K.S. Murthy (2020) Int. J. Appl. Sci. Biotechnol. Vol 8(4): 410-416
DOI: 10.3126/ijasbt.v8i4.32445

ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

Molecular Identity of Subterranean Termites

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Article Information

Received: 06 November 2020
Revised version received: 18 December 2020
Accepted: 22 December 2020
Published: 28 December 2020

Cite this article as:
K.S. Murthy (2020) Int. J. Appl. Sci. Biotechnol. Vol 8(4): 410-416. DOI: 10.3126/ijasbt.v8i4.32445

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Peer reviewed under authority of IJASBT
© 2020 International Journal of Applied Sciences and Biotechnology

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Abstract

Identification of termites is a challenging task due to variable morphological differences among species and their eusocial behaviour with different caste system. A method for easy and accurate species-level identification at any life stage is required. In this study, a 658-base pair region of the mitochondrial cytochrome oxidase I (COI) gene was used to explore its utility in the identification of important subterranean termites. Termite specimens collected from 25 locations from various states, were characterized using specific primers for their identification. Sequence analysis and divergence among the species was assessed. The composition of the mitochondrial sequence of the COI gene in the present study was expectedly AT biased. GenBank accession numbers were obtained for the species. Sequence analysis and divergence among the species was assessed, which revealed significantly high percentage of A+T base composition. Molecular sequence information from NCBI revealed relatedness in all the collected termites, accurately as revealed by their morphological characters. The studies indicate the relevance of DNA sequencing to match different forms of termites and address ambiguities in morphological identification. The information on species diversity would help plan strategies for management of termites.

Keywords: COI gene; Genbank accession; Molecular characterisation; Termites

Introduction

Termites, referred to as "white ants" (Isoptera) are considered as the most abundant invertebrates and represent up to 95% of soil insect biomass show an elaborated morphology and complex behaviour (Wang *et al.*, 2009) Termites are the serious pests of agricultural and horticultural crops that mainly destroy the roots and above ground parts and feed on paper, wood and timber (Murthy *et al.*, 2015). They are classified in about 280 genera, and over 2800 species within 14 subfamilies (Eggleton and Tayasu, 2001). In India about 300 species within seven families have been reported (Kumar and Pardeshi, 2011; Pardeshi and Prusty, 2010). Termites are often referred as "ecosystem engineers" (Jouquet *et al.*, 2006; Ali *et al.*, 2013) as they play a vital role in recycling of plant materials and wood, modifying and improving the soil condition and composition, and providing food for other animals (Ackerman *et al.*, 2009; Sugimoto *et al.*, 2000). Termites are also considered as potent catalysts due to their role involved in converting lignocellulose into biofuels (Deivendran, 2013) and contribute to gas exchange,

nitrogen fixation, and soil stability and quality (Bignell, 2000; Hemachandra *et al*., 2010).

The most important group of termites considered as pest, belongs to the group of subterranean species which have their colonies underground consisting millions of individuals from where they voyage a long distance in search of their food such as paper, timber etc) These colonies mostly consists worker termites (Chan *et al*., 1993) that are morphologically uniform and show few traditional taxonomic characteristic for identification as a reason it lacks the understanding of diversity in these termites.

Since termites are social insects with different caste system it contributes to ambiguity in morphological identification and it turns out to be a challenging task for diagnosis of species. Termite taxonomy is mostly based on the soldier caste or the worker caste as they are largely encountered in fields since they scavenge away from nest and are associated in damaging wood (Kirton, 2005).

Morphological identification of termite species can be difficult as diagnostic morphological markers can be rare and are often restricted to soldiers or alates. For such taxa, sequencing of gene fragments (DNA barcoding) is now an important molecular tool widely used to elucidate phylogenetic relationships between taxa and to identify species The use of taxonomic keys often requires proficiency to avoid inaccuracy for those similarities which cannot be easily deciphered. Under these circumstances, DNA analysis appears promising to solve the species identification problem owing to the durability and stability of the DNA **(**Wallman and Donnellan, 2001). It also can solve the problems of morphological identification with damaged specimens (Judith and Nicola, 2008).

Molecular identification of the species with the help of various mitochondrial genome sequences such as the 16S rDNA, cytochrome oxidase genes and AT-rich region have shown as an efficient alternative for species identification and phylogenetic studies (Wells and Sperling, 2001; Roy *et al.,* 2006) The mitochondrial DNA evolves more rapidly than nuclear DNA and is inherited maternally and the substitution of nucleotides occurs at neutral site (Behura, 2006). Among the molecular markers used for sequence analysis of termites, the COII gene is often used since it has proved to be a good marker and has been extensively used to evaluate population structures, gene flow, phylogeny, and taxonomy of termites (Kambhampati *et al.,* 2000; Lo *et al.*, 2000; Thompson *et al.,* 2000; Ohkuma *et al.*, 2004).

In our present study the identification of subterranean termites collected from different locations in India was established using the mitochondrial cytocrome oxidase 1 gene (COI) as molecular marker.

Materials and Methods

Collection and Identification of Termite Samples

Termite specimens (Soldiers and workers) were collected from the various locations listed in Table 1, by adopting the belt transect method **(**Eggleton *et al.,* 1997; Davies *et al.,* 2003; Anantharaju *et al.,* 2014**).** Collections were made at three months intervals from August 2016 to July, 2018. The sampling also included the micro habitat (mounds, leaf litter, stump, tree bark, tree logs, bamboo fencing and vegetation) apart from the crop canopy. Contiguous sections of the transect, measured 10 sq. m each. The collected specimens were taken to the laboratory at the Division of Genomic Resources, NBAIR-ICAR Bangalore, The labelled specimens were preserved in 75% (v/v) ethyl alcohol and stored in laboratory freezer at -80°C, prior to analysis. Taxonomical identification of these specimens was done at the Division of Entomology Indian Agricultural Research Institute, New Delhi, Institute of Wood Science Technology Bangalore, and Centre for Insect taxonomy, University of Agricultural Sciences, Bangalore, by using the keys of Roonwal and Chottani (1989), Chottani (1997) and Kalleshwaraswamy *et al.* (2013).

Extraction of Genomic DNA

The genomic DNA was extracted using modified DNeasy blood tissue kit protocol. The samples were washed with distilled water as they were stored in absolute alcohol which was then followed by dissection of head region. The dissected head was collected in 1.5ml autoclaved micro centrifuge tube to which 150 µl of digestion buffer was added and crushed using autoclaved micro pestle in order to release the genomic DNA from cells. 20 µl proteinase k was added to it and incubated overnight in water bath at 56^0C. It was further incubated for 10minutes after the addition of lysis buffer. 100 µl of 100% ethanol was added and the samples were transferred to mini spin columns containing silica gel and centrifuged at 8000rpm for 5 minutes. The supernatant collected in flow-through was discarded and further two wash was given to it with AW1 and AW2 buffer and centrifuged 8000rpm for 5 minutes simultaneously. The columns were then transferred to fresh autoclaved micro centrifuge tubes in order to elute the DNA using 50 µl of distilled water and centrifuged at 8000rpm for 5 minutes. The extracted DNA was then checked in 1.5% agarose gel. It was then quantified using nanodrop spectrophotometer. The DNA nitrogenous bases showed at strong absorption at a wavelength of 260nm. The samples were then stored at -20^{o}C until further processing of PCR was done.

Amplification and Sequencing of Partial CO1 Gene

A region of 658bp cytochrome oxidase 1 gene fragment was amplified using the universal primers COI- forward (LCO1490) 5'-GGTCAACAAATCATAAAGATATTGG-3' and COI- reverse (HCO2198) 5'-TAACTTCAGGCTGACCAAAAAATCA-3'. PCR reaction was carried out in 0.2 ml of PCR tubes with a

reaction mixture of 25 µl consisting 2.5 µl 10x Taq buffer, 0.5 µl GENie TM 10mM dNTP mix, 1 µl of forward and reverse primer each, 1 µl GeNieTM Taq DNA polymerase (1U/µl), 5 µl of template DNA and 14 µl of sterile water.

The PCR cycles set up were initial denaturation at 95ºC for 2 minutes, denaturation at 95ºC for 30 seconds, annealing at 50ºC for 1 minute, extension at 72ºC for 2 minutes followed by 34 cycles and final extension at 72ºC for 7 minutes were carried out in thermal-cycler (BioRad, USA). The amplified products were confirmed by running on 1.5% agarose gel with 250bp ladder and visualized in INGENIUS^3 gel dock. The amplified products were sequenced using Dideoxy method or chain termination method at M/S. Eurofins Pvt Ltd, Bangalore.

Sequence Analysis and Data Interpretation

The most commonly used method of DNA sequencing is the dideoxy method or chain termination method. The amplified products of COI gene were got sequenced at M/s. Eurofin Pvt Ltd, Bangalore. The COI gene sequence data was retrieved in the form of Chromatograms. Several individuals from each species were sequenced and chromatograms were subjected to VSQual to evaluate the reliability of the data, and good quality fragments were used to construct a consensus sequence for each sample. 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 sequence data was submitted to NCBI and accession numbers were obtained. Consensus sequences of COI partial gene were multiple aligned using Clustal W (ver. 1.83) **(**Thompson *et al*., 1994; Tauz *et al.*, 2003).

Results and Discussion

Study Area

The study area included different locations from the states of Andhra Pradesh, Arunachal Pradesh, Karnataka, Kerala, Meghalaya, Tamilnadu and Uttar Pradesh. The soil type in these areas was of red loamy and red sandy with varied cropping pattern that comprised of rice, small millets, maize, groundnut, sorghum, sugarcane, castor, gauva, arecanut, grapevine and plantation crops.

Molecular Identification

The genomic DNA was isolated from the populations of termites collected from different states viz, Andhra pradesh, Himachal Pradesh, Karnataka, Kerala, Meghalaya, Tamilnadu and Uttar Pradesh. Perusal of the data on the collection of termites from the different geographical locations and crops and their identification had revealed the diversity of termites in the country. An array of subterranean termites (Table 1) belonging to the subfamilies (Macrotermitinae, Nasutitermitinae Apicotermitinae and Kalotermitidae) were collected. Ten species of Macrotermitinae, three species of Nasutitermitinae and one each of Apicotermitinae and Kalotermitidae were recorded. Species belonging to Microtermitinae was more predominant than others under variable conditions.

Soldier castes was utilised for identification in the present studies, the isolated genomic DNA of termites from various locations was characterised through COI gene fragment (648-656 bp size) was successfully sequenced for all the specimens, and the alignment of all specimens considered in this study lacked any insertion or deletion. A total of **15** species were sequenced over COI regions and the Blast done with NCBI database to decipher the identity of the termites from various locations is given in **Table 1**.

Such studies were earlier carried out by Austin *et al.*, (2002), Marini and Mandovani, (2002)., Manjula *et al*., (2011)., Patel Preethi and Jadav, (2019).

The sequence data was retrieved in the form of chromatograms and submitted to genbank for obtaining the accession numbers. Chromatograms were edited in order to remove the ambiguous bases and then aligned using Basic Local Alignment Search Tool (BLAST), with the sequences of same or related genera retrieved from the nucleotide database (PUBMED) of National Centre for Biological Information (NCBI). The COI nucleotide sequences of the termite species included in our present study were aligned and compared with the species obtained from PUBMED, using CLUSTAL.

Molecular sequence information from NCBI revealed relatedness in all the collected termites, accurately as revealed by their morphological characters.

The utility of DNA data in taxonomy and species diagnosis in the termites was reported by Kambhampati *et al.* (2000); Lo *et al.* (2000) and Ohkuma *et al.* (2004), based on the sequence variation in DNA based groups which were highly structured. The population of termites from various locations were characterised using Cytochrome C oxidase subunit I (*COI)* gene, which has been recognised as an effective marker not only for species identification but also for phylogenetic relationship (Thompson *et al.*, 2000; Singla *et al*., 2013; Parween *et al*., 2016).

Nucleotide Analysis

The sequence of species under investigation were characterised based on the frequencies of A-T base composition. The complete gene analysis of nucleotide sequence for each of the collected termite species was done in order to find out the MCL (Maximum Composite Likelihood) estimate of the pattern of nucleotide substitution, AT%, GC% and the AT content at first, second and third codon position. MCL pattern indicated the likelihood of substitution from one base (row) to another base (column) (Tamura et al. 2007). Rate of different

transitional in substitutions were 10.86, 14.06, 17.05 and 8.24 and the rate of transversional substitutions were 6.52, 6.07, 7.36 and 4.95 (Table 2).

Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 411 positions in the final datasets. The overall result of nucleotide analysis showed AT-rich gene which is common in insects. This pattern suggests that the sequence divergence is often accompanied by high AT mutation pressure or less probably resulting from the selection for the incorporation of amino acids encoded by AT rich codon families.

Table 1: Molecular Identity of subterranean termites collected from various locations and their GenBank accession numbers

S.N.	Species (NCBI Blast result)	Subfamily	Location	Latitude/Longitude	GenBank Acc. No.
1	*Odontermes obesus*	Macrotermitinae	Anakapalle	17 .38° N, 83.2° E	174056
			Samarlakota	17 .5° N, 82.2° E	KU 687341
			Tirupathi	13.65° N, 79.42° E	KU687342
			Kapatganj (UttarPradesh)	26.920° N, 83.77° E	KM657477
2	*Macrognathotermes errator*		Phasighat (Arunachal Pradesh)	28.069° N, 95.32° E	KM657477
3	*Odontermes mathurai*		Phasighat	25.341°N, 91.592°E	KM657487
			Shillong (Meghalaya)		KM647487
4	*O. gurdaspurensis*		Bangalore	12.97° N., 77.57° E	KM657483
			Bangalore	12.97° N, 77.57° E	KM657481
			Yellahanka	12.97° N, 77.57° E	KM657480
5	*Microtermes mycophagus*		Bangalore	12.97° N, 77.57° E	KM657479
6	*Odontotermes longignathus*		Attur		KT254244
			Thrissur (Kerala)	10.52° N, 76. 2° E	KT719274
			IIHR		KU687338
			Kolar		KX611498
			Chikkaballapur		KX583491
7	*Hypotermes xenotermitis*		Marathahalli		KT274764
			Mysore		KT224387
			Attur		KX646190
			Uddanpatti (TN)	Eucalyptus	KU687340
8	*Hypotermes makhamensis*		Sivaganga	Vegetable	KT274765
			Aizawl (Mizoram)		KX444138
			Aizawl (Mizoram)		KX495578
9	*Odontotermes -wallonesis*		Thirtahalli	Arecanut	KT224388
			Chikmagalur	Arecanut	KT224394
10	*Odontotermes holmgren*		Belgaum	Arecanut	KT224389
			Mudhigeri	Grapevine	KT224392
			Mudhigeri	Arecanut	KT224393
			Kannur (Kerala)	Arecanut	KT719275
			Kalpetta (Kerala)	Arecanut	KT719276
11	*Dicuspiditermes Krishna*		Mudigere	13.1365° N, 75.64° E	KT224391
12	*Microtermes obesi*		Udupi	Neem	KM657488
13	*Nasuitermes sp.*	Nasutitermitidae	Sirsi	Arecanut	KT224390
	Nasuitermes		Sringeri	Arecanut	KT224395
			Belgaum	15.51° N., 74.29° E	KU665432
			Kannur (Kerala)	11.8° N, 75.32° E	KU665432
	Nasutitermes octopilis		Ooty	11.41° N, 70.58° E	KM657478
	Nasutitermes exitiosus		Ooty		KM 015487
			Theni	15.51° N, 77. 79° E	KM657488
	Trinervitermes togoensis		Valparai		KX711183
14	*Neotermes koshunensis*	Kalotermitidae	Dindigul	Guava	KM657485
15	*Euhamitermes hamatus*	Apicotermitinae	Bangalore	12.97° N, 77.57° E	KM657484

Table 2. Maximum composite likelihood estimates of the pattern of nucleotide substitution of COI sequence

	A	**T**	**C**	**G**
A	-	6.07	7.36	**8.24**
T	6.52	-	**17.05**	4.95
C	6.52	**14.06**	-	4.95
G	**10.86**	6.07	7.36	-

Nucleotide frequencies of A, T, G and C were found to be 26.19%, 24.37%, 29.55% and 19.89% respectively. The transition/ transversion rate ratios are k_1 = 1.665 (purines) and k_2 = 2.318 (pyrimidines). The overall transition/ transversion bias is R = 1.021, where R = [A*G* k_1 + T*C* k_2]/[(A+G)*(T+C)].

The composition of the mitochondrial sequence of the COI gene in the present study was expectedly AT biased and this was generally observed in several previous studies (Williams *et al.* 1990, Foster *et. al.*, 2004). In general, the frequency of transitional substitutions is known to be higher than transversion substitutions in the genome (Tauz *et al*, 2003), According to 10X rule the percentage of nucleotide divergence between the intraspecies should be less than 3% and that of interspecies should be more than 3%. Hence the sequences analyzed in the present study exhibited high inter species variability on the basis of nucleotide sequences. Therefore, the intra specific divergence was higher enough to discriminate between the individuals.

Results indicated that the COI-based pest identification was extremely effective for the termites based on the COI marker profile. DNA sequence data have been employed successfully to elucidate the relationships of many groups of insect species at generic level. Molecular sequence information from NCBI revealed relatedness in all the collected termites, accurately as revealed by their morphological characters. Our observations, corroborate with the reports of (Monti *et al*, 2005). Qiu , *et al,* (2009)**, s**uggested that where sequence information is available in Genbank for morphologically defined species, which can be matched with some DNA based clusters, close relationship can be identified readily in sequence variation in field collected field samples and these clusters are likely to correspond to previously described unknown species. Similar observation was made by Vidyashree *et. al.* (2018)

Our studies indicate the relevance of DNA sequencing to match different forms of termites and address the issues of having to depend exclusively on morphological features and avoid misdiagnosis. The information generated on the basis of this data can be used for molecular identification of Indian termites species and their classification to differentiate on the basis of morphological parameters.

The species diversity is influenced by the cropping pattern, the climatological factors and the altitude. The distribution and diversity will depend upon various factors like food, habitat, vegetation and soil type Our findings contribute to a better understanding of the identification of pests by CO1 genes and aid in formulating better management strategies.

Conclusion

The diversity of subterranean termites from various geographical locations of India occurring in crops were morphologically identified and characterised using molecular tools. Molecular sequence information from NCBI revealed relatedness in all the collected termites, accurately as revealed by their morphological characters. The relevance of DNA sequencing to match different termites and address limitations in morphological identification is indicated. Knowledge on species diversity, through surveys would be helpful in planning strategies for conservation of natural enemies, habitat management, design and develop pest management strategies.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

Acknowledgements

The authors are thankful to the Director, ICAR-NBAIR for providing the facilities. Identification of the termites by Dr C. A. Viraktamath, Department of Entomology, University of Agricultural Sciences, Bangalore, Dr Sachin, Division of Entomology, Indian Agricultural Research Institute, New Delhi and Dr Rashmi Shanbhag, Institute of Wood Science Technology, Bangalore is also duly acknowledged.

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