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PCR-SSCP and sequence analysis of three populations of *Microtermes obesi* (Order: Isoptera; Family: Termitidae) from Chandigarh (India) on the basis of partial *16Sr RNA* and *ND1* gene

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Abstract

In the present work three populations of Microtermes obesi collected from Chandigarh, India were studied. PCR-SSCP and sequencing techniques have been applied to characterize the partial sequences of two mitochondrial genes *i.e.*, 16Sr RNA and 16Sr RNA tRNA leu ND1 of these populations. SSCP analysis revealed two types of conformational patterns for each gene. Types-I and –II were found in 419 bp long 16Sr RNA and Types-A and -B in 532 bp long 16Sr RNA tRNA leu ND1. A+T content was seen to be high for each gene which was above 60%. Stretches of As were more in 16Sr RNA, while in 16S rRNA tRNA leu ND1, stretches of Ts were more. For 16Sr RNA the percent diversity was found to be zero within M. obesi individuals. In case of 16Sr RNA tRNA leu ND1 it ranged between 0.2 to 0.7 %. Thus, in this study ND1 gene was found to be evolving faster than 16Sr RNA.

Keywords: *16Sr RNA tRNA leu ND1* | PCR - SSCP analysis | mitochondrial genes | conformational patterns | percent diversity | sequence variations

Introduction

Termites of the genera *Odontotermes* and *Microtermes* cause the heaviest destruction of seasoned timber both within buildings and extramurally. Losses due to termites run to several millions of rupees in agricultural crops alone. About 10-25 per cent loss is estimated in most field and forest crops. Severe loss in different regions of India has been recorded on highly susceptible crops such as wheat and sugarcane in northern India, maize, groundnuts, sunflower and sugarcane in southern India, tea in North-Eastern India and cotton in western India. Termite problems in agriculture in Southeast Asia largely affect

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perennial tree crops. The most economically important genera throughout Southeast Asia are *Microtermes, Coptotermes, Odontotermes, Macrotermes, Trinevitermes* and *Heterotermes.*

Though a good deal of work has been carried out on the taxonomy of Indian termites based on their morphological aspects, identifying workers and separating soldiers of different species is very difficult and in spite of using precise measurements, overlap may occur (Scheffrahn and Su, 1994). They, thereby, need to be characterized at molecular level. Nowadays, molecular methods have revolutionized insect systematics (Roderick, 1996; Caterino *et al*., 2000), and they are increasingly being applied to diverse groups of insects.

The population structure of *Coptotermes gastroi* (Wasmann) was characterized by using microsatellite markers by Yeap *et al.* (2011). Mitochondrial DNA is a valuable marker that is being used to study the insects phylogeny. Sharma *et al.* (2013) and Singla *et al.* (2013) used the partial fragments of mt. genes *COI* and *COII* and *12S* to determine the phylogenetic positions of various Indian termites. Similarly, partial fragments of two mitochondrial genes *viz*., *16SrRNA* and *ND1* were used in present study to find the extent of genetic relatedness/variations in three populations of *Microtermes obesi* (Isoptera: Termitidae). *16Sr RNA* is specific to a given species. Black and Piesman (1994) using *16Sr RNA* investigated the phylogeny of ticks at the family level and concluded that this gene is useful for phylogenetics of ticks at or below this level. They also suggested that *16S* in combination with another gene would give a more fully resolved tree. In 2010, Yeap *et al.* (2010) used the information from partial sequence of mitochondrial genes *16S, 12S and COII* together with morphometric measurements to determine the relationship between *Coptotermes heimi* and *C. gastroi* and found these species to be conspecific.

CP analysis of genome is another approach introduced by Orita *et al*. (1989). The technique can detect single base pair mutations in a PCR product without any prior sequence knowledge beyond that needed for the PCR amplification. The product is denatured to form single stranded DNA and snapcooled to form folded structure, which affects the mobility of the strands in a non-denaturing gel. Hence two bands are expected from homozygotes and four from heterozygotes. Two identically migrating bands cannot be assumed to have identical sequences, because not all mutations will affect mobility.

Molecular diagnosis, therefore, is just one way that might gain insight as to the origin of newly introduced species of termites, so that intervention may be directed in the most economically effective manner. The information so obtained can further be used to denitrify the source of introduction of termites, to discriminate the species for the application of corrective treatment measures and for cataloguing species with the intent to correctly classify them that would likely be discovered later. Molecular diagnostics are expected to yield genetic information from the collections, which will further be used as an integral

component of phylogenetic studies.

Materials and Method

Collection and storage

Termites were collected from three locations separated by about 10-15 km of distance within Chandigarh and its surrounding areas from North-Western part of India (Table 1) and preserved in 100% ethanol. Voucher specimens were put in 70% ethanol mixed with a few drops of glycerol and maintained in the Department of Zoology, Panjab University, Chandigarh (UT), India.

Identification of termites

Soldier specimens of all the populations collected (packed in scintillation vials in 70% ethanol) were sent to the Isoptera Section of the Zoological Survey of India (ZSI), Kolkata, for their identification. The details of collection site, date of collection, source and name of the authors were mentioned on each vial.

Isolation of genomic DNA

Genomic DNA was extracted from termites regardless of their castes by using standard phenol: chloroform extraction method (Sambrook *et al.,* 1989). The whole insect was homogenized in 1.5 µl eppendorf tube in 500 µl of TE (Tris EDTA, pH 8) with hand pestle and the homogenate was centrifuged at 7000-10,000 rpm for 10 minutes in cooling centrifuge (4°C). The supernatant was discarded and the pellet dissolved in 500 µl of lysis buffer (400 µl of TE and 100 µl of 10% SDS) followed by the addition of 6 µl of Proteinase K and the solution was incubated at 65°C for 1 hour in water bath. After the addition of 120 µl of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), the tubes were vortexed for 30 seconds and then centrifuged for 5 min at 10,000 rpm in cooling centrifuge. The upper aqueous layer was carefully transferred to fresh tube and 500 µl of isopropanol was added to it and stored at 4°C for overnight and then centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The alcohol was drained out, the pellet dried and dissolved in 30 µl of TE and stored at – 20°C after checking in 0.8% agarose gel. The concentration of DNA was then quantified by using-UV visible scanning spectrophotometer.

Amplification of *16SrRNA* gene

The region between nucleotides 7902 and 8321 in *Bombyx mori* (GenBank, AB070264) (Yukuhiro *et al*., 2002) of *16SrRNA* was amplified by using the universal primers LR-J-13007 and LR-N-13398 in the same termites to give a 419 bp long fragment (Table 2, Fig.1).

Amplification of *16Sr RNA-tRNA leu-ND1* gene

A 532 bp long fragment of *16Sr RNA-tRNA leu-ND1* gene from nucleotides 136 to 668 in *Reticulitermes banyulensis* (GenBank, AY510537) (Kutnik *et al*., 2004) was amplified by using the appropriate primers (Table 2, Fig. 2).

Single strand conformation polymorphism for *16Sr RNA* AND *ND1* genes of termites

SSCP analysis was carried out by using protocol of Vega *et al.* (1997) with slight

modifications. Five microlitres of PCR product was resuspended in an equal volume of formamide loading buffer in separate 0.2ml PCR tubes.

Electrophoresis to detect SSCPs

Electrophoresis was carried out in a vertical unit (BioRad Protean II system) by casting non-denaturing polyacrylamide gels using well clean glass plates. Gels were made from 12% acrylamide solution and 0.5X TBE; polymerization was initiated by adding 40µl of TEMED and 400µl of ammonium per sulphate (10% w/v). Gels were electrophoresed in 1X TBE buffer. Pre run was done and after that, the upper lid was removed and the shark toothcomb was inserted between the glass plates taking care that it just pierces the top of the gel and complete wells were formed. The samples were denatured by placing them in thermocycler at 95°C for 5 minutes and the tubes were cooled by keeping them immediately on the ice. Denatured samples (3.0µl each) were loaded in each well and the apparatus was run at 350V for 12 hours at 15°C. These were ideal conditions for detection of variations in *16SrRNA* and *ND1* genes. These conditions in fact allowed detection of fragments up to 550 bp length.

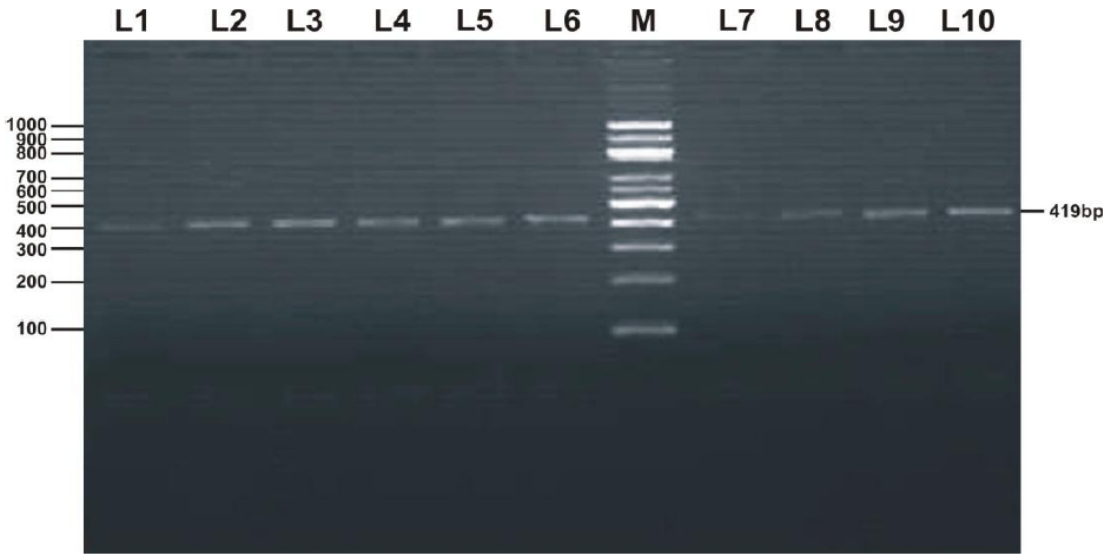
S. No.	Date	Species	Collection Site	Source	Sample Code*
1.	24.06.2004	*Microtermes obesi*	Hallomajra, Chandigarh	Tree	H
2.	26.06.2004	-do-	Darba, Chandigarh	Dampwood	D
3.	22.08.2004	-do-	Outskirts of Sukhna Lake, Chandigarh	Dead Tree Trunk	S

*H, D and S designate the initials for the name of places from where collections were made

Table 1: Collection data of termite species and their populations

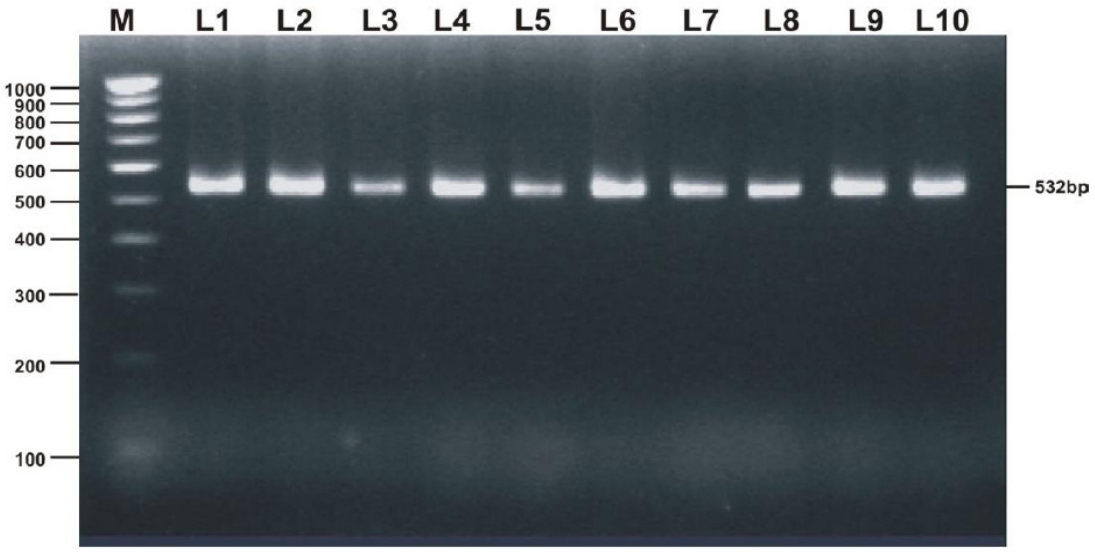
Primers	Primer Sequences	Length Melting	Annealing Temperature (Tm)	Temperature (Ta)
LR-J-13007-F(*16S*)	5'TTACGCTGTTATCCCTAA-3'	18	58.4°C	49°C
LR-N-13398-R(*16S*)	5'-CGCCTGTTTATCAAAAACAT-3'	20	57.3°C	49°C
ND1-F	5'TATTTTGGCAGATAAGTGCGTTAG -3'	24	53°C	53°C
ND1-R	5'AAAATAAAGGCCAATCTTACCTC A-3'	24	53°C	53°C

Table 2: Sequences and the conditions of the primers used to amplify mitochondrial DNA genes i.e. *16Sr RNA* and *ND1* in termites



Lane M: 100bp DNA leader
Lane L1-L10: Amplified products (419bp)

Fig. 1: PCR amplified products of *16Sr RNA* gene in various populations of termites.



Lane M: 100bp DNA ladder
Lane L1-L10: Amplified products

Fig. 2: PCR amplified products of *16Sr RNA-tRNA leu-ND1* gene in various populations of termites

Species	Populations	Total number of individuals studied	Pattern Type	Number of individuals with a haplotype	Frequency (%)
Microtermes obesi	P1	10	I	10	100
-do-	P2	10	I	10	100
-do-	P3	10	I	8	80
			II	2	20

Table 3: Frequencies of conformational pattern types obtained in various populations of *Microtermes obesi* studied for *16Sr RNA* gene

Silver staining

Gels were stained in clean shallow plastic trays. They were fixed in 2000 ml of 10% glacial acetic acid for 20 minutes. Washed thrice with double distilled water with agitation and dipped in 2000 ml of silver nitrate solution (2g silver nitrate and 3 ml formaldehyde in 2000 ml of double distilled water) for 30 minutes. The gels were agitated in between twice or thrice. They were removed from staining solution, kept in double distilled water for 10 seconds and then in 2000 ml of developer (60 gm sodium bicarbonate, 400µl sodium thiosulphate and 3 ml 37% formaldehyde in distilled water) with agitation until the first band appeared. They were then immediately kept in stopper or fixative *i.e.*, 10% glacial acetic acid and rinsed with double distilled water and kept for drying in cellophane sheet for preservation and further analysis.

Sequencing

Sequence interpretation

After the completion of the electrophoresis (run), chromatograms drawn by data collection software were used to extract the sequences of each individual. The sequences were subjected to multiple alignments with CLUSTAL V and further adjusted manually. For CLUSTAL V alignment 'Meg Align' program of 'Lasergene' software package (DNASTAR Inc., USA) was used. Edited sequences were compared against non-redundant nucleotide database from NCBI using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) algorithm from National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The edited nucleotide sequences were aligned and compared amongst the various haplotypes obtained across the species/populations of the order Isoptera included in the present study. Percent divergence was determined by computing the number of base differences for the total length of the gene sequence. The sequences were aligned in pairs, followed by aligning between pairs. Phylogenetic tree based on neighbor-joining method and divergence/similarity matrix were also drawn using the same Lasergene software.

Results and Discussion

For these species/populations, no prior genomic information is available. Fragments of two mitochondrial genes *i.e.*, *16Sr RNA* and *16Sr RNA tRNA leu ND1* genes were subjected to PCR amplification by using appropriate primers across all three populations (Table 3). Each individual yielded fragments of specific base pair length (Fig.1 and Fig.2). The amplified fragments were than subjected to polyacrylamide gel electrophoresis (SSCP analysis) in order to find variants within each group of species (Fig.3 and Fig.4). This helped in screening the samples for sequencing.

Single strand conformation polymorphism

After optimization of the parameters that affect the detection of SSCPs, the PCR products from 30 individuals (10 from each of the 3 populations studied were run on polyacrylamide gel) were analyzed for *16Sr RNA* and *16Sr RNA tRNA leu ND1* gene under modified conditions.

Single strand conformation polymorphism detection of 16Sr RNA gene

Figure 3 shows the SSCP analysis of 419 bp fragment of the *16Sr RNA* gene. Two conformational patterns (I, II) were detected for this fragment (Table 3).

All the three populations of this species showed very high frequency *i.e.,* 100, 100 and 80% of Type-I pattern for P10, P11, P12 respectively. Type-II was seen only in P12 with frequency of 20% only.

Single strand conformation polymorphism detection of 16Sr RNA tRNA leu ND1 gene

Figure 4 shows the SSCP analysis of 532 bp long fragment of *16Sr RNA tRNA leu ND1* gene.

In three populations (P10, P11 and P12) of *M. obesi* studied, two types of conformational patterns *i.e.*, Types-A and -B were observed. All the individuals of populations P1 and P2 revealed Type-A pattern only. This pattern also had high frequency in P3 (80%). Twenty percent of the individuals showed Type-B pattern.

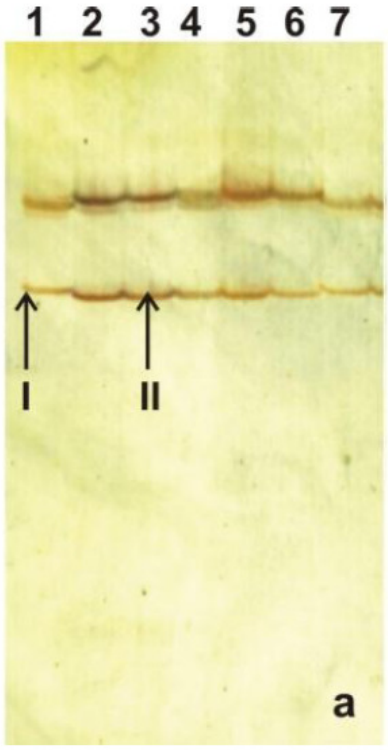


Fig. 3: SSCP analysis of 419bp fragment of *16Sr RNA* gene. Electrophoresis was performed by running 3 l of denaturated samples in a 12% acrylamide gel at 350V and 15°C for 12 hours. The frequencies of the conformational patterns detected were 93.33% for pattern I, 6.67% for pattern II in *Microtermes obesi*.

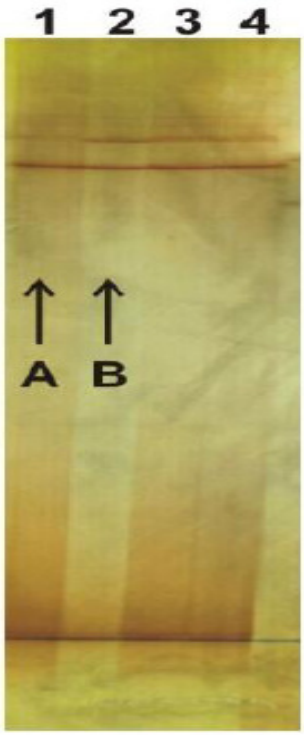


Fig. 4: SSCP analysis of 532bp fragment of *16Sr RNA-tRNA leu-ND1* gene. Electrophoresis was performed by running 3 l of denaturated samples in a 12% acrylamide gel at 350V and 15°C for 15 hours. The frequency of Type-A, 93.33% i and Type-B was 6.67% in *Microtermes obesi*.

Species	Populations	Total number of individuals studied	Pattern Type	Number of individuals with a haplotype	Frequency (%)
Microtermes obesi	P1	10	A	10	100
-do-	P2	10	A	10	100
-do-	P3	10	A	8	80
			B	2	20

Table 4: Frequencies of conformational pattern types obtained in various populations of *Microtermes obesi* studied for *16Sr RNA-tRNA leu-ND1* gene

Sequence analyses of 16Sr RNA gene

One individual of each haplotype pattern was sequenced and the variation was observed only at one position in the form of indel. The A+T content was 63.1% and that of G+C, it was found to be 36.9%. The percent diversity between the two haplotypes was zero. The average base frequencies were observed as A (0.420%); G (0.104%); T (0.217%) and C (0.257%). Stretches of As were more commonly seen in the sequences of the amplified fragment.

The sequence divergence of the Indian haplotypes of *Microtermes obesi*-I and *M. obesi*-II (Family: Termitidae) with both outgroup members of the same family *i.e. N. ephratae* and *N.costalis* from Guadeloupe (Schefrahn *et al*., 2005), retrieved from NCBI public database (Table 5), was found to be 14.6 and 15.3% respectively (Table 6).

Species	Family	Subfamily	Isolates/Country	Accession Number
Nasutitermes ephratae	Termitidae	Nasutitermitinae	Guadeloupe	AY623089
Nasutitermes costalis	Termitidae	Nasutitermitinae	Guadeloupe	AY623099

Table 5: List of species whose sequences were retrieved from Gene Bank public database and included in the analysis

	***M. obesi*-I**	***M. obesi*-II**	***N. ephratae***	***N. costalis***
M. obesi-I	0			
M. obesi-II	0.0	0		
N. ephratae	14.6	15.3	0	
N. costalis	14.6	15.3	1.4	0

Table 6: Pair wise data matrix showing percent diversity with out group taxa

Sequence analyses of 16Sr RNA tRNA leu ND1 gene

From the sequence alignment data and data matrix showing pairwise percentage divergence, it was apparent that the sequence divergence of all *Microtermes obesi* haplotypes was low. The sequences of all the three individuals were seen to be almost identical

showing approximately 0.64% variations among themselves. 0.43% transition substitutions were revealed, while transversions were 0.21% (Table 7). The A+T content was 65.03% and that of G+C, it was 34.97%. The average base frequencies were observed as A (0.153%); G (0.241%); T (0.499%) and C (0.109%). In this case Stretches of T_s were more common.

M. obesi-A1 and -A2 showed 0.2 % divergence among themselves. *M. obesi*-B had the divergence value of 0.7% from *M. obesi*-A1 and 0.4 % from *M. obesi*-A2 (Table 8).

The sequence divergence within the Indian haplotypes of *Microtermes obesi* ranged from 0.2-0.7%, while with outgroup members of the family Rhinotermitidae *i.e.*, *R. flavipes* and *R. grassei*, retrieved from NCBI public database (Table 9), the percent divergence was found to be 16.6-16.9 and 15.6-15.8 % respectively (Table 10).

Haplotypes	**Variations**		
M. obesi-A1	G	G	C
M. obesi-A2	G	G	T
M. obesi-B	A	T	T

Table 7: Variant sites in *Microtermes obesi* haplotypes for *16Sr RNA-tRNA leu-ND1* gene

	***M. obesi*-B**	***M. obesi*-A1**	***M. obesi*-A2**
M. obesi-B	0		
M. obesi-A1	0.7	0	
M. obesi-A2	0.4	0.2	0

Table 8: Matrix showing pairwise percentage divergence amongst two haplotypes of *M. obesi* of North-West India (Isoptera: Termitidae) in *16Sr RNA-tRNA leu ND1* gene.

Species	**Family**	**Subfamily**	**Isolates/Country**	**Accession Number**
R. flavipes	Rhinotermitidae	Rhinotermitinae	Raleigh (USA)	AY101831
R. grassei	-do-	-do-	Aranda de Duero (Spain)	AY101828

Table 9: List of species from Rhinotermitidae family whose sequences were retrieved from Gene Bank public database and included in the analysis.

	***M. obesi*-B**	***M. obesi*-A1**	***M. obesi*-A2**	***R. flavipes***	***R. grassei***
M. obesi-B	0				
M. obesi-A1	0.7	0			
M. obesi-A2	0.4	0.2	0		
R. flavipes	16.9	16.9	16.6	0	
R. grassei	15.8	15.8	15.6	6.6	0

Table 10: Pair wise data matrix showing percent diversity with out group taxa.

Among the three collections, P3 revealed two types of conformational patterns for each gene, while no variations were observed in the rest of the two populations in each case. The existence of multiple mtDNA haplotypes within a single collection was an unexpected result as mtDNA is maternally inherited in animals (Brown *et al.*, 1983) and cooperative colony found by multiple females is not common in termites and if such cases are there then the multiple founders appeared to involve sisters, which maintain the single maternal lineage (Thorne, 1982). The observed polymorphism might be due to the heteroplasmic nature of the mtDNA for more than one haplotype. Tokuda *et al.* (2012) analyzed the complete mitochondrial genome sequence of *C. formosanus* collected from three isolated islands in the Ryukyu Archipelago of Japan and found 99.9% similarity among these populations.

Nucleotide analyses of *16Sr RNA* gene

Because of its moderate size and range of evolutionary rates across sequences, *16Sr RNA* has great importance in phylogenetic studies across wide range of insects (Simon *et al.*, 1994). The sequences of the two *Microtermes obesi* individuals examined in the present study were almost 100% identical within this region. In Similar study carried out by Jenkins *et al.* (2002), it was observed that Formosan subterranean termites did not show any genetic polymorphism for *16S* marker. This lack of variations in *16Sr RNA* made it ideal for molecular diagnosis. The application of the *16Sr RNA* had been applied to identify *Reticulitermes* populations from the south-central United States (Austin *et al.*, 2004a, b, c) and across North America (Austin *et al.*, 2005a). This marker has tremendous potential for molecular diagnosis of *Reticulitermes*, with increased accuracy of positive species identifications (Szalanski *et al.*, 2003) and clarifying the identities of exotic introductions around the world (Austin *et al.*, 2005a, b). The genome of *Microtermes* has a high A+T contents (63.1%). Having high A+T content in mitochondrial genome is a general observation in insects (Xiang and Kocher, 1991; Kambhampati, 1995). The application of *16Sr RNA* proved to be reliable and easy to use for clarification between numerous *Reticulitermes* groups, particularly within *R. flavipes* from North America (Austin *et al.*, 2004a,b,c) and from Europe (Marini and Mantovani, 2002; Luchetti *et al.*, 2004).

Nucleotide analysis of *16Sr RNA tRNA leu ND1* gene

The diversity among various haplotypes of *Microtermes* individuals ranged between 0.2-0.7% revealing close association among different colonies. By using the *16Sr RNA tRNA leu ND1* gene, Uva *et al.* (2003) found association among colonies of Italian populations of *R. lucifugus*. They had detected four haplotypes (A, B, C, D) in the individuals of 13 colonies and reported two evolutionary groups within the species studied. Like *16S* gene fragment *ND1* gene fragment too had high A-T contents. It was between 60.13-65.52% for all the individuals studied.

From the present study it has been revealed that no information of any gene sequences

from *M. obesi* is available in the data bank till now. Thus, the sequence data generated and submitted to the Gene Bank in this study will act as baseline data for future studies regarding comparison and diagnosis of this species.

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