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Reconstructing phylogenetic relationship among Indian termite species inferred from COII gene sequences (Blattodea: Isoptera: Termitidae)

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Abstract

To analyze the phylogenetic relationship among nine species of Indian termites, 675 base pairs fragment of complete COII gene was utilized. Neighbor-joining (NJ) and Maximum Likelihood (ML) methods were used in Molecular Evolutionary Genetic Analysis (MEGA 5.2) software to define the molecular phylogeny. Both the trees revealed almost identical topology among the species and paraphyly of *Microcerotermes* and monophyly of the species belonging to subfamily Macrotermitinae of family Termitidae were strongly supported by two reconstruction methods.

Keywords: Termitidae, COII, phylogeny

Introduction

Termites are group of social insects classified under Infraorder Isoptera, holding unresolved evolutionary origin for more than half a century [1-7] and include the most primitive insects of the terrestrial Neoptera, which also comprises cockroaches and mantids often embodied in a Superorder Dictyoptera and order Blattodea. Kambhampati *et al.* [8] carried out first molecular studies in Isoptera on a portion of the mitochondrial large ribosomal subunit gene (16SrRNA), in ten genera of five families to draw interfamilial relationships. Although different molecular markers were considered, the results obtained were similar [9]. The sequences of the mitochondrial genes have been widely used to estimate phylogenetic relationships at different taxonomic levels in insects [10-12].

Till now, the complete nucleotide sequences of the mitochondrial cytochrome oxidase II gene of 13 species of insects, representing 10 orders has been studied. The gene ranges from 673 to 690 bp in length encoding 226 to 229 amino acids. Several insertion or deletion events involving one or two codons can be observed. The 3' end of the gene is extremely variable in both the length and sequence [13]. The usefulness of COII region of the mitochondrial DNA (mt DNA) has been well demonstrated in studying the phylogenetic relationship of termites by some scientists [8, 14-24]. Mitochondrial genes are known to evolve more rapidly than nuclear genes and are therefore good markers to analyze relatively close relationships, such as species relationships within a genus [15, 25-28]. The protein coding COII gene of mitochondrial DNA has been used for many phylogenetic studies of termites from different geographical areas resulting in a large number of sequence data appearing in GenBank makes it is the most sequenced gene so far for the termites. We investigated the phylogenetic relationships among Indian termites of family Termitidae by using complete COII gene sequences.

2. Materials and Methods

2.1 Materials for molecular analysis

Termites for the present study were collected randomly from various regions of India as described in Table 1. Specimens were preserved in rectified alcohol and maintained in the Department of Zoology, Panjab University, Chandigarh (India). Soldier specimens were got identified from Zoological Survey of India, Kolkata and Forest Research Institute, Dehradun employing keys and descriptions of Chottani [29]. 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 varied from 2010 to 2013.

Table 1: Collection data of the Indian termite species (Family: Termitidae)

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

2.2 DNA extraction, PCR and sequencing

Genomic DNA was extracted from worker termites by performing modified phenol: chloroform extraction method [30]. Polymerase Chain Reaction was conducted by applying protocol of Williams *et al.* [31] followed with some modifications. Approximately 780 bp fragment of COII gene flanking with tRNA's was developed. Details of primers are as follows:

1. TL2-J-3037 (5-ATGGCA-GATTAGTGCAATGG-3) was designed by Liu and Beckenbach [13] and described by Simon *et al.* [32] and Miura *et al.* [14]. This is the 3037 primer, so named because of its location in the mt genome; this is partially overlapping with and complimentary to the COI primer 3041.
2. TK-N-3785 (5-GTTTAAAGAGACCAGTACTTG-3) from Simon *et al.* [32], described by O'Grady *et al.* [33] and O'Grady [34]. This is the same as 3771 or 3791 based on its location in the mt genome.

These primers, originally published in Liu and Beckenbach [13], have been very useful for studying species and genus [34] divergence in insects. Primers are anchored in transfer RNA genes flanking COII and are able to amplify the entire gene of approximately 673-690 in length.

The amplified products were got sequenced directly from Chromous Biotech. Pvt. Ltd., Bangalore (India).

2.3 Phylogenetic analysis

The sequences were first edited manually for discarding the ambiguous and skipped bases and files were converted into FASTA format. The edited sequences were compared with related sequences from the nucleotide database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>), using Basic Local Alignment Search Tool (BLAST N) algorithm [35] to make sure that correct target sequences were amplified. Prior to tree building, all sequences were aligned automatically using the multiple alignment algorithm in Clustal omega [36] with the default settings. The evolutionary distances were computed using the Kimura 2-Parameter (K2P) method [37] and Evolutionary analyses were performed in MEGA 5.2 [38]. Phylogenetic analyses was performed employing the Neighbor-Joining (NJ) method based on the Kimura 2-Parameter distance with the uniform rate substitution among sequences and Maximum likelihood (ML) method by MEGA 5.2. Bootstrap analysis using 1000 pseudo replications [39] was included 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

Average amplicon size resulting from DNA sequencing was 780 base pairs (bp). To facilitate genetic comparisons with

existing GenBank DNA sequences, tRNA flanking region from the 5' end and 3' end of the amplicon was excluded, and the remaining 675 bp COII portion was used for further analyses.

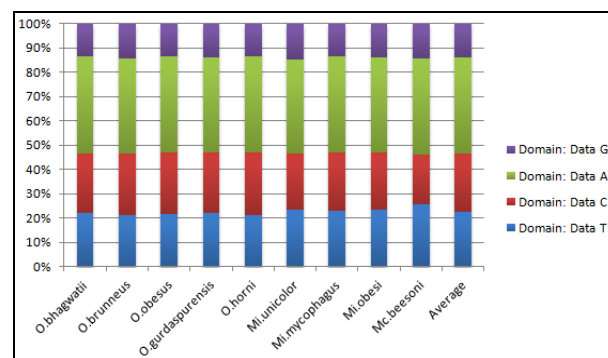
3.1 Nucleotide analysis

The composition of the different nucleotides in the COII gene fragment of the nine species under study was noted and their percentages were calculated (Table 2).

Table 2: Percentage of nucleotides in COII gene fragment in termites species under study

S. No.	Identified Species	A+T (%)	G+C (%)
1.	<i>Microcerotermes besoni</i>	60.75	39.25
2.	<i>Microtermes obesi</i>	60.17	39.83
3.	<i>Microtermes unicolor</i>	60.02	39.98
4.	<i>Microtermes mycophagus</i>	60.72	39.28
5.	<i>Odontotermes horni</i>	59.65	40.35
6.	<i>Odontotermes obesus</i>	59.72	40.28
7.	<i>Odontotermes gurdaspurensis</i>	60.15	39.85
8.	<i>Odontotermes brunneus</i>	60.86	39.14
9.	<i>Odontotermes bhagwati</i>	59.87	40.13

The COII gene fragment showed high percentage of A+T that varied from 59.72 to 60.86. Nucleotide variation showed an adenine thymine bias in their composition that is consistent with data on other insect COII mitochondrial gene studies [40-42]. Figure 1 clearly indicates the same observations.

**Fig 1:** Graph indicating the nucleotide composition in

3.1.1 Species of termites under study

As proven by other researchers, a phylogenetic analysis of Australian dry wood termites was done by Thompson *et al.* [43] utilizing 624 bp long fragment of COII gene which showed an adenine thymine bias in the nucleotide composition (A+T=64.20%). Based on the DNA sequence of a portion of the mitochondrial COII gene of *Reticulitermes* including 21 species and subspecies from three continents, the average base

frequencies were found to be A+T=63% and G+C=37% [22]. Four species belonging to the genus *Odontotermes* (*O. obesus*, *O. horni*, *O. bhagwatii* and *Odontotermes* sp.) and one of *Microtermes* (*M. obesi*), a high A + T content was observed [44]. A + T content was found to be 59.55% and G + C = 40.45% for *Microtermes obesi*.

3.2 Pairwise genetic distance

The sequences of the complete fragment of COII gene from the termites species under study were used to calculate Pairwise genetic distance values (Kimura 2 parameter) using MEGA 5.2 (Table 3).

Table 3: Pairwise genetic distances (Kimura 2-parameter) in species under study

S. No.	Identified Species	1	2	3	4	5	6	7	8	9
1.	<i>Odontotermes bhagwatii</i>									
2.	<i>Odontotermes brunneus</i>	0.063								
3.	<i>Microtermes unicolor</i>	0.188	0.191							
4.	<i>Odontotermes gurdaspurensis</i>	0.072	0.060	0.196						
5.	<i>Odontotermes horni</i>	0.057	0.025	0.174	0.044					
6.	<i>Odontotermes obesus</i>	0.061	0.056	0.187	0.058	0.043				
7.	<i>Microtermes mycophagus</i>	0.127	0.134	0.054	0.139	0.117	0.128			
8.	<i>Microtermes obesi</i>	0.157	0.165	0.030	0.167	0.145	0.161	0.028		
9.	<i>Microcerotermes beelsoni</i>	0.194	0.212	0.229	0.200	0.199	0.199	0.181	0.192	

The K2P distance in congeneric species of genus *Odontotermes* varies from 0.025 to 0.072. It was found to be lowest (0.025) between *O. brunneus* and *O. horni* and highest (0.072) between *O. bhagwatii* and *O. gurdaspurensis*. The interspecies K2P distance of genus *Microtermes* was highest (0.054) between *M. unicolor* and *M. mycophagus* and lowest (0.028) between *M. unicolor* and *M. mycophagus* and *M. obesi*. *Microcerotermes beelsoni* gave highest value with the other species under study. K2P distance of *M. beelsoni* was highest (0.229) with *M. unicolor* and lowest (0.181) with *M. mycophagus*.

3.3 Phylogenetic inferences

Phylogenies are helpful for organizing knowledge of biological diversity, describing structuring classifications and providing insight into the events that occurred during evolution. The sequences of complete COII gene fragment were compared with the sequences available in the database (Table 4) retrieved from National Centre for Biotechnology Information (NCBI). The species retrieved from GenBank were given in both the trees with their accession numbers in parentheses. By using MEGA 5.2 software, phylogenetic trees were drawn by NJ (Fig. 2) and ML (Fig. 3) methods.

Table 4: Species data summary included in the phylogenetic analysis; References are given for sequences obtained from GenBank

S. No.	Taxon	Family	Subfamily	Locality	Accession number	Author
1	<i>Odontotermes bhagwatii</i>	Termitidae	Macrotermitinae	India	EU242525	Sobti <i>et al.</i> [44]
2	<i>Odontotermes horni</i>	Termitidae	Macrotermitinae	India	EU242523	Sobti <i>et al.</i> [44]
3	<i>Hypotermes makhamensis</i>	Termitidae	Macrotermitinae	Thailand	AB109521	Ohkuma <i>et al.</i> [45]
4	<i>Hypotermes xenotermitis</i>	Termitidae	Macrotermitinae	Thailand	AB011409	Miura <i>et al.</i> [14]
5	<i>Odontotermes ceylonicus</i>	Termitidae	Macrotermitinae	Sri Lanka	DQ442207	Inward <i>et al.</i> [46]
6	<i>Odontotermes obesus</i>	Termitidae	Macrotermitinae	India	EU242524	Sobti <i>et al.</i> [44]
7	<i>Odontotermes escherichi</i>	Termitidae	Macrotermitinae	Sri Lanka	DQ442208	Inward <i>et al.</i> [46]
8	<i>Odontotermes species</i>	Termitidae	Macrotermitinae	China	JQ518446	Unpublished
9	<i>Odontotermes species</i>	Termitidae	Macrotermitinae	India	EU242521	Sobti <i>et al.</i> [44]
10	<i>Odontotermes formosanus</i>	Termitidae	Macrotermitinae	China	HQ012032	Huang <i>et al.</i> [47]
11	<i>Odontotermes formosanus</i>	Termitidae	Macrotermitinae	China	JQ518437	Unpublished
12	<i>Odontotermes Formosanus</i>	Termitidae	Macrotermitinae	China	HQ012034	Huang <i>et al.</i> [47]
13	<i>Odontotermes formosanus</i>	Termitidae	Macrotermitinae	Thailand	AB300694	Brandl <i>et al.</i> [48]
14	<i>Odontotermes hainanensis</i>	Termitidae	Macrotermitinae	China	HQ012040	Huang <i>et al.</i> [47]
15	<i>Odontotermes hainanensis</i>	Termitidae	Macrotermitinae	China	JQ518444	Unpublished
16	<i>Odontotermes hainanensis</i>	Termitidae	Macrotermitinae	China	JQ518439	Unpublished
17	<i>Pseudocanthotermes militaris</i>	Termitidae	Macrotermitinae	Africa	DQ442233	Inward <i>et al.</i> [46]
18	<i>Pseudocanthotermes spiniger</i>	Termitidae	Macrotermitinae	Africa	AB304490	Brandl <i>et al.</i> [48]
19	<i>Ancistrotermes cavithorax</i>	Termitidae	Macrotermitinae	Netherland	JF302860	Nobre <i>et al.</i> [49]
20	<i>Ancistrotermes crucifer</i>	Termitidae	Macrotermitinae	Africa	DQ442069	Inward <i>et al.</i> [46]
21	<i>Microtermes obesi</i>	Termitidae	Macrotermitinae	India	EU242522	Sobti <i>et al.</i> [44]
22	<i>Microtermes obesi</i>	Termitidae	Macrotermitinae	Thailand	AB109523	Ohkuma <i>et al.</i> [45]
23	<i>Microtermes pakistanicus</i>	Termitidae	Macrotermitinae	Pakistan	AY940138	Unpublished
24	<i>Microtermes sp.</i>	Termitidae	Macrotermitinae	Africa	JF923190	Hausberger <i>et al.</i> [50]
25	<i>Microtermes sp.</i>	Termitidae	Macrotermitinae	Africa	JF923188	Hausberger <i>et al.</i> [50]
26	<i>Microtermes sp.</i>	Termitidae	Macrotermitinae	Africa	JF923192	Hausberger <i>et al.</i> [50]
27	<i>Microtermes sp.</i>	Termitidae	Macrotermitinae	Africa	JF923191	Hausberger <i>et al.</i> [50]
28	<i>Microtermes sp.</i>	Termitidae	Macrotermitinae	Africa	JF923166	Hausberger <i>et al.</i> [50]
29	<i>Microcerotermes parvus</i>	Termitidae	Termitinae	Africa	DQ442167	Inward <i>et al.</i> [46]
30	<i>Microcerotermes dubius</i>	Termitidae	Termitinae	Borneo	DQ442165	Inward <i>et al.</i> [46]
31	<i>Microcerotermes sp.</i>	Termitidae	Termitinae	China	HQ012038	Huang <i>et al.</i> [47]
32	<i>Microcerotermes serratus</i>	Termitidae	Termitinae	Australia	DQ442168	Inward <i>et al.</i> [46]
33	<i>Microcerotermes newmani</i>	Termitidae	Termitinae	Australia	DQ442166	Inward <i>et al.</i> [46]

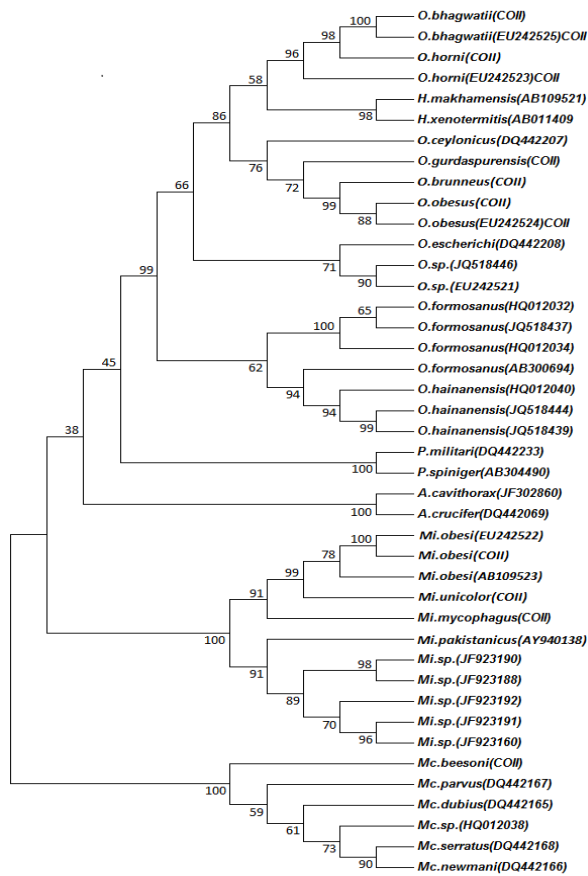


Fig 2: Neighbor-joining (NJ) phylogenetic tree of termite species inferred from *COII* gene fragment

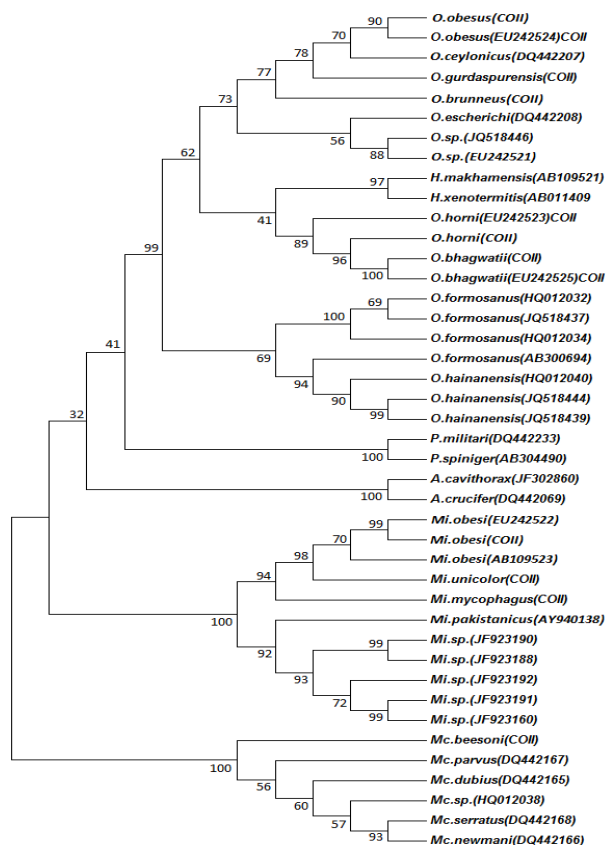


Fig 3: Maximum likelihood (ML) phylogenetic tree of termite species inferred from *COII* gene fragment

The topology of both the trees (NJ, ML) was nearly identical, differing basically in supporting values of nodes. Our phylogenetic analyses defined four robust clades: one (cluster I) included 21 sequences representing all members of genus *Odontotermes* and two species of *Hypotermes*. The second (Cluster II) comprised of *Pseudocanthotermes* and third (Cluster III) comprised *Ancistrotermes*. Fourth (cluster IV) included 11 sequences representing all members of genus *Microtermes*. All the four clusters comprised of the species belong to subfamily Macrotermitinae of family Termitidae.

Within first cluster, two strongly supported subclades were resolved as indicated by high bootstrap value. One subclade was mostly composed of the oriental species of genus *Odontotermes* and *Hypotermes* while the second comprised the Palearctic genera *Odontotermes* with one Oriental species.

In cluster II and III, species respectively belonging to the genus *Pseudocanthotermes* and *Ancistrotermes* formed their own clusters.

In cluster IV, two subclades were resolved belonging to genus *Microtermes*: one subclade comprising of Oriental species and other comprising of all Afrotropical species with one species of oriental origin (*Microtermes pakistanicus*).

A sharp cluster representing subfamily Termitinae of genus *Microcerotermes* was observed at the bottom of both the trees indicating Termitinae to be paraphyletic.

All the congeneric species supported a good bootstrap value indicating genetic relationship between them. The conspecific individuals of genus *O. horni*, *O. obesus*, *O. bhagwatii* and *M. obesi* were supported by more than 85% bootstrap values. All the congeneric species and conspecific individuals clustered together indicating higher bootstrap values.

A comprehensive phylogenetic analysis of the family Rhinotermitidae as a whole has been carried out by Austin *et al.* [51] by using the DNA sequences of a portion of mitochondrial *COII* gene revealing sufficient variation in many species from North America, Europe and Asia [45]. The amount of genetic differentiation among several *Reticulitermes lucifugus* populations was also determined [52] and this study has supported the view that Mastotermitidae is the basal lineage among extant termites and the family Rhinotermitidae is polyphyletic, given the familial status of Serritermitidae. Moreover, distinct groups were established within this family by studies of other scientists [46, 53-58].

Also morphological identifications of Malaysian termites in the family Termitidae (Isoptera) were verified by Lee *et al.* [59] by phylogenetic analyses of *COII* gene sequence. The extent of genetic relatedness/variations within and between various populations of the different species of Indian termites belonging to the family Termitidae was observed by using 12SrRNA, 16S rRNA, COI, COII, NDI genes resulting in dissonant results indicates the usefulness of complete genome analysis of these species [44, 60-66].

Also discussed by Lo *et al.* [67], some Australian termites to be more closely related to Asian termites than to other species native to Australia, again suggesting the possibility of over water movement for a short distance. *Coptotermes formosanus* population of Taiwan, Japan and China and *C. gestroi* populations of Taiwan showed near resemblance with those of Philippines and Hawaii as observed by Li *et al.* [68]. Phylogenetic tree drawn on the basis of combined nucleotide matrices of 12S, 16S and *COII* gene among 11 subterranean termite species of *Coptotermes* from East Asia and Australia revealed two major clades with six subclades [69] showing relatedness among these species.

Thus, it can be inferred that these phylogenetic trees have been

an essential tool in the study of evolution since the time of Charles Darwin. It clearly depicts the utility of such genetic tool in establishing the overall picture of relationship and taxonomic positioning of the lesser known species [70-71]. As expected, the present analysis revealed phylogenetic relatedness either on the basis of genera, species or geographical location and it seems that all these species had common origin but later got diversified with the change in geographical location. Today, similarities in lower termite species across continents such as Australia, South America, and Africa indicated that they retain a genetic link from the time they were all joined as Gondwana [72].

4. Conclusion

The analysis will be valuable in studying molecular taxonomy particularly for those species that are difficult to identify using morphologic characters. Phylogeny can help indicating the identity of the unidentified species but can further be confirmed by morphological analysis using DNA barcoding. Our construction of phylogeny emphasizes the strikingly different species of termites showing genetic relationship with each other belonging to different geographical regions. This profound evolutionary relationship of Indian termites can be further exploited for comparison with other gene sequences.

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