022	0.006	0.028						
5.	*Odontotermes horni*	0.076	0.102	0.069	0.103					
6.	*Microtermes unicolor*	0.088	0.092	0.088	0.084	0.139				
7.	*Microtermes obesi*	0.116	0.120	0.116	0.112	0.171	0.025			
8.	*Microtermes mycophagus*	0.095	0.118	0.087	0.119	0.069	0.107	0.137		
9.	*Microcerotermes beesoni*	0.131	0.140	0.140	0.132	0.179	0.119	0.141	0.149	

Congeneric interspecies distance ranged from 0.006 to 0.103 in genus *Odontotermes* while in genus *Microtermes*, it ranged from 0.025 to 0.137. The highest congeneric interspecies genetic distance of 0.103 was observed between O. *gurdaspurensis* and *O. horni* while 0.137 was found to be highest between *M. mycophagus* and *M. obesi*. The intergeneric genetic distance was found to be maximum of genus *Microcerotermes* with other species under observation ranging from 0.119 to 0.149.

3.3 Phylogenetic Inferences

In order to put forth the holistic picture of relationships of Indian species with different geographical species belonging to family Termitidae and also to other families, the sequences of the selected species were retrieved from NCBI public database (Table 4). As the ending region of *COI* is not individually studied much for the genera under study in the literature, the species depicting whole genome related to amplicon region of COI gene fragment of study were included in the analysis. The species included for studying the phylogeny and their accession numbers (given in parentheses) are shown in fig. 2 and 3. Both NJ and ML trees revealed similar topology among the species.

Table 4: List of species whose sequences were retrieved from GenBank public database and included in the analysis

S. No.	Taxon	Family	Subfamily	Locality	Accession number	Author
1	*Reticulitermes santonensis*	Rhinotermitidae	Heterotermitinae	France	JN615370	Djernaes *et al.* (2012) [28]
2	*Reticulitermes santonensis*	Rhinotermitidae	Heterotermitinae	North America	EF206315	Cameron and Whiting (2007) [29]
3	*Reticulitermes flavipes*	Rhinotermitidae	Heterotermitinae	North America	EF206314	Cameron and Whiting (2007)
4	*Reticulitermes hageni*	Rhinotermitidae	Heterotermitinae	North America	EF206320	Cameron and Whiting (2007)
5	*Reticulitermes virginicus*	Rhinotermitidae	Heterotermitinae	North America	EF206319	Cameron and Whiting (2007)
6	*Reticulitermes virginicus*	Rhinotermitidae	Heterotermitinae	North America	EF206318	Cameron and Whiting (2007)
7	*Heterotermes tenuis*	Rhinotermitidae	Heterotermitinae	–	AY553153	Unpublished
8	*Heterotermes sp.*	Rhinotermitidae	Heterotermitinae	Australia	JX144936	Cameron *et al.* (2012) [30]
9	*Coptotermes lacteus*	Rhinotermitidae	Coptotermitinae	Australia	JX144934	Cameron *et al.* (2012)
10	*Macrotermes subhyalinus*	Termitidae	Macrotermitinae	Australia	JX144937	Cameron *et al.* (2012)
11	*Macrotermes barneyi*	Termitidae	Macrotermitinae	China	JX050221	Wei *et al.* (2012) [31]
12	*Microcerotermes sp.*	Termitidae	Termitinae	–	AY553147	Unpublished
13	*Macrognathotermes errator*	Termitidae	Macrotermitinae	Australia	JX144939	Cameron *et al.* (2012)
14	*Drepanotermes sp.*	Termitidae	Macrotermitinae	Australia	JX144938	Cameron *et al.* (2012)
15	*Nasutitermes triodiae*	Termitidae	Macrotermitinae	Australia	JX144940	Cameron *et al.* (2012)
16	*Nasutitermes parvonasutus*	Termitidae	Macrotermitinae	–	AY553146	Unpublished
17	*Odontotermes formosanus*	Termitidae	Macrotermitinae	–	AY553149	Unpublished

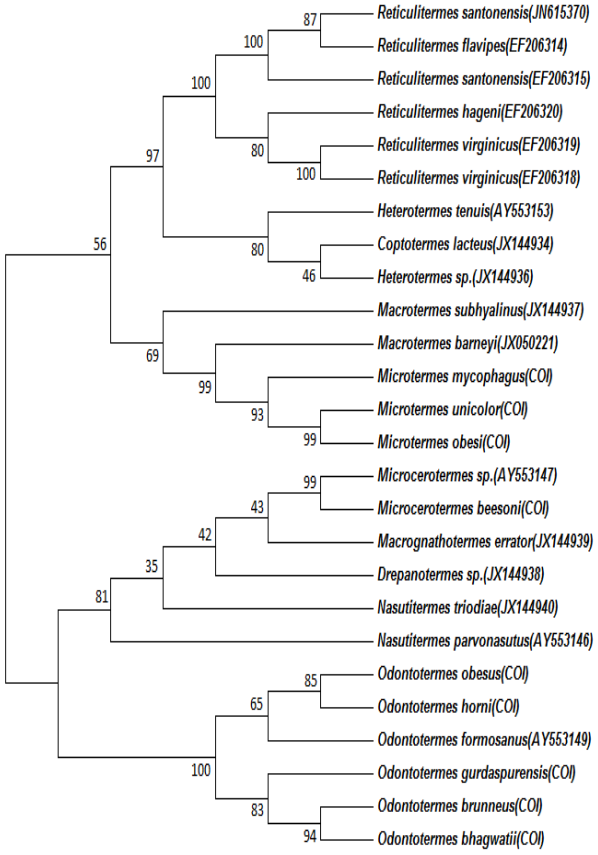

Fig 2: Neighbor-joining (NJ) phylogenetic tree of termite species inferred from *COI* gene fragment

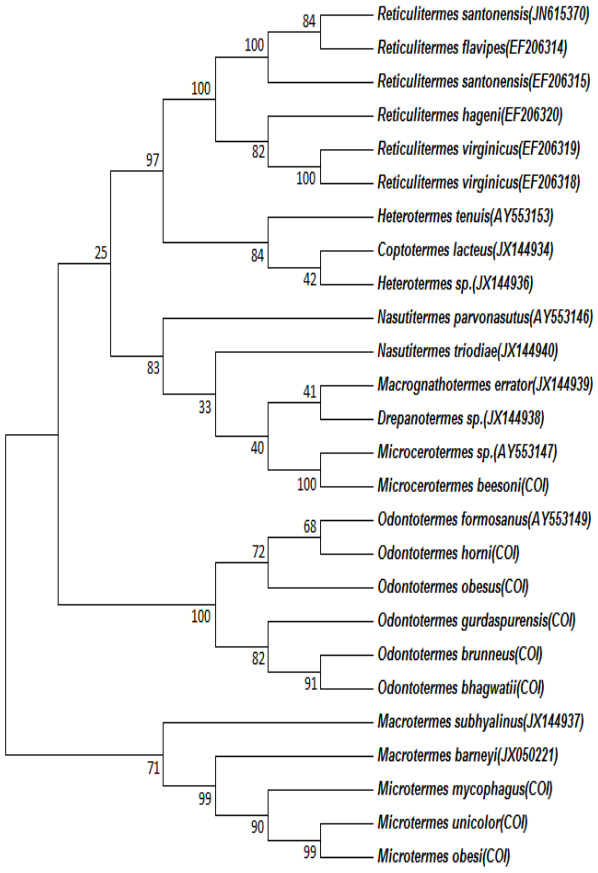

Fig 3: Maximum likelihood (ML) phylogenetic tree of termite species inferred from *COI* gene fragment

Both the phylogenetic trees revealed the similar phylogeny and there was the formation of four major clusters. The grouping of all the *Odontotermes* species under study with the *O. formosanus* was observed which is also listed from an Asian subcontinent. The species belonging to family Rhinotermitidae (*Reticulitermes*, *Heterotermes*, *Coptotermes*) formed a separate clade from the species of family termitidae. The congeneric species of genus *Microtermes* showed relatedness to the species of genus *Macrotermes*. In another major cluster, intergeneric species from the subfamily Termitinae (*Microcerotermes*, *Macrognathotermes*, *Drepanotermes*, *Nasutitermes*) formed a separate cluster and *Microcerotermes beesoni* shows close relatedness to *Microcerotermes sp.* from NCBI database. The congeneric species of genus *Odontotermes* formed a separate cluster showing close relationship amongst them. ML tree also revealed similar observations with minor differences. Bootstrap values were found to be significant for both the trees. The low bootstrap values of 46 indicated the paraphyly of Rhinotermitidae revealing genetic differences.

First comprehensive combined molecular and morphological analysis of the major group of termites was inferred by Inward *et al.* [32]. Their analysis showed the monophyly of Hodotermitidae, Kalotermitidae and Termitidae, while Termopsidae and Rhinotermitidae were observed to be paraphyletic. This was further proved by Legendre *et al.* [33] while studying DNA sequence data of seven gene fragments (*12S* rDNA, *16S* rDNA, *18S* rDNA, *28S* rDNA, *COI*, *COII* and *cytb*) to show phylogenetic relationship amongst the termite species.

The taxonomy, gene flow and evidence for introduction dynamics of two subterranean termite species, *Reticulitermes lucifugus grassei* and *R. flavipes*, by phylogenetic analysis of two mitochondrial genes (*COI*, *COII*) and it was observed that genetic partitioning is strong for all *Reticulitermes* species groups [34].

It has been observed that Rhinotermitid genera *Coptotermes*, *Heterotermes* and *Reticulitermes* are the sister group to the Termitidae, thus making the Rhinotermitidae paraphyletic with respect to the Termitidae, but there has been no support for it being paraphyletic with respect to the Serritermitidae. It has been indicated by Lo *et al*. (2004) [35] by identifying six main lineages, investigating the relationships among genera in the termite family Rhinotermitidae and their relationship to the families Termitidae and Serritermitidae based on analysis of three mitochondrial genes: *COI*, *COII* and *16S* rDNA. Based on the aligned dataset of 929bp of *COI* region, the monophyly of Malagasy *Microtermes* was seen by Nobre *et al.* [36].

It can, therefore, be concluded that the phylogenetic analysis based on the mitochondrial *COI* sequence mirrored the homology between the different termite species, as the degree of difference among them contains the information about their relationships. They group close together according to their family and subfamily as only few species of genus *Odontotermes*, *Microtermes* and *Microcerotermes* have been sequenced for this region of *COI* gene amplified in the present study.

4. Conclusion

The molecular studies carried out on termites species are being extended to include the mitochondrial genes *16S*, *12S*, *NDI* etc. to study different strains of species. The analysis will be valuable in studying molecular taxonomy particularly for those species that are difficult to identify using morphologic characters. Sequence comparison of different geographic region will give estimate of their genetic relatedness when

compared with other genes. Sequence oriented genomics and the correlation of sequence data has established the base for functional genomics and proteomics.

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