Singla et al., Int J Evol 2013, 2:1
http://dx.doi.org/10.4172/2324-8548.1000101

International Journal of Evolution

Research Article A SCITECHNOL JOURNAL

Genetic Relationship Among Indian Termites Based on DNA Sequence of Mitochondrial *12S* Ribosomal RNA Gene

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Abstract

Partial *12S* gene fragments were amplified by using specific primers in nine species of termites of the genus *Odontotermes, Microtermes* and *Microcerotermes* (*Isoptera: Termitidae: Macrotermitinae*), and the PCR products were subjected to sequence analysis. The sequences obtained were characterized to see the frequencies of each nucleotide bases, and high A+T content was observed. The divergence of the species was found be lower within the same family, and highest with species from the family *Rhinotermitidae*. Phylogenetic tree drawn on the basis of distance Neighbour-joining method revealed clustering of individuals according to their genera and families.

Keywords

Macrotermitinae; Phylogeography; Termites; 12SrRNA

Introduction

Insect molecular systematics has complemented and enhanced the value of morphological and ecological data, making substantial contributions to evolutionary biology in the process. During the past decade, our understanding of the relationship among organisms at various levels of taxonomy has advanced greatly with the aid of DNA molecular systematic techniques and phylogenetic theory. However, the advent of molecular taxonomy provides more sensitive techniques for examining complex identification issues. Advances in method objects have led to the accumulation of large amounts of DNA sequence data from most major insect groups [1].

Molecular taxonomy based on mitochondrial DNA has proved to be an efficient alternative to species identification and their phylogenetic relationships. Infact, mitochondrial markers have been used with a number of insects for systematic and identification purposes [2,3]. The use of mitochondrial genome sequence is further supported by the occurrence of cladistically informative gene order rearrangement events. Mitochondrial sequence data have, therefore, been extensively used in the past 10 years to evaluate the population structure, gene flow, phylogeny, phylogeography and taxonomy of termites [4]. Indeed, several studies based on mitochondrial genome sequences such as the cytochrome oxidase genes and the AT rich region have thrown a great deal of light on termite taxonomy.

Termites are also very important ecological players in tropical ecosystem, having been described as "ecosystem engineers", due to their important role in providing soil ecosystem services [5]. Termites adapt to arid environment and play an important role in decomposition, where common decomposers such as micro bacteria and fungi cannot function. Despite their importance, our understanding of a number of their basic biological processes in termites is extremely limited. Developing a better understanding of termite biology is closely dependent upon reliable species identification. The use of molecular markers may be helpful in estimating phylogenetic relatedness between the termite species and estimating genetic differentiation among local populations within each species. *12SrDNA* is highly conserved, and has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. In general, *12S* and 16S *rDNAs* are the most conserved regions among the mitochondrial genes. Mitochondrial *12S* and *16SrRNA* genes are useful for the phylogenetic studies because of their slow evolutionary rate, and the existence of universal insect primers and ease of reliable PCR amplification.

In the present study, the molecular characterization of nine species of termites of family *Termitidae* has been described, because very scarce information is available in relation to the mitochondrial *12SrRNA* in termite species. The data on this gene in Indian termites is also not available.

Materials and Methods

Collection and storage of samples

The specimens of the termites under study were collected from various locations in India (Table 1). The voucher specimens were preserved in absolute ethanol mixed with a few drops of glycerol, and maintained in the Department of Zoology, Panjab University, Chandigarh (UT), India, till the extraction of genomic DNA.

Identification of termites

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 the keys or descriptions of Roonwal and Chhotani [6]. The details of collection site, date of collection, source and name of the collector were mentioned on each vial.

Isolation of genomic DNA (gDNA)

gDNA was extracted from worker termites using the modified phenol: chloroform extraction method [7]. The whole insect was homogenized in 1.5 ml eppendorf tube in 500 µl of TE (Tris EDTA pH 8), with hand pestle, and the homogenate was centrifuged at 10,000 rpm for 10 min in cooling centrifuge (-4°C). The supernatant was discarded, and the pellet was dissolved in 500 µl of lysis buffer (400 µl of TE and 100 µl of 5% SDS), followed by the addition of 6 µl of Proteinase K, and the solution was incubated at 65°C for 1 and a half hour in the incubator. A mixture of 120 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and the tubes were vortexed

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Received: December 07, 2012 **Accepted:** February 20, 2013 **Published:** February 23, 2013

Table 1: Collection data of termite species belonging to family Termitidae and their accession numbers from the Genbank sequence submission for partial 12S rRNA gene sequences.

S.No	Identified Species	Collection Site	Source	Family	Subfamily	Accession number
1.	*Microcerotermes beesoni* (Snyder)	South Goa	Cashew nut tree	Termitidae	Termitinae	JX263665
2.	*Microtermes obesi* (Holmgren)	Hallu majra and Outskirts of Sukhna Lake, Chandigarh	Damp wood	Termitidae	Macrotermitinae	EU551158
3.	*Microtermes unicolor* (Snyder)	Village Kothe Radha, Jagraon, Ludhiana Distt. (Punjab)	Log of wood	Termitidae	Macrotermitinae	JX263667
4.	*Microtermes mycophagus* (Desneux)	Badehar, Hamirpur Distt. (Himachal Pradesh)	Tree trunk	Termitidae	Macrotermitinae	JX045651
5.	*Odontotermes horni* (Wasmann)	Sec.11, Chandigarh	Damp wood, tree	Termitidae	Macrotermitinae	EU551159
6.	*Odontotermes obesus* (Rambur)	Malak Road, Jagraon (Punjab)	Mound	Termitidae	Macrotermitinae	EU551160
7.	*Odontotermes gurdaspurensis* (Holmgren and Holmgren)	Mandi (Himachal Pradesh)	Mound	Termitidae	Macrotermitinae	JX263666
8.	*Odontotermes brunneus* (Hagen)	Pipli, Haryana	Wood	Termitidae	Macrotermitinae	JX263664
9.	*Odontotermes bhagwatii* (Chatterjee and Thakur)	Sec. 10, Chandigarh	Tree	Termitidae	Macrotermitinae	EU551161

for 30 s, and then centrifuged for 10 min at 10,000 rpm in cooling centrifuge. The upper aqueous layer was carefully transferred to a fresh tube, without disturbing the protein layer at the interphase. 500 µl of isopropanol was added to this aqueous layer and stored at -4°C overnight, and then centrifuged at 7,000 rpm for 10 min. The supernatant was discarded and 70% ethanol was added to the pellet. The alcohol was drained out; the pellet was dried and dissolved in 30 µl of TE. It was stored at –20°C, after checking on 0.8% agarose gel.

Quantification of DNA by nanodrop spectrophotometer

The concentration of extracted DNA was determined by nanodrop spectrophotometer. All organic compounds have characteristic absorption spectra. The nitrogenous bases in double stranded DNA exhibited a strong absorption minimum at a wavelength of 260 nm.

PCR amplification of *12SrRNA* gene fragment

The DNA obtained was used for amplifying a portion of mitochondrial *12S rRNA* gene fragment, using universal primer sequences 12S-F (SR-J-14199) 5' TACTATGTTACGACTTAT 3' and 12S-R (SR-N-14594) 5'AAACTAGGATTAGATACCC 3' [8], and a product of 430 bp length was obtained in termites (Figure 1). The primers were procured from Chromous Biotech Pvt. Ltd., Bangalore. The protocol of Williams et al. [9] was followed. Each reaction mixture of 25 µl consisted of 2.5 µl of 10X PCR buffer, 2.0 µl $MgCl_2$ (2.5 mM), 0.2 µl dNTPs (200 µM), 1 µl of *Taq* Polymerase (1U/ µl), 1 µl of each of forward and reverse primer sequences, 1 µl of DNA, and 16.3 µl of distilled water. The amplification was carried out in thermal-cycler (BioRad, USA), following PCR conditions as denaturation at 94°C for 5 min, annealing and extension at 49°C for 45 seconds, and 72°C for 1 min, respectively, and final extension was carried out at 72°C for 5 min. The whole process was carried out in a total of 31 cycles. The amplified products were run on 2% agarose gel (stained with ethidium bromide) with DNA ladder (100 bp). Gels were visualized in a gel doc system.

Sequencing

The most commonly used method of DNA sequencing is the dideoxy method or chain termination method [10]. The key to the method is the use of modified bases called dideoxy bases, *i.e.* ddNTPs. The amplified products of *12SrRNA* were got sequenced from Chromous Biotech Pvt. Ltd., Banglore. The *12SrRNA* sequence data was retrieved in the form of Chromatograms. The sequence data so obtained was submitted to nucleotide public database (PUBMED), and accession numbers are given in table 1.

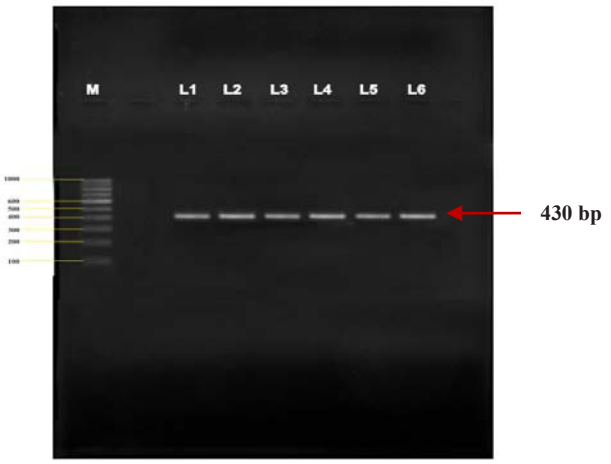

Figure 1: PCR amplified products of *12S rRNA* gene in various species of termites. Lane M: 100 bp DNA ladder, Lanes L1- L6: amplified products.

Sequence analysis and data interpretation

Chromatograms were edited to discard ambiguous bases, and edited sequences were aligned by using the Basic Local Alignment Search Tool (BLAST) [11], with the sequences of same or related genera retrieved from the nucleotide database (PUBMED) of National Centre for Biotechnology Information (NCBI) [12] (Table 2). The *12SrRNA* nucleotide sequences of the entire termite species included in the present study were aligned and compared with the species obtained from PUBMED, using the CLUSTAL W alignment [13]. Neighbor joining phylogenetic tree was drawn by using 'Meg Align' program of 'Lasergene' software package (DNASTAR Inc., USA).

Table 2: List of species from Termitidae and Rhinotermitidae families whose sequences were retrieved from GenBank public database and included in the analysis.

S. No.	Species	Family	Subfamily	Accession numbers
1	*O. mathuri*	Termitidae	Macrotermitinae	DQ441780
2	*O. ceylonicus*	Termitidae	Macrotermitinae	DQ441778
3	*O. escherichi*	Termitidae	Macrotermitinae	DQ441779
4	*O. hainanensis*	Termitidae	Macrotermitinae	EU253723
5	*M. arboreus*	Termitidae	Termitinae	DQ441734
6	*M. dubius*	Termitidae	Termitinae	DQ441735
7	*Reticulitermes arenicola*	Rhinotermitidae	Reticulitermitidae	AY168215
8	*Reticulitermes tibialis*	Rhinotermitidae	Reticulitermitidae	AY168221

Results

Nucleotide analysis

The amplification and sequence of *12SrRNA* gene of nine species of termites under study yielded 430 base pair long fragment. The detailed nucleotide sequence analysis of gene revealed significantly high percentage of A+T base composition content in each of the studied species. The sequence base composition of various species of termites depicted average composition of A+T=65.15% and G+C=34.84% (Table 3). The predominance of transitions over transversions was observed in the gene sequence data of *12SrRNA*.

Divergence and percent identity

Based on sequence alignment, the divergence and percent identity was calculated using laser gene software. The minimum divergence of 1.2 was observed between two in group species of genera *Odontotermes, i.e. O. escherichi* and *O. bhagwatii* showing close resemblance to each other. The maximum divergence of 26.1 was observed between *O. horni* and *Microcerotermes beesoni.* The divergence of the species belonging to family *Rhinotermitidae* was found to be highest with species from family *Termitidae* (Figure 2).

Phylogenetic inferences

A phylogenetic tree of the species using Neighbour joining method was drawn on the basis of multiple sequence alignment of *12SrRNA* gene (Figure 3). The similarity data and constructed phylogeny revealed the formation of two major clusters. The two *Rhinotermitidae* species, *i.e. R. arenicola* and *R. tibialis,* formed an entirely separate cluster from the other *Termitidae* species. The two species of *Microcerotermes, i.e. M. arboreous* and *M. dubius,* formed a clade with the species under study, *i.e. Microcerotermes beesoni,* while the other species belonging to the genus *Microtermes* and *Odontotermes* originating from the major cluster showed a close relatedness to the species belonging to their respective genera. The phylogenetic relationships of various termite families of Indian origin have been well demonstrated by using *12SrRNA* region of mitochondrial DNA.

The precise grouping of the different *Isopteran* species in the present study is in accordance with the geographical location, and it clearly depicts the utility of such genetic tool in establishing the overall structure of relationship and taxonomic positioning of the lesser known species.

Table 3: A+T and G+C content (%) for partial *12SrRNA* of the different termite species belonging to family Termitidae.

S.No.	Identified Species	A+T (%)	G+C (%)
1.	*Microcerotermes beesoni*	64.70	35.30
2.	*Microtermes obesi*	65.58	34.42
3.	*Microtermes unicolor*	65.27	34.73
4.	*Microtermes mycophagus*	64.72	35.28
5.	*Odontotermes horni*	65.65	34.35
6.	*Odontotermes obesus*	64.98	35.02
7.	*Odontotermes gurdaspurensis*	64.52	35.48
8.	*Odontotermes brunneus*	65.38	34.62
9.	*Odontotermes bhagwatii*	65.60	34.40

Discussion

The use of mitochondrial DNA in population genetic studies has been popular due to the extensive intraspecific polymorphism it exhibits, and it has become increasingly clear that genetic variability among and within the populations of pests, affects the success of biological control agents [14]. The classification based on morphological features poses problems because of their small size, morphological attributes that change as a function of environment and prevalence of biotypes and species that cannot be easily differentiated by morphological criteria. Comparisons among mitochondrial DNA sequences of closely related species showed that one type of substitution is more likely than others. The gene sequence data of *12SrRNA* gene showed a predominance of transitions over transversions. The most frequent transversions were of the A-T type. Other types were very rare, and accounted for only few percent of the transversion differences. The difference could be due to deficient mitochondrial DNA repair mechanism and tautomeric base pairing.

Sequence comparison of a fragment of *12S* mitochondrial rRNA gene was also used to infer phylogenetic relationship among six species of *Reticulitermes,* namely *R. flavipes*, *R. arenincola*, *R. tibalis*, *R. hageni*, *R. virginicus* and *R. hesperus* [15]. The study revealed that *R. flavipes* and *R. arenincola* to be possibly conspecific, while other clade included *R. tibalis* and *R. hesperus* in one sister group. Phylogenetic relationship studies on *COII*, *16S* and *12S* genes were carried out on the six species of termites belonging to the genus *Reticulitermes* (*Rhinotermitidae*). The average nucleotide composition in *12S* rRNA fragment of various species of *Reticulitermes* reveals 43.98% (A), 22.29% (T), 12.45% (G) and 21.27% (C), as with average AT bias of 66%. The present *Isopteran* species under study also have the same

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		100.0	97.7	89.3	100.0	89.9	84.5	97.5	91.3	98.0	97.2	97.2	87.9	88.2	96.6	34.6	35.8	**1**	M.mycophagus
2	0.0		97.7	89.3	100.0	89.9	84.5	97.5	91.3	98.0	97.2	97.2	87.9	88.2	96.6	34.6	35.8	**2**	M.unicolor
3	2.4	2.4		88.2	97.7	89.0	83.7	96.9	89.9	96.9	96.6	96.6	86.5	87.3	96.3	34.6	35.8	**3**	O.gurdaspurensis
4	11.4	11.4	12.8		89.3	90.1	76.9	88.5	83.4	89.3	87.9	88.7	95.8	96.1	88.2	32.7	33.8	**4**	M.beesoni
5	0.0	0.0	2.4	11.4		89.9	84.5	97.5	91.3	98.0	97.2	97.2	87.9	88.2	96.6	34.6	35.8	**5**	O.brunneus
6	9.2	9.2	10.2	7.8	9.2		78.9	89.3	85.4	89.6	89.3	89.9	87.9	89.3	89.9	31.8	33.0	**6**	M.obesi
7	16.1	16.1	17.3	26.1	16.1	21.4		84.8	82.8	83.1	83.9	84.5	76.9	77.7	84.2	29.3	30.7	**7**	O.horni
8	2.1	2.1	2.8	11.8	2.1	9.2	15.0		89.9	97.2	97.2	97.5	87.0	87.9	97.5	34.4	35.5	**8**	O.bhagwatii
9	7.0	7.0	8.7	15.9	7.0	12.4	15.1	8.0		91.0	90.4	90.4	83.1	83.1	90.4	33.2	34.4	**9**	O.obesus
10	1.8	1.8	3.1	11.1	1.8	9.2	17.6	2.8	7.0		96.6	96.6	87.3	87.9	96.6	34.4	35.5	**10**	O.mathuri
11	2.5	2.5	3.1	12.5	2.5	9.9	16.2	1.8	8.0	2.8		98.0	86.2	87.9	96.6	33.5	34.6	**11**	O.ceylonicus
12	2.1	2.1	2.8	11.1	2.1	8.8	15.0	1.2	7.7	2.5	1.8		86.8	88.5	96.3	34.4	35.5	**12**	O.escherichi
13	11.5	11.5	13.3	3.1	11.5	8.9	24.9	11.9	14.6	11.9	13.0	11.9		95.8	87.0	32.4	33.5	**13**	M.arboreus
14	11.9	11.9	13.0	3.4	11.9	8.6	24.5	11.6	16.1	11.9	11.6	10.5	3.5		88.2	32.1	33.2	**14**	M.dubius
15	3.1	3.1	3.4	12.2	3.1	8.5	15.8	2.1	7.3	3.4	2.5	2.5	12.0	11.2		34.1	35.2	**15**	O.hainanensis
16	162.0	162.0	162.0	180.7	162.0	204.3	245.5	163.0	176.1	169.0	178.1	165.2	181.3	192.6	165.7		95.8	**16**	R.arenicola
17	142.6	142.6	142.6	156.0	142.6	168.1	182.4	143.5	153.4	147.4	153.5	145.0	156.6	162.6	145.5	3.5		**17**	R.tibialis
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**		

Figure 2: Matrix showing the divergence and percent identity among the nine species under study and the eight species of termites retrieved from the database which acts as an out-group taxa

(*O* denotes *Odontotermes* in *O. horni*, *O. obesus*, *O. gurdaspurensis*, *O. brunneus*, *O. bhagwatii*, *O. mathuri*, *O. ceylonicus*, *O. escherichi*, *O. hainanensis*
M denotes *Microtermes* in *M. unicolor*, *M. mycophagus*, *M. obesi*
M denotes *Microcerotermes* in *M. beesoni*, *M. arboreous*, *M. dubius*
R denotes *Reticulitermes* in *R. arenicola*, *R. tibialis*)

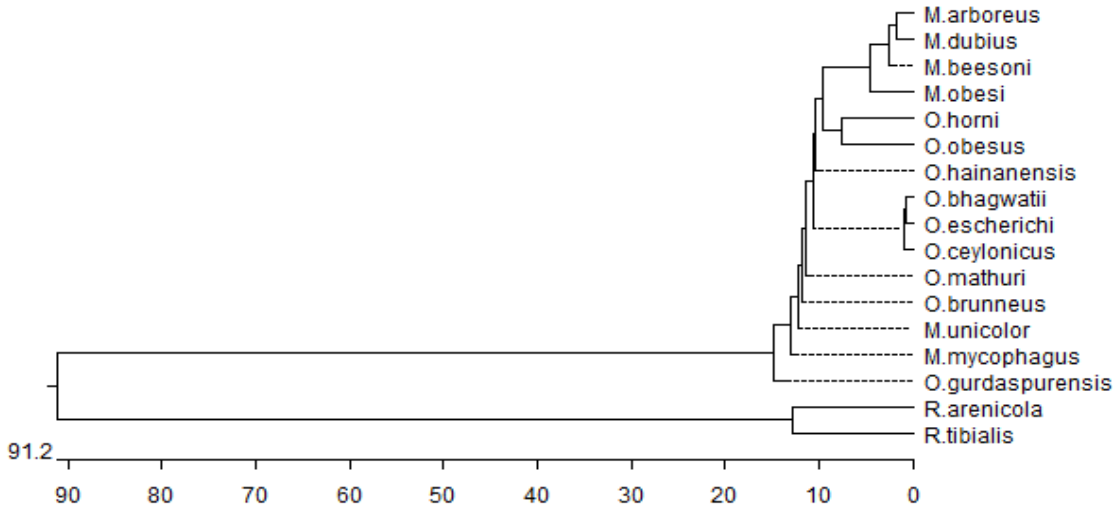

Figure 3: A phylogeny of Indian termite species of Termitidae family in relation to species of Rhinotermitidae and Termitidae families from different parts of the world.

(*O* denotes *Odontotermes* in *O. horni*, *O. obesus*, *O. gurdaspurensis*, *O. brunneus*, *O. bhagwatii*, *O. mathuri*, *O. ceylonicus*, *O. escherichi*, *O. hainanensis*
M denotes *Microtermes* in *M. unicolor*, *M. mycophagus*, *M. obesi*
M denotes *Microcerotermes* in *M. beesoni*, *M. arboreous*, *M. dubius*
R denotes *Reticulitermes* in *R. arenicola*, *R. tibialis*)

range of AT content. It had also been showed that Californian *R. flavipes* closely resembled the Chilean *R. flavipes,* on the basis of their combined mitochondrial DNA sequences. It also suggested that both Chilean and Californian *R. flavipes* have the same origin in North America [16].

Molecular genetic techniques have made contributions to studies on subterranean termites at all levels of biological organization. As additional molecular tools and genomic resources become available, and as more termite researchers incorporate molecular techniques into their approaches, accelerating advances are expected in all aspects of the biology of this group [17].

Conclusion

This kind of information is valuable in studies related to characterization and molecular comparisons between the sequences

of different species of Indian termites. The information generated on the basis of this data can be used for the molecular identification of Indian termite species, their prevalence and classification, which are difficult to differentiate on the basis of morphological parameters.

This data can also be exploited to study the degree of genetic relationships amongst Indian species, and also with the species from other regions of the world. The information generated from *12SrRNA* gene in combination with other genes, such as *16S, COI, COII* and *COIII*, commonly used for the phylogenetic studies, will provide high resolution among most ambiguous species of termites.

Acknowledgements

We acknowledge University Grants Commission (UGC), New Delhi for financial support; Department of Zoology and Department of Biotechnology, Panjab University, Chandigarh, for required laboratory facilities.

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