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Genetic diversity among Indian termites based on mitochondrial *12S rRNA* gene

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ABSTRACT

Genetic relationship among termites collected from various locations was characterized based on 12S rRNA gene using specific primers. Sequence analysis and divergence among the species was assessed. Genbank accession numbers were obtained for the different species. Phylogenetic tree based on Maximum-Likelihood method was drawn on the basis of multiple sequence alignment , which revealed clustering of individuals according to the genera. Among the species, Microtermes obesi and Neotermes koshonensis were distinct from others.

Keywords: Termite, PCR, Phylogeny, *12S rRNA*, Sequence

INTRODUCTION

Termites (Order: Isoptera) are serious pests of agricultural, horticultural and plantation crops including forest trees, especially in the semi arid and sub-humid tropics and cause significant yield losses (over one billion dollars in the United States alone). They attack roots and above ground parts , attack wooden structures, timber and paper. More than 2800 species in about 200 genera distributed over nine families and fourteen subfamilies is presently recognized [1,2]. In India approximately 300 species, 37 genera from seven families are reported [3]. Termites are often described as "ecosystem engineers" [4], due to their role in recycling of soil organic matter and decomposition [5,6],which make up to 95% of the soil insect biomass [7].

Agriculturally important termites include genera from the following families: Hodotermitidae (Anacanthotermes, Hodotermes), Kalotermitidae (Neotermes), Rhinotermitidae (Copotermes, Heterotermes and Psammotermes) and Termitidae (Amitermes, Aneistrotermes, Cornitermes, Macrotermes, Microcerotermes, Microtermes, Odontotermes, Procornitermes and Syntermes) [8, 9].

Lack of taxonomic understanding has been a major impediment to the study and management of termites. Identification of termite species is a challenging task due to the ambiguity in their morphological characters and crypto-biotic social structure **[**10]. Molecular tools have come in handy to complement the value of morphological taxonomy and to understand the evolutionary relationships among the species. Molecular taxonomy based on mitochondrial DNA has proved to be an efficient alternative to species identification and their phylogenetic relationships [11]. DNA sequences of the mitochondrial genes cytochrome oxidase subunit II (CLII) , ribosomal RNA (r RNA) large subunit (16S) and the r RNA small subunit (12S) have been extensively used by [12], [13-19]

for molecular diagnostics and to conduct comparative genetic analyses to study the taxonomy, gene flow, colony characterization and genetic variations. Genetic diversity in subterranean termites was also studied using RAPD markers [20,21].

The use of molecular markers may be helpful in estimating phylogenetic relatedness between the insect species and estimating genetic differentiation among local populations within each species. Hence, studies were contemplated to estimate the genetic differentiation among the population of termites and assess their phylogeny using the most conserved mitochondrial gene, *12S rRNA.*

MATERIALS AND METHODS

Collection and identification of termite populations

The termite specimens were collected from different geographical locations in India. The specimens were preserved in absolute alcohol and stocked at the Division of Molecular Entomology, National Bureau of Agricultural Insect Resources, Bangalore, India. The specimens were identified at the ZSI, Kolkata, Department of Entomology, GKVK, Bangalore and Institute of Wood Science and Technology, Bangalore. The termites collected were preserved in 90% ethanol and stored at -80˚C.

Molecular characterization

Isolation of genomic DNA

DNA was extracted from 10-15mg of alcohol preserved specimens using DNeasy Qiagen Kit. DNA samples were stored in the Qiagen elution buffer at 4˚C until subjected to PCR. The genomic DNA was extracted from worker termites collected from different locations and host plants adopting the phenol chloroform extraction method in 500 [22]. The whole insect was homogenized in 1.5 ml appendorf tube in 500 µl of TE (Tris-EDTA-pH 8), with hand pestle and the homogenate was centrifuged at 10,000 rpm for 10 minutes in cooling centrifuge (-4^{0}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^{0}C for one and half hours in the incubator. A mixture of 120 µl phenol chloroform isomyl alcohol (25:24:1) was added and the tubes vortexed for 30 seconds and then centrifuged for 10 minutes at 10,000 rpm in cooling centrifuge. The upper aqueous layer was carefully transferred in to fresh tube, without disturbing the protein layer at the interphase. 500 µl of isopraponal was added to this aqueous layer and stored at -4^{0}C overnight and then centrifuged at 7000 rpm for 10 minutes. 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.I was stored at -20^{0}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’.. The primers were procured from M/s Eurofins Limited, Bangalore. The protocol of [23] was followed. Each reaction mixture of 25 µl consisted of 2.5 µl of 10X PCR buffer, 2.0 µl MgCl2 (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 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 at M/s. Eurofin Pvt Ltd, Bangalore. The *12SrRNA* sequence data was retrieved in the form of Chromatograms. The sequence data’s was submitted to NCBI and accession numbers

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), with the sequences of same or related genera retrieved from the nucleotide database (PUBMED) of National Centre for Biotechnology Information (NCBI). 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 [24]. Neighbor joining phylogenetic tree was drawn by using 'Meg Align' program of 'Lasergene' software package (DNASTAR Inc., USA).

Phylogenetic Analysis

The Blast search analysis (http://www.ncbi.nlm.nih.gov) was done to compare all the sequences of 12S rRNA gene sequences available in the Gene bank data base. Phylogenetic tree was constructed using character based Maximum-Likelihood method based on the Tamura-Nei model [25]. MEGA-6 bioinformatics tool was used to construct phylogenetic tree and the genetic relatedness between the isolates was analyzed. The bootstrap analysis using 1000 iterations was done to test the accuracy of phylogeny. Constructed phylogenetic tree was visualized using tree viewer program. The gamma distribution shape parameters and substitution rates were used in phylogenetic analysis.

RESULTS AND DISCUSSION

The genomic DNA was isolated from 15 populations of termites and the 12S rRNA gene was characterized, using universal primer sequences *12S*-F (SR-J-14199) 5' TACTATGTTACGACTTAT 3' and 12S-R (SR-N-14594) 5'AAACTAGGATTAGATACCC 3' and a product of 650 bp length was obtained in termites.

Nucleotide analysis

The detailed nucleotide sequence analysis of gene revealed that nucleotide frequencies are 27.86% (A), 37.90% (T/U), 16.15% (C), and 18.10% (G). The transition/transversion rate ratios are k_1 = 4.841 (purines) and k_2 = 2.698 (pyrimidines). The overall transition/transversion bias is R = 1.647, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. Based on sequence alignment, the divergence and percent identity was calculated using Mega 6 software. The amplified products of *12SrRNA* were got sequenced at M/s. Eurofin Pvt Ltd, Bangalore. The *12SrRNA* sequence data was retrieved in the form of Chromatograms. The sequence data's was submitted to NCBI and accession numbers are given in Table 1.

Table 1: Place of collection of termite species and accession numbers of gene bank

Sr. NO	Code	Place of Collection	Organism	Accession number
1	DAST-2	Dasarahali	*Microtermes obesi*	KM657488
2	Phas-t-2	Phasighat	*Odentotermes mathuri*	KM647487
3	Dast-3	Dasarahali	*Euhamitermes hamatus*	KM657484
4	Then-TE-1	Thengudi	*Neotermes keshonensis*	KM657485
5	OOTY-TE-1	Ooty	*Odentotermes bhagwatii*	KM523662
6	OOTY-TE-3	Ooty	*Odentotermes bhagwatii*	KM523663
7	IBS-M-3	GKVK Bangalore	*Odentotermes gurdaparensis*	KM523664
8	IBS-M-9	GKVK Bangalore	*Odentotermes gurdaparensis*	KM253665
9	IBS-M-7	GKVK Bangalore	*Odentotermes gurdaparensis*	KM523666
10	IBS-M-10	GKVK Bangalore	*Odentotermes gurdaparensis*	KM523667
11	IBS-M-4	GKVK Bangalore	*Odentotermes gurdaparensis*	KM657483
12	IBS-M-1	GKVK Bangalore	*Odentotermes gurdaparensis*	KM657482
13	IBS-M-8	GKVK Bangalore	*Odentotermes gurdaparensis*	KM657481
14	Yelahanka	Yelahanka	*Odentotermes gurdaparensis*	KM657480
15	Rajan-T-2	Rajankunte	*Microtermes mycophagus*	KM657479

Earlier, Sequence comparison of a fragment of 12S mitochondrial rRNA gene was used by [16] to infer phylogenetic relationship among six species of *Reticulitermes*, namely *R. flavipes*, *R. arenincola*, *R. tibalis*, *R. hageni*, *R. virginicus* and *R. Hesperus*. 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. Studies by [26, 27 and 28], on *COII*, *16S* and *12S* genes of six species of termites belonging to the genus *Reticulitermes* (*Rhinotermitidae*), indicated average nucleotide composition of 43.98% (A), 22.29% (T), 12.45% (G) and 21.27% (C), with average AT bias of 66% in *12S rRNA* fragment of various species of *Reticulitermes.*

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 difference could be due to deficient mitochondrial DNA repair mechanism and tautomeric base pairing [29].

Phylogenetic analysis

A Phylogenetic tree of the species using Maximum-Likelihood method was drawn on the basis of multiple sequence alignment of *12SrRNA* gene (Fig. 1). The constructed phylogeny revealed the formation of two major clusters. The genus belonging to *Microtermes* and *Odontotermes* originating from the major cluster showed a close relatedness to the species belonging to their respective genera. The first clade had separated from the second cluster with strong bootstrap value of 100%.

In the present study the three *Odentotermus* sp formed the separate clade along with *Neotermes keshonensis* and *Reticulitermes arenicola.* The two *Odentotermes gurdaparensis* collected from Bangalore separated from reset of *Odentotermes gurdaparensis* collected from the same place. The grouping of the different species is in accordance with the geographic locations and their taxonomic positions based on the 12S r RNA. Our observations, broadly corroborate with the reports of [29], who reported genetic relationship of Indian termites based on 12S rRNA and those by [7] and [30] and on phylogeny of Asian termites based on COII gene of 31 genera from families Termitidae and Rhinotermitidae. A more extensive sampling of the genera may be required to confirm our studies. Phyloegentic analysis of termites illuminates key aspects of evolutionary biology (mapping of biological traits, nesting type, feeding groups and family relationships) [1,31, 32]. Phylogenetic analysis of evolutionary relationships may further lead to greater accuracy of pest management with insecticides on the basis that species closely related, are likely to share similar physiology [33]. [34] reported that phylogenetic analyses of mt DNA sequences corroborate taxonomic designations based on cuticular hydrocarbons in termites. Qualitative and quantitative differences in CHC profiles aid in identification of putative taxa. Our results demonstrate that DNA sequences of genes that are not likely to vary in function are useful for inferring termite phylogeny. However, the present studies do not lead to exclusive inference, since a large number of termite groups are yet to be analyzed.

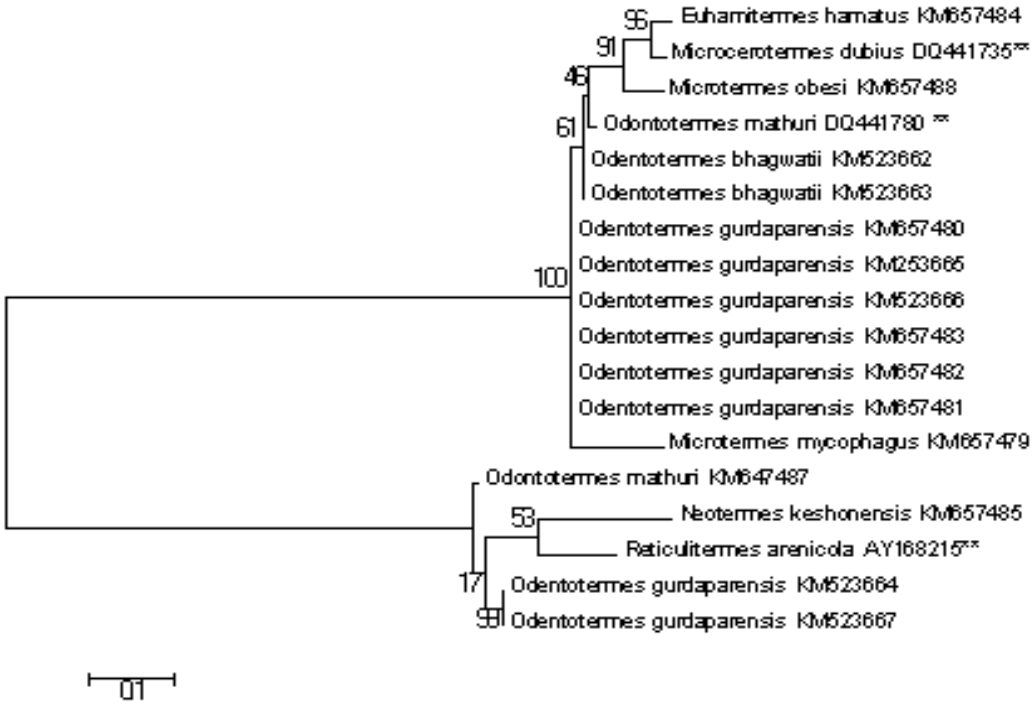

Fig 1: A phylogeny of Indian termite species of Termitidae family

*Note: Where ** marked indicate sequences from the NCBI*

CONCLUSION

The *12S rRNA* gene was characterized for assessing the genetic diversity among the common subterranean termites of India collected from various locations. Phylogenetic analysis and evolutionary divergence matrix indicated differences among the species. The information can be utilized to study the systematics of Indian termites, characterization and molecular comparisons between the sequences of different species of Indian termites, their evolution and also address the ambiguities in morphological taxonomy.

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