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continued on the back inside cover

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COMMUNICATION

Identification and phylogenetic analysis of various termite species distributed across southern Haryana, India

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Abstract: Termites are highly abundant and vital insects that directly and indirectly influence local soils. The present study investigated the morphological and molecular phylogenetics of termite species collected from study fields in southern Haryana, India, from 2020 to 2021. Samples were subjected to integrated systematic analyses, taking into account the mandible features of soldiers for morphological systematics and 16S rRNA gene-based phylogeny for molecular systematics. Based on the external phenotype and relations to reference sequences in NCBI GenBank, 21 species were identified; these included five species each from *Odontotermes* and *Microcerotermes*, four species from *Coptotermes*, two species each from *Microtermes* and *Eremotermes*, and one species each from *Amitermes*, *Angulitermes,* and *Neotermes*. 16S rRNA gene sequences were utilized to construct phylogenetic trees to explore the relatedness among identified species. The results of the different molecular phylogenetic approaches including maximum parsimony, maximum likelihood, and neighbor-joining revealed nearly identical topological relations between the species and grouping of individuals in relation to their genera. The maximum parsimony tree based on mandible features has been found to be effective in clustering multiple species of a given genus in a clade in at least three termite genera.

Keywords: 16S rRNA gene, Kalotermitidae, mandible, molecular analysis, morphological taxonomy, phylogenetic tree, Rhinotermitidae, Termitidae.

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Author details: Bhanupriya is presently working on Diversity and Phylogeny of Termite Species (Insecta: Isoptera) in southern Haryana as part of PhD research. Dr. Shubhankar Mukherjee, an assistant professor, has published dozens of papers in the reputed journals. His field of research includes plant molecular biology, biotechnology and physiology, and molecular evolution. Dr. Nidhi Kakkar, a research associate, has published dozens of papers in the national and international journals and a book chapter on termite gut microbes. Her field of research includes biodiversity and entomology. Dr. Sanjeev K. Gupta, a professor, has published dozens of papers in the national and international journals and few book chapters. His field of research includes cell biology and biodiversity.

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INTRODUCTION

Isopterans are one of the most significant insect groups, serving as key decomposers of wood and other materials, and termites can also be serious pests of wood and other crops (Bignell & Eggleton 2000; Ackerman et al. 2007; Pooja et al. 2017; Govorushko 2019; Korb et al. 2019). Termites are eusocial insects, with colonies exhibiting caste specialization and division of labour. Around 3,106 species are listed worldwide, of which 337 have been reported from India and 39 from Haryana State (Bignell et al. 2010; Krishna et al. 2013; Pooja et al. 2017; Paul et al. 2018; Effowe et al. 2021; Bhanupriya et al. 2022a,b). Since termites are highly diverse it is important to identify them properly and classify them into well-defined groups, typically via multiple approaches.

Classical systematics has been used to classify termite species using morphological features of worker and soldier castes (Donovan et al. 2000; Aldrich et al. 2007; Rocha et al. 2019), which is useful to the genus level. To accurately discern species, a combination of molecular and morphological approaches have proven useful (Wallman & Donnellan 2001; Austin et al. 2005; Yeap et al. 2007), especially in cases where only partial or damaged samples are available (Judith & Nicola 2008). Molecular systematics based on mitochondrial DNA sequences has proven especially effective in unravelling termite taxonomy (Wells & Sperling 2001; Roy et al. 2006). Studies have been carried out using a variety of mitochondrial genes, including those for cytochrome oxidases and ribosomal RNAs (e.g., 12S and 16S rRNA; Murthy 2020). Mitochondrial genes tend to vary more rapidly than those in nuclear DNA, and they are inherited maternally (Behura 2006). 16S rRNA-based trees have been used to understand the taxonomy and evolution of termite species (Kambhampati et al. 1996; Vidyashree et al. 2018).

The present study was designed to characterize termite species collected from southern Haryana, India, based on morphological and molecular data. Termites were classified using phylogenetic trees built on the basis of 16S rRNA gene sequences, and maximum parsimony trees based on soldier mandible features. The results of molecular identification and morphological assessments are compared.

METHODS AND METHODS

Collection of Termite Samples

A total of 168 termite samples (soldiers and workers) were collected from several localities of southern Haryana, India, situated between 28.25° N & 76.29° E during a 2-year study period (Table 1; Figure 1), via random sampling (Bhanupriya et al. 2022a,b) of microhabitats that included dung cakes, common rush, vegetation, leaf litter, tree bark, stumps, mounds, bamboo fencing and tree logs. Collections were completed at three-month intervals from March 2020 to November 2021. Each sample contained around 50 individuals and their distribution in study sites was mapped based on the collective data of the current study. The voucher specimens were well-maintained in 10ml of 70% ethanol mixed with 2–3 drops of glycerol in 20 ml glass vials for morphological and molecular analysis. Samples kept in the vials were labeled with the day and date of collection, name of the study sites and source of isolation, and retained in the Department of Zoology, IIHS, Kurukshetra University, Kurukshetra, India.

Morphological identification of termite species

Identification of specimens was done using identification keys (Roonwal & Chhotani 1989; Chhotani 1997; Krishna et al. 2013) based upon different diagnostic characteristics of soldier caste: head length, head width, head shape, mandible length, mandible plus head length, body length, body width, body-colour, tibial spur, tarsal segments and antennae segments (Wang et al. 2009; Bhanupriya et al. 2022a,b). These measurements were examined under the light compound microscope and photographs were also collected. And complete analysis of body measurements was performed by calculating mean and SD.

Parsimony tree based on mandible features

For construction of parsimony tree based on mandible characters, observations were made on features like mandible without a tooth, mandible with serrations, serrated mandibles without any large tooth, mandibles strongly incurved at distal half, mandible with incurved apex, cylindrical mandibles, tooth present at mid of the mandible, tooth present at the near tip of the mandible, tooth present at near base of the mandible, left mandible with a single tooth, right mandible with a single tooth, left mandible with six marginal teeth, right mandible with two teeth, left mandible with four crenulations, right mandible with two crenulations, right mandible with three crenulations and right mandible

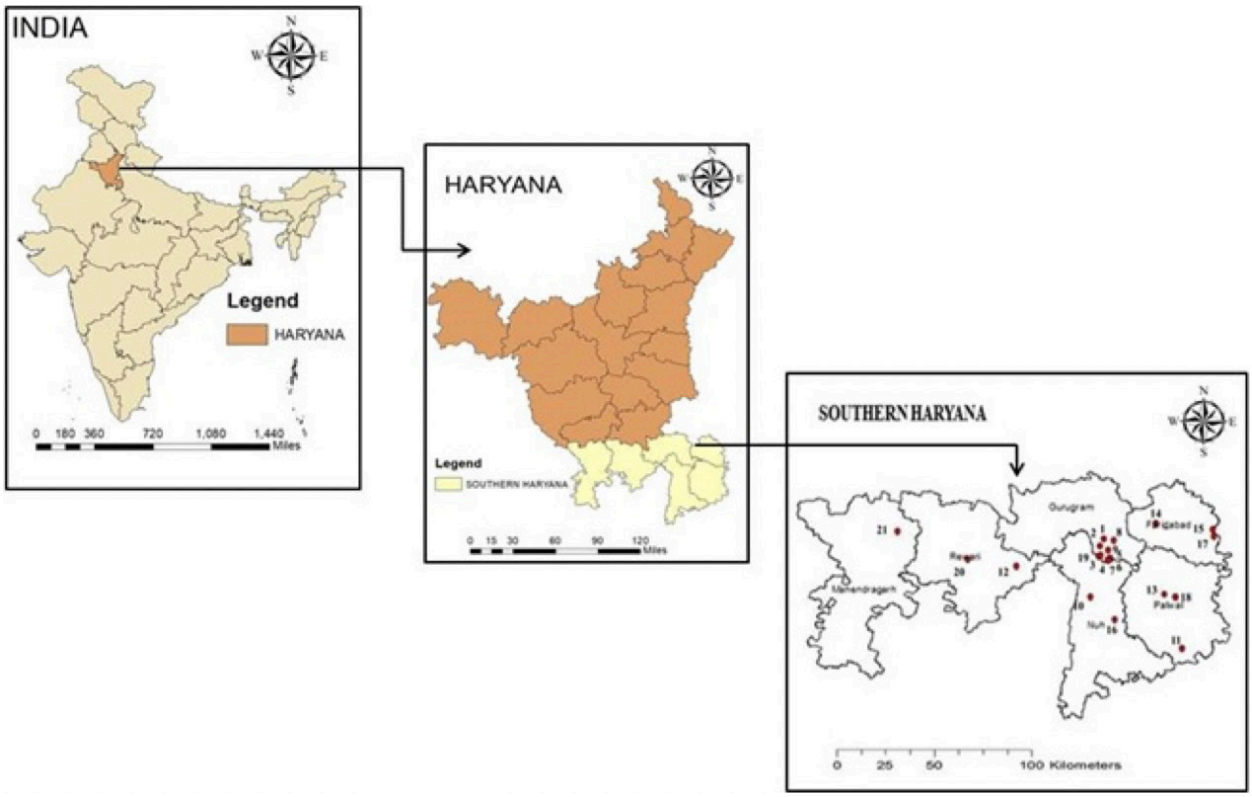

Figure 1. Various locations where termite samples included in this study were collected. Numbers (depicting species) mentioned in Table 1.

with four crenulations. The presence and absence of the above mentioned characters were assigned values of 1 and 0 respectively, and the strings of 1's and 0's were used as vectors to define a particular termite species. Maximum parsimony tree was constructed based on these strings of 1's and 0's using PAST 4.10 software.

Molecular identification

Extraction of Genomic DNA, PCR and Sequencing

The genomic DNA of termites was isolated from worker castes using Qiagen mini kits as per manufacturer instructions. The polymerase chain reaction was conducted using 16S rRNA gene-specific forward and reverse primers (16SF 5'-CGCCTGTTTATCAAAAACAT-3' 16SR 5'- CCGGTCTGAACTCAGATCACGT-3') by the protocol of Szalanski et al. (2004) with some modifications. Approximately, 500 bps amplicon of 16S rRNA gene was obtained for each termite species. The PCR reaction was performed in 0.2 ml of PCR tubes with a 25 µl reaction mixture consisting of 5 µl genomic DNA, 12.5 µl PCR mix, 1.0 µl primers (16SF & 16SR), and 6.5 µl of nuclease-free water. The PCR reactions were repeated in thermal cycles 40 times. In thermal cycles initial denaturation occurred at 95° C for 5 minutes, denaturation, annealing, and extension at 94° C, 55° C, and 72° C, respectively, for 30 seconds each, followed by final extension at 72° C for 7 minutes. The sequence of amplified DNA segments was approved using both the primers using Applied Biosystems BigDye Terminator V3.1 Cycle Sequencing kit. These sequences were further copied and analyzed using ChromasPro v 1.34.

Data Analysis

The 16S rRNA gene sequences were assembled using ChromasProV3.1 sequence assembly software. The assembled sequences were edited to remove uncertain bases and revised sequences were subjected to BLAST analysis in the NCBI database, for molecular identification. Since these were partial sequences, the overall identification of termites relied on both molecular as well as morphological characters. Hereafter, these 16S rRNA gene sequences were submitted to NCBI to get the accession numbers (Table 1). The 21 sequences were aligned using MAFFT (Katoh et al. 2019) and trimmed and edited in Jalview (Procter et al. 2021). *Cryptocercus* (cockroach) mtDNA sequences were included to be used as the outgroups. To explore the genetic relatedness of given termite species, phylogenetic (NJ, ML and MP) trees were constructed using the PHYLIP package version 3.695 (Felsenstein 2008).

Table 1. Source of collection of termites, geographical coordinates of the location, and GenBank accession numbers of the sequenced 16S rRNA gene fragments.

	Source of collection	Latitude & Longitude	Date of collection	Molecular identity	Accession No.
1	Kikar tree	28.12352 N 77.89224 E	28.vi.2020	*Amitermes belli*	MZ269706
2	Mango tree	28.225932 N 77.082438 E	24.iv.2020	*Coptotermes gestroi*	OK606100
3	Woody thing	28.226763 N 77.084916 E	22.vi.2020	*Microtermes mycophagus*	OK606129
4	Sheesam wood	28.2288656 N 77.0883974 E	23.vi.2020	*Coptotermes kishori*	OL335912
5	Wood	28.2287955 N 77.0885184 E	23.vi.2020	*Coptotermes heimi*	OK606090
6	Woody thing	28.23808 N 77.04488 E	25.vi.2020	*Angulitermes akhorisainensis*	OL780326
7	Wood log	28.2233547 N 77.0803697 E	02.vi.2020	*Eremotermes paradoxalis*	OL335913
8	Common rush	28.39208 N 77.01185 E	28.vi.2020	*Microcerotermes newmani*	ON385997
9	Peepal tree	28.39208 N 77.28196 E	28.vi.2020	*Coptotermes emersoni*	OK181907
10	Woody thing	28.09059 N 77.01185 E	30.viii.2021	*Odontotermes obesus*	OL721750
11	Cattle dung	27.45184 N 77.82596 E	03.vii.2020	*Eremotermes neoparadoxalis*	OL335910
12	Soil mound	28.35496 N 77.2624 E	11.vii.2020	*Odontotermes redemanni*	OL454814
13	Wooden block	28.22636 N 77.80092 E	06.viii.2020	*Odontotermes guptai*	OL335911
14	Common rush	28.8804 N 77.4924 E	17.iii.2021	*Microcerotermes raja*	OL470522
15	Common rush	28.3324 N 77.4812 E	23.v.2021	*Microcerotermes cameroni*	OL470529
16	Common rush	28.0088 N 77.1048 E	18.iii.2021	*Microcerotermes baluchistanicus*	OL454819
17	woody logs	28.3072 N 77.4848 E	23.v.2021	*Microtermes obesi*	OL454826
18	Kikar tree	28.239467 N 77.051106 E	29.viii.2021	*Odontotermes parvidens*	OL454829
19	Sheesham tree	28.201364 N 76.72994 E	04.ix.2021	*Neotermes kemneri*	OL780345
20	Kikar tree	28.225001 N 76.545022 E	04.ix.2021	*Odontotermes assmuthi*	OL721753
21	Common rush	28.325773 N 76.277785 E	14.x.2021	*Microcerotermes beesoni*	OM241964

500 replicates of the DNA sequence alignment were generated using Seqboot. For constructing MP tree, the output of Seqboot was fed to the program Dnapars and the resultant MP trees were obtained. The majority rule consensus tree was built from MP trees using Consense program. For constructing ML tree, the output of Seqboot was fed to the program Dnaml and ML trees were obtained from the datasets which were fed to Consense to arrive at majority rule consensus ML Tree. For constructing NJ tree, the output of Seqboot was fed to the program Dnadist to compute the distance matrices for the given datasets. The output of Dnadist was fed to the program Neighbor to obtain the NJ trees from the given datasets. The output of Neighbor was fed to Consense to construct the majority rule consensus NJ tree.

RESULTS AND DISCUSSION

Taxonomic Account of Termites:

Based on aforementioned morphological keys, 168 termite samples were identified into 21 species (Image 1) belonging to three families (Termitidae, Rhinotermitidae, and Kalotermitidae), four subfamilies (Amitermitinae, Termitinae, Macrotermitinae, and Coptotermitinae) and eight genera (*Amitermes, Eremotermes, Microcerotermes*, *Angulitermes, Odontotermes, Microtermes, Coptotermes*, and *Neotermes*) as shown in Table 2. Species *M. baluchistanicus* is an arid zone species that is restricted to only Rajasthan (Rathore & Bhattacharyya 2004). Parihar (1981) reported that this species destroyed the guar crop. In the Nuh region of Haryana, *M. baluchistanicus* has been discovered for the first time.

Morphological tree

Investigation of the intra and intergenic relatedness in termites was carried out on the basis of mandible features of soldier castes by using parsimony analysis (Image 1). The mouthparts (mandibles) of termites are sclerotized structures that are adapted according to the substrate on which they feed. These adaptations are helpful for mechanically breaking down the hardwood substrates of their diet (Wilson & Jessica 2019). Therefore, mandibles are significantly important in

Table 2. Body parameters of the soldier castes of the studied termite species (n = 5).

	TBL**	HL	HML	ML	HW	BW	Mandible features
Amitermes belli	5.02±0.24*	1.13±0.11	1.9±0.14	0.77±0.08	1.18±0.26	1.04±0.12	single tooth on each mandible
Coptotermes gestroi	4.87±0.31	1.47±0.03	2.38±0.08	0.91±0.06	1.18±0.06	1.09±0.06	right mandible with 3 crenulations
Microtermes mycophagus	4.3±0.6	0.92±0.08	1.48±0.11	0.58±0.05	0.83±0.12	0.84±0.05	single tooth on each mandible
Coptotermes kishori	4.5±0.45	1.15±0.05	1.93±0.05	0.78±0.05	1.01±0.09	1.02±0.08	right mandible with 4 weaker crenulations
Coptotermes heimi	5.58±0.42	1.33±0.05	2.14±0.20	0.82±0.16	1.18±0.08	1.04±0.04	right mandible with 4 crenulations
Angulitermes akhorisainensis	4.56±0.19	1.34±0.04	2.69±0.05	1.36±0.04	0.94±0.05	0.9±0.03	Mandibles long, rod like, distally pointed and incurved
Eremotermes paradoxalis	3.84±0.24	0.95±0.08	1.76±0.15	0.81±0.07	0.68±0.07	0.72±0.08	single tooth on each mandible
Microcerotermes beesoni	4.8±0.4	1.32±0.13	2.13±0.2	0.81±0.12	0.84±0.12	0.82±0.08	finely serrated, a prominent denticle present near the base of each mandible
Coptotermes emersoni	5.18±0.55	1.1±0.1	1.96±0.12	0.86±0.05	0.96±0.12	1.08±0.08	2 teeth on right mandible
Odontotermes assmuthi	5.64±0.4	1.57±0.12	2.3±0.16	0.75±0.07	1.21±0.05	1.18±0.08	Left mandible with single tooth
Microcerotermes newmani	4.56±0.4	1.2±0.13	2±0.2	0.8±0.12	0.9±0.12	0.8±0.08	coarsely serrated
Eremotermes neoparadoxalis	3.84±0.22	0.77±0.06	1.75±0.11	0.88±0.04	0.73±0.11	0.67±0.06	single tooth on each mandible
Odontotermes redemanni	4.64±0.41	1.21±0.07	1.92±0.15	0.79±0.07	1±0.1	0.87±0.05	Left mandible with single tooth
Neotermes kemneri	8.04±0.27	2.55±0.13	4.19±0.13	1.64±0.04	1.92±0.11	2.46±0.12	right mandible with 2 and left with 6 teeth
Odontotermes guptai	4.92±0.08	1.04±0.12	1.64±0.18	0.6±0.08	0.95±0.05	0.91±0.06	Left mandible with single tooth
Microcerotermes baluchistanicus	4.72±0.58	1±0.07	1.96±0.05	0.96±0.05	0.67±0.08	0.88±0.08	coarsely serrated, with a larger tooth-like serration in the middle
Microcerotermes raja	4.76±0.28	1.18±0.11	2.01±0.14	0.83±0.05	0.81±0.01	0.8±0.1	Coarsely serrated, without any large tooth.
Microcerotermes cameroni	4.98±0.22	1.52±0.16	2.41±0.18	0.89±0.04	0.94±0.09	0.82±0.08	coarsely serrated with one larger tooth-like serration near the middle
Microtermes obesi	4.3±0.35	0.98±0.08	1.52±0.09	0.54±0.04	0.83±0.08	0.88±0.08	single tooth on each mandible
Odontotermes parvidens	6±0.38	1.99±0.07	3.17±0.25	1.23±0.11	1.76±0.21	2.34±0.24	Left mandible with single tooth
Odontotermes obesus	5.14±0.70	1.32±0.13	2.2±0.16	0.88±0.11	1.15±0.05	1.14±0.09	Left mandible with single tooth

*All values represented in mm | **TBL—Total Body Length | HL—Head Length | HML—Head Mandible Length | ML—Mandible Length | HW—Head Width | BW—Body Width.

feeding biology, i.e., pulling, cutting, scraping, pounding, and grinding the wooden structures (Matsuoka et al. 1996; Wilson & Jessica 2019).

As termites are cryptic species, hence for identification, soldiers' mandible features were counted as a valuable or noticeable parameter for their characterization (Donovan et al. 2000; Engel et al. 2009). This is also because of the higher range of disparity displayed by soldier caste in their conspicuous morphological characters associated with the head and mandibles compared to either the alate or worker castes (Ishikawa et al. 2008; Wang et al. 2009; Ahmed et al. 2011; Ke et al. 2017). Wang et al. (2009) identified five species of the genus *Reticulitermes* (*R. flavipes, R. virginicus, R. arenincola, R. tibialis,* and *R. hageni*) by utilizing soldiers and alates body features. One species, i.e., *Heterotermes indicola* (Mahapatro & Kumar 2013), two species of the genus *Neotermes* (*N. koshunensis* and *N. sugioi*) (Yashiro et al. 2019) and seven species of three genera (*Odontotermes, Macrotermes,* and *Microtermes*) (Kassaye et al. 2021) were also identified with soldiers and Imago's morphological features. Vidyashree et al. (2018) also utilized soldiers' features and characterized 12 species of termites from the Western

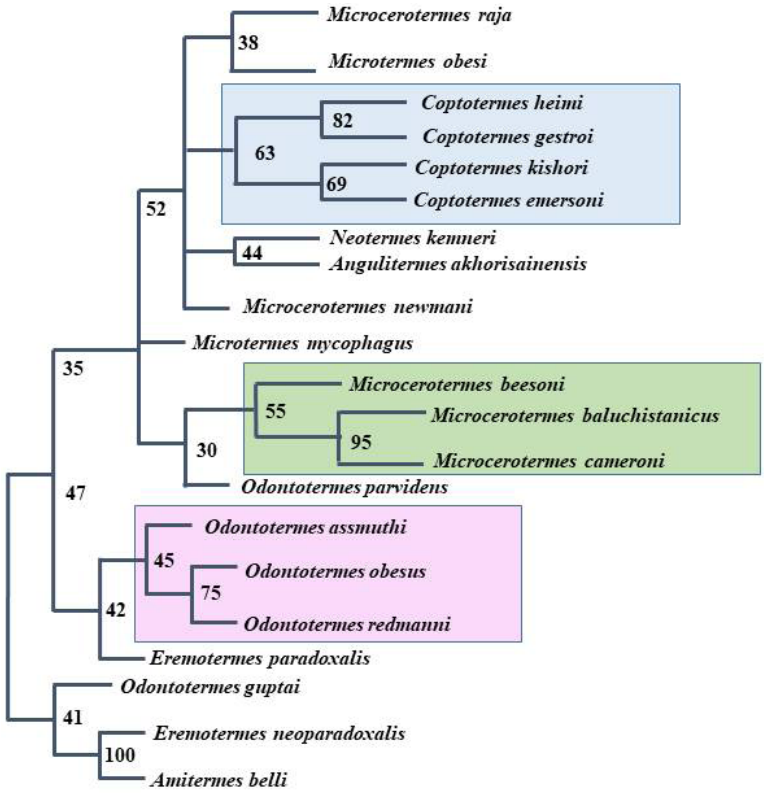

Figure 2. Maximum Parsimony tree based on mandibular features of termites soldiers. (*Bootstrap values obtained from 100 replicates datasets displayed at nodes; 100=100%)

Ghats of Karnataka, India.

In earlier phylogenetic studies, imago-worker's mandible characters were utilized for parsimony analysis. However, this usually resulted in inconsistencies in the results, which were more apparent in the family Termitidae (Ahmad 1950). Similarly, mandibular forms seem to have changed numerous times with modifications in feeding strategies like in the case of the *Nasutitermes* group (Donovan et al. 2000; Chiu et al. 2018; Govorushko 2019). Hence, traditional morphological phylogenies are difficult to rely upon always, since they have been built on restricted subsets of characters, e.g., the mandibles of worker-imago caste (Ahmad 1950) and the worker gut (Johnson 1979; Noirot 1995; Donovan et al. 2001). Rocha et al. (2017) also had the same view that workers' mandible characters alone are not good enough as phylogenetic markers. But according to Carrijo et al. (2020) termite species can be easily identified with the combination of the soldier as well as worker caste characters, as they showed in the classification of *Heterotermes longiceps* species.

Termites can also be classified on the basis of soldier's mandibles into different types of defense categories including Biting/Crushing, Slashing (Rhinotermitidae, Serritermitidae, and Termitidae), Slashing/ Snapping, Symmetrical Snapping (*Termes, Cavitermes, Homallotermes, Dentispicotermes,* and *Orthognathotermes*), Asymmetrical Snapping (*Capritermes* and *Neocapritermes*), Piercing (*Amitermes, Armitermes, Rhynchotermes,* and *Rhinotermes*), Vestigial (Nasutitermitinae and Rhinotermitidae), Ejected Secretion (*Nasutitermes*) and Vestigial Labral Brush (*Acorhinotermes*) (Prestwich 1984). But Rocha et al. (2017) reconstructed the classification of termites into three categories (biting/crushing, piercing and slashing) based on their defense behavior.

In the present study, the importance of mandibular features in soldiers was investigated in determining the taxonomy of termite species. The soldier caste is the main caste on which consistent amount of termite taxonomic work has been focused (Seid et al. 2008; Kuan et al. 2020; Amina et al. 2020). The MP tree based on mandible features exhibited certain clusters which were consistent with the DNA based tree, as it can be observed that the species of *Microcerotermes, Odontotermes*, and *Coptotermes* are falling in their respective clades with significant bootstrap values (Figure 2); earlier investigators have also emphasized on the importance of mandible features in the determination of taxonomic position of different termite species (Donovan et al. 2000; Carrijo et al. 2020). So, the tree based on mandible features though not completely defined the relationship

between different termite families, but at genus level, proper clustering of three genera was obtained. First cluster comprised species of genus *Coptotermes* (*C. kishori, C. heimi, C. gestroi*, and *C. emersoni*), second comprised *Odontotermes* species (*O. obesus, O. assmuthi*, and *O. redemanni*) and the third comprised species of the genus *Microcerotermes* (*M. beesoni, M. baluchistanicus*, and *M. cameroni*). The clustering of rest of the species included in our study was chiefly dependent on the morphometric features.

Rocha et al. (2017) also notified such anomalies when he reported that, based on head morphology, *Rhynchotermes nasutissimus* and *Uncitermes teevani* came closer, though, phylogenetically, *U. teevani* is closer to *Labiotermes labralis*. Inward et al. (2007) commented that the defense morphologies in termites vary from species to species and evolved autonomously among all Isopterans. Similarly, Hare (1937) stated that soldiers of a few genera—*Microcerotermes, Termes*, and *Nasutitermes* (Termitidae)—lack a marginal tooth in their mandibles, while in some others, including *Amitermes, Eremotermes*, and *Odontotermes*, a distinct tooth is present at the edge of the soldier mandible (Chhotani 1997). Such observations point to the fact that there might have been a convergent evolution in the case of mandible features where similarities are indicative more of similar defense behavior and other habits rather than phylogenetic.

Molecular Identification

Sequence analysis

About 500 bps of PCR products of 16S rRNA gene were sequenced for 21 species. These sequences were BLAST-searched in NCBI databases to determine the identity of termite samples. The sequences of these 21 species were deposited to the NCBI GenBank and the accession number of each termite species was obtained (Table 1).

Nucleotide-composition analysis

The nucleotide composition in the mt16S rRNA gene fragment was calculated for the 21 termite species using MEGA11 software (Tamura et al. 2021). It exhibited considerably high frequencies of A+T base composition that ranged from 61.08 to 68.56% compared to G+C composition (31.44–38.92 %). These nucleotide arrangements showed bias towards adenine and thymine in their composition which is consistent with data on 16S rRNA mitochondrial gene studies in various insects (Kambhampati et al. 1996; Vidyashree et al. 2018). The individual nucleotide frequencies for each species have been plotted in Supplementary Figure S3. High AT frequencies have also been reported by other groups like Kambhampati et al. (1996); Vidyashree et al. (2018), Austin et al. (2002), Ohkuma et al. (2004), and Murthy (2020).

Table 3. Maximum composite likelihood estimates the pattern of nucleotide substitution.

	A	T	C	G
A	-	5.34	3.69	**9.54**
T	6.75	-	**12.52**	2.85
C	6.75	**18.08**	-	2.85
G	**22.61**	5.34	3.69	-

* transitional substitutions were 22.61, 18.08, 12.52, and 9.54, and transversional substitutions were 6.75, 5.34, 3.69, and 2.85.

The entire gene analysis of investigated termite species was done using the maximum composite likelihood (MCL) estimates method. The MCL estimates calculate the probability of substitution of one base with another base (Tamura et al. 2021). Substitution rates were assessed using MEGA11 (Tamura et al. 2021). The rates of different transitional substitutions were 22.61, 18.08, 12.52, and 9.54, and the rates of transversional substitutions were 6.75, 5.34, 3.69, and 2.85 (Table 3). The nucleotide frequencies were found to be 36.24% (A), 28.65% (T), 19.84% (C), and 15.28% (G), respectively. The transition and transversion rate ratios were obtained as $k1$ = 3.35 (purines) and $k2$ = 3.388 (pyrimidines). The overall transition/transversion bias (R) came out to be 1.513, where $R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]$.

Distance analysis

Based on sequence alignment, the divergence was calculated to investigate the intergenic variations among termite species by using MEGA11 software. The sequences of 16S rRNA gene from the termite species under this study were used to calculate pairwise genetic distance values (Kimura 2 parameter) using MEGA11 (Table 4). The K2P distance matrix values in species of the *Odontotermes* genus ranged from 0.031 to 1.256. It was found to be lowest (0.031) between *O. redemanni* and *O. obesus* and highest (1.256) between *O. parvidens* and *O. obesus*. The K2P interspecific distances in the genus *Coptotermes* ranged 0.005–1.015, recorded maximum (1.015) between *C. gestroi* and *C. emersoni,* and minimum (0.005) between *C. kishori* and *C. heimi*. However, divergence in genus *Microcerotermes* was ranged highest (0.081) between *M. raja* and *M. beesoni* and lowest (0.0)

Table 4. Pairwise genetic distances (Kimura 2-parameter) between species under study.

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	-																				
2	0.173	-																			
3	0.219	0.176	-																		
4	1.128	0.830	1.106	-																	
5	1.166	0.839	1.138	0.005	-																
6	0.974	0.715	0.96	0.145	0.151	-															
7	1.078	0.836	1.066	0.167	0.171	0.134	-														
8	0.943	0.719	0.983	0.143	0.137	0.013	0.123	-													
9	1.268	1.015	1.36	0.014	0.033	0.166	0.2	0.160	-												
10	1.162	0.924	1.235	0.159	0.171	0.163	0.186	0.159	0.159	-											
11	0.941	0.72	0.965	0.138	0.136	0.007	0.123	0.014	0.161	0.156	-										
12	1.072	0.804	1.044	0.15	0.164	0.124	0.047	0.120	0.17	0.168	0.122	-									
13	1.305	0.43	1.23	0.128	0.121	0.114	0.128	0.107	0.128	0.031	0.114	0.13	-								
14	1.167	0.760	1.133	0.260	0.252	0.24	0.251	0.238	0.292	0.314	0.230	0.243	0.272	-							
15	0.151	0.145	0.23	1.193	1.144	0.984	1.181	1.047	1.318	1.21	1.04	1.127	1.21	1.257	-						
16	0.946	0.688	0.982	0.154	0.154	0.076	0.123	0.065	0.179	0.162	0.068	0.116	0.137	0.246	1.077	-					
17	0.991	0.669	0.965	0.167	0.166	0.087	0.134	0.081	0.197	0.12	0.078	0.133	0.178	0.244	1.129	0.025	-				
18	0.984	0.719	0.946	0.138	0.150	0.007	0.118	0.014	0.175	0.155	0.0	0.122	0.114	0.228	1.048	0.070	0.077	-			
19	1.089	0.798	1.111	0.165	0.158	0.135	0.188	0.145	0.173	0.140	0.143	0.154	0.135	0.229	1.103	0.142	0.16	0.144	-		
20	0.162	0.167	0.228	1.216	1.218	1.047	1.15	1.023	1.343	1.256	1.056	1.170	1.238	1.197	0.045	1.037	1.072	1.066	1.15	-	
21	0.144	0.187	0.227	1.17	1.190	1	1.125	0.985	1.288	1.167	1.00	1.125	1.216	1.182	0.062	1.016	1.055	1.031	1.105	0.041	-

1—*Amitermes belli* | 2—*Coptotermes gestroi* | 3—*Microtermes mycophagus* | 4—*Coptotermes kishori* | 5—*Coptotermes heimi* | 6—*Angulitermes akhorisainensis* | 7—*Eremotermes paradoxalis* | 8—*Microcerotermes beesoni* | 9—*Coptotermes emersoni* | 10—*Odontotermes obesus* | 11—*Microcerotermes newmani* | 12—*Eremotermes neoparadoxalis* | 13—*Odontotermes redemanni* | 14—*Neotermes kemneri* | 15—*Odontotermes guptai* | 16—*Microcerotermes baluchistanicus* | 17—*Microcerotermes raja* | 18—*Microcerotermes cameroni* | 19—*Microtermes obesi* | 20—*Odontotermes parvidens* | 21—*Odontotermes assmuthi.*

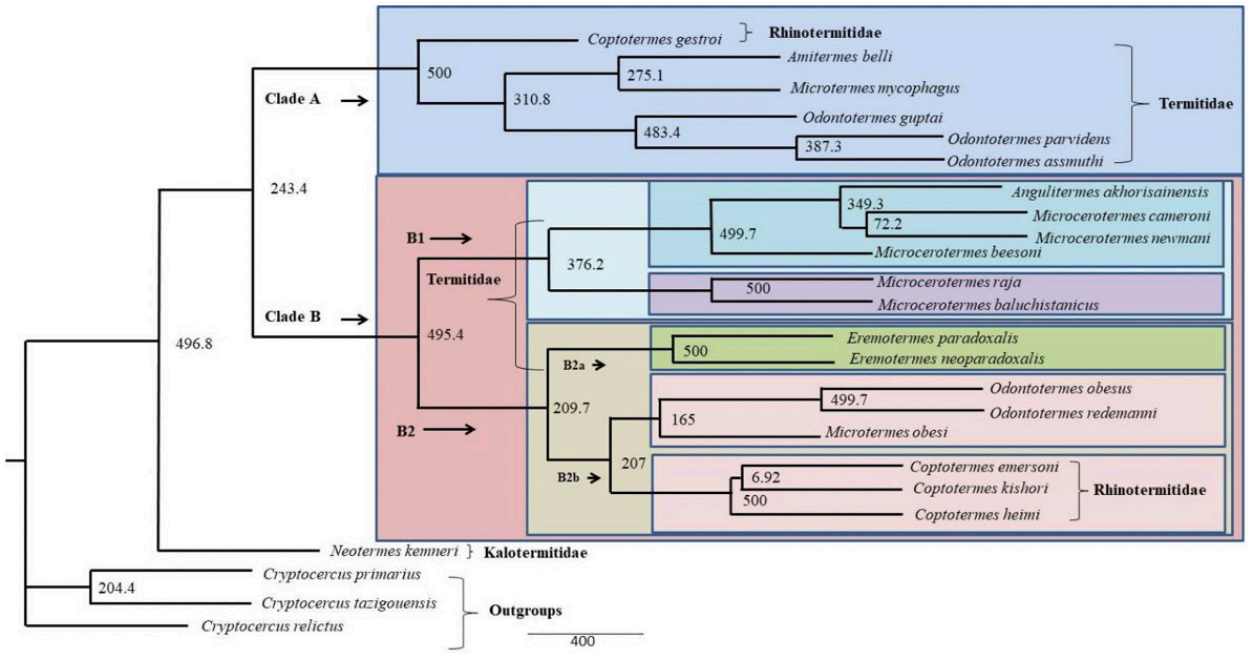

Figure 3. Phylogenetic tree built on Maximum Parsimony method to know the relatedness between isopterans; *Cryptocercus* sequences being taken as an outgroups. (*Bootstrap values obtained from 500 replicates datasets displayed at nodes; 500 = 100%).

between *M. cameroni* and *M. newmani* (Table 4).

Phylogenetic analysis

The molecular phylogenetic trees were constructed from the aligned sequences of mt16S rRNA gene using maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) methods taking *Cryptocercus* mt gene sequences as an outgroup. All the methods yielded highly similar results and genetic relatedness between different termite species was established (Figure 3, Supplementary Figures S1 & S2).

As evident in the maximum parsimony tree, all the termite sequences are distinctly different from the *Cryptocercus* sequences, which cluster separately at the base of the tree. *Amitermes belli*, *C. gestroi*, *M. mycophagus*, *O. guptai*, *O. parvidens,* and *O. assmuthi* together form a well-bootstrap value (500) supported clade (Clade A) in the MP tree as well as in ML and NJ trees. *Neotermes kemneri* associates with this clade in both NJ and ML trees, which could be indicative of relatedness between *Neotermes kemneri* and various members of this clade. In clade A, species *O. guptai, O. parvidens, O. assmuthi, M. mycophagus,* and *A. belli*, all belong to the same family Termitidae, and *C. gestroi* belongs to the family Rhinotermitidae; their clustering being strongly supported by 100% bootstrap value. Our findings were broadly consistent with those of Vidyashree et al. (2018) (based on 16S rRNA) who stated that the species of genera *Microtermes* and *Odontotermes* (belonging to family Termitidae and subfamily Macrotermitinae) form a major cluster together as they showed higher resemblance with each other on morphological basis.

The rest of the termite sequences are clustered together in a large clade (Clade B) which is well supported with bootstrap values in all the trees examined. This clade could be further subdivided into two subclades, i.e., B1 and B2 with a 495.4 bootstrap value at the node joining them. Subclade B1 having six members of two genera (*Microcerotermes* and *Angulitermes*) that belong to the same family Termitidae, i.e., *Angulitermes akhorisainensis*, *M. cameroni*, *M. newmani*, *M. beesoni*, *M. raja,* and *M. baluchistanicus*. Species *M. raja* and *M. baluchistanicus* are highly related with a 500 bootstrap value, while *Angulitermes akhorisainensis*, *M. cameroni*, *M. newmani,* and *M. beesoni* are also clustered together at 499.7 bootstrap value. The same relationships are observed in ML and NJ trees as well.

Subclade B2 having members, i.e., *E. paradoxalis*, *E. neoparadoxalis*, *O. obesus*, *O. redemanni*, *M. obesi*, *C. emersoni*, *C. kishori,* and *C. heimi*. Therefore, subclade B2 comprises species from three subfamilies (Amitermitinae, Coptotermitinae, and Macrotermitinae) and four genera (*Eremotermes, Coptotermes, Odontotermes,* and *Microtermes*) of two families, Termitidae and Rhinotermitidae. B2 is further subdivided

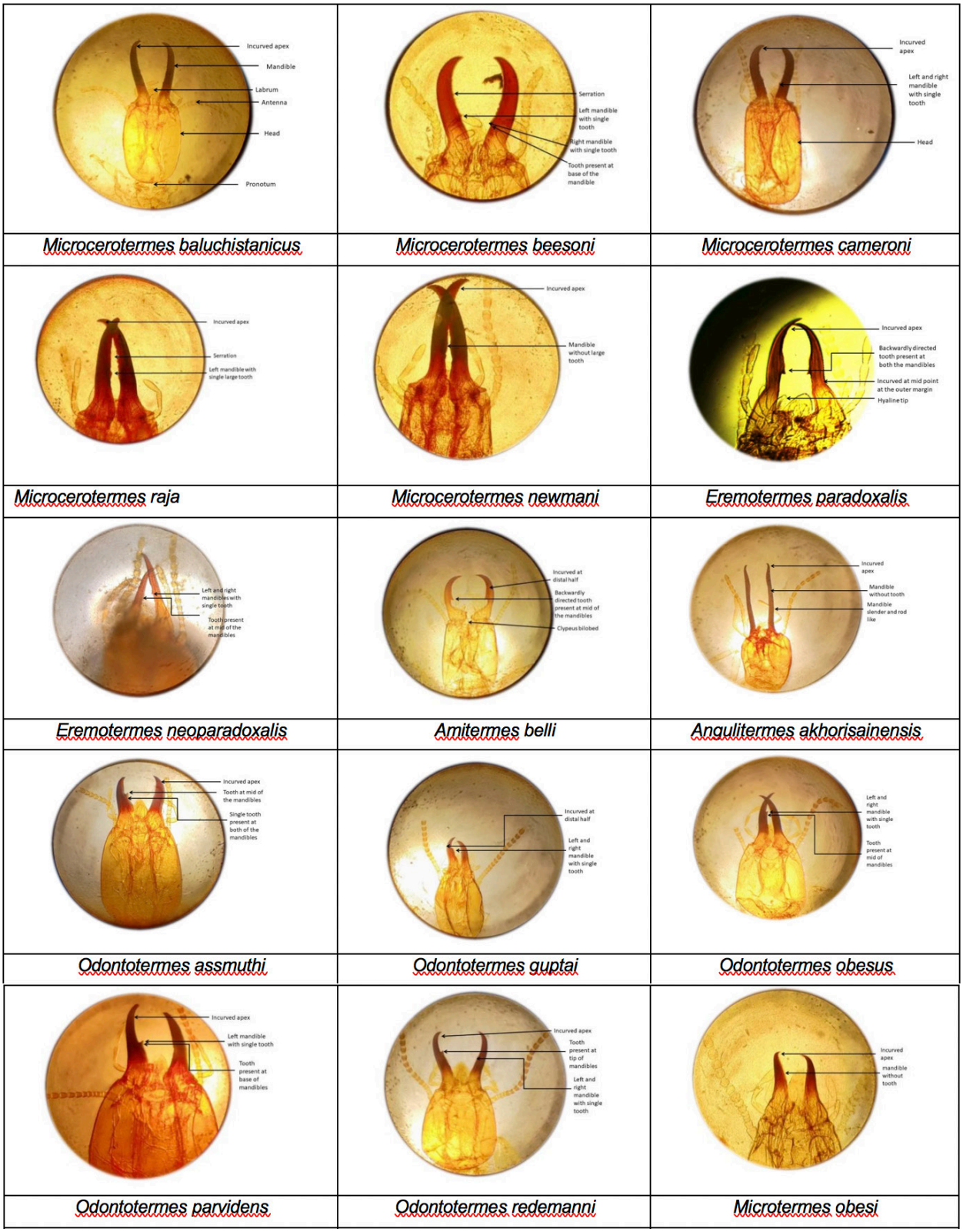

Image 1. Micrographs of mandibles of soldier caste of 21 morphologically identified species.

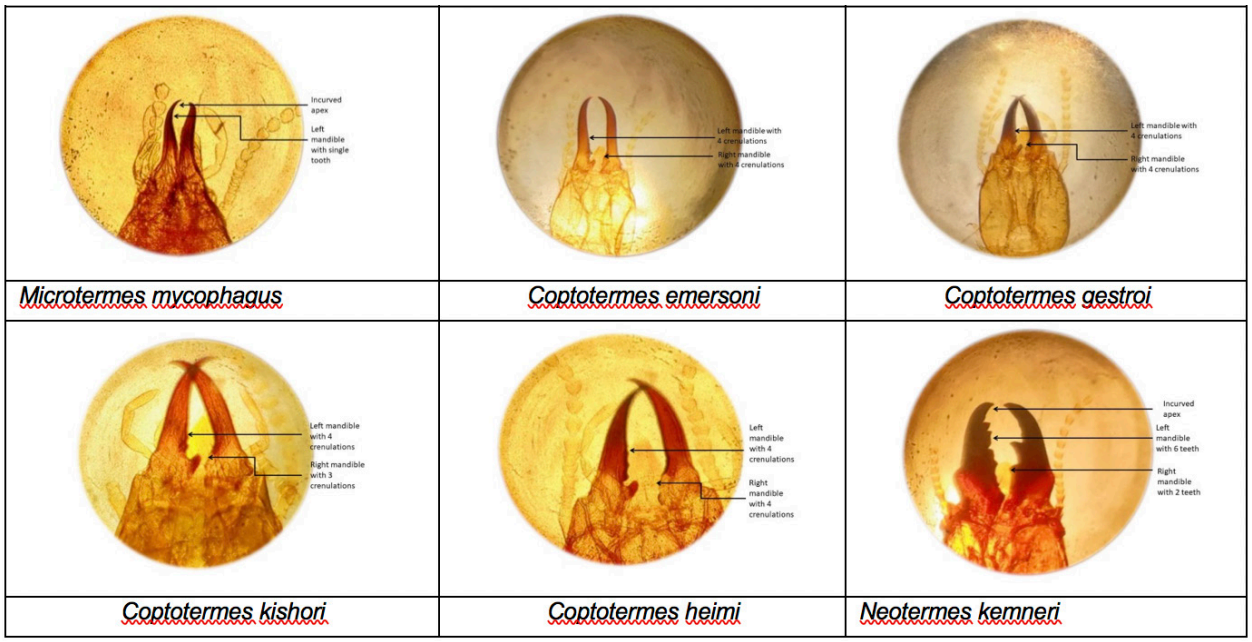

Image 1 contiued. Micrographs of mandibles of soldier caste of 21 morphologically identified species.

into two sub-subclades, i.e., B2a and B2b. In the subclade B2a, *E. paradoxalis* and *E. neoparadoxalis* are clustered together with 100% bootstrap value. However, in B2b, members of two families named Rhinotermitidae and Termitidae clustered together with almost 42% bootstrap value. Rhinotermitidae family is represented by *C. emersoni, C. kishori,* and *C. heimi,* whereas the Termitidae family is represented by *O. redemanni, O. obesus,* and *M. obesi.* Species *O. obesus*, *O. redemanni,* and *M. obesi* could also be considered related to each other since this clustering is common in both MP and ML trees, and the cluster is well supported with bootstrap values in the ML tree.

Species of genera *Coptotermes*, *Odontotermes*, and *Microtermes* were clustered on separate clades, i.e., A and B2. One possible explanation of this separate clustering comes from the morphological features of the members of these two groups, which differ in the location of tooth on the either side of mandibles and shape of the head. In *O. redemanni* and *O. obesus,* mandibles are sickle shaped and head is oval shaped. *M. mycophagus* possesses one tooth like projection near the tip of the mandibles while *M. obesi* don't have tooth. *O. obesus* is always found to cluster with the species *M. obesi* (Vidyashree et al. 2018), whereas, species of the genus *Microcerotermes* of subfamily Amitermitinae tend to fall in a separate cluster (Bourguignon et al. 2014; Vidyashree et al. 2018).

Findings from the present investigation broadly validate the results of Thompson et al. (2000) and Ohkuma et al. (2004) who described Asian termite's phylogeny, based on COII gene, taking 31 genera of Termitidae and Rhinotermitidae families.

The present work was designed to study the morphology and carry out genetic analysis of different termite species belonging to the family Termitidae, Rhinotermitidae and Kalotermitidae based on the mt16S rRNA gene. This integrated analysis was done to solve the problem that occurs in the identification of these puzzling species (Austin et al. 2005, 2012; Yeap et al. 2007; Ke et al. 2017; Ghesini et al. 2020). Significant similarities were observed in certain cases regarding the clustering of individual species in both the phylogenetic tree and the tree based on mandible features, for example, species of genus *Microcerotermes* and *Coptotermes* formed separate clades in mandible-based tree just like in the phylogenetic tree. Rhinotermitidae family clustered distinctly from Termitidae which is in equivalence with morphological identification (Vidyashree et al. 2018).

Lee et al. (2005) also verified morphological and phylogenetic analyses of Malaysian termites of the Termitidae family (Isoptera) with COII gene sequence. The first few combined studies at both the molecular and morphological levels between the major groups of isopterans were performed by Lo et al. (2004) and Inward et al. (2007). Their analysis showed Kalotermitidae, Hodotermitidae and Termitidae to be monophyletic, while Rhinotermitidae and Termopsidae

were found to be paraphyletic. This was further verified by Legendre et al. (2008) using seven gene sequences (12S rDNA, 16S rDNA, 18S rDNA, 28S rDNA, COI, COII, and cytb) establishing phylogenetic connections between the termite species. Their findings revealed that the genera of Rhinotermitidae (*Heterotermes, Reticulitermes* and *Coptotermes*) forms a sister group with the Termitidae. Rhinotermititdae and Termitidae members exhibited sister relations in our investigation as well (Figure 3; Clade B). In another study, Rhinotermitidae family shared paraphyletic relations with the family Termitidae whereas Kalotermitidae was found to be monophyletic with the Rhinotermitidae, Serritermitidae and Termitidae (Bourguignon et al. 2014). In the present research with limited members of termite species, members of Kalotermitidae, Rhinotermitidae and Termitidae also showed common ancestry (Figure 3; Clade A)

Overall, from our studies, it could be concluded that morphological and molecular systematics both considered together generates a better template for termite identification and classification.

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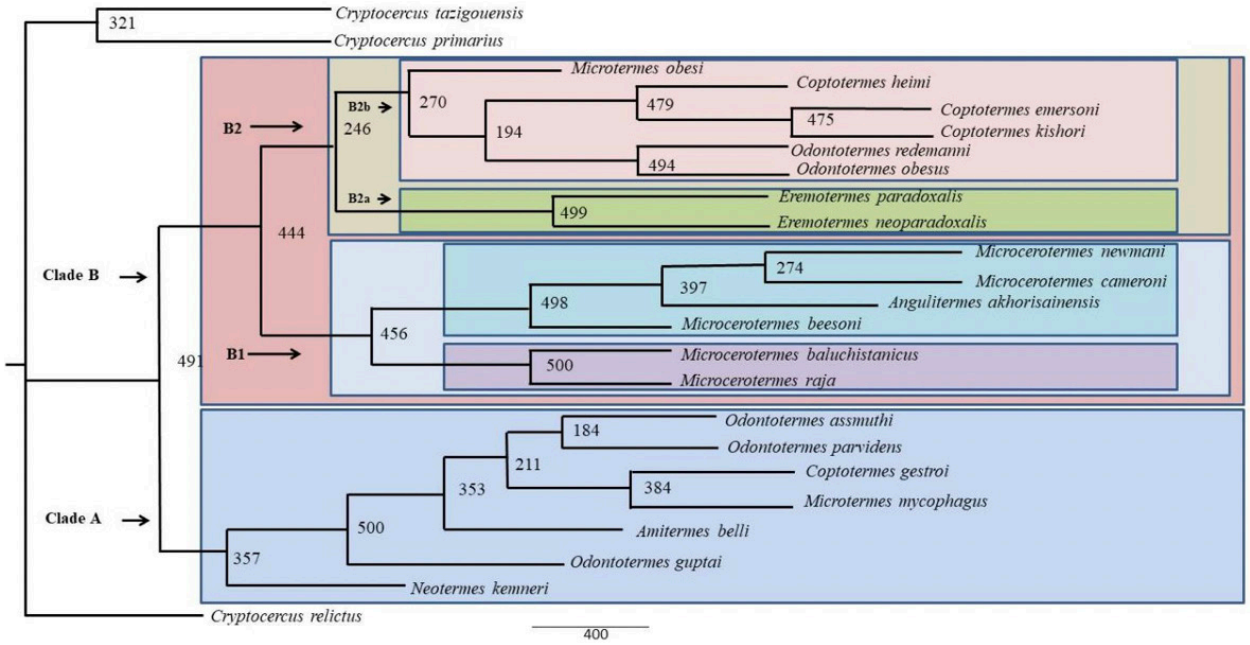

Figure S1. Phylogenetic tree built on neighbor joining method to know the relatedness between isopterans; *Cryptocercus* sequences being taken as an outgroups. (*Bootstrap values obtained from 500 replicates datasets displayed at nodes; 500 = 100%)

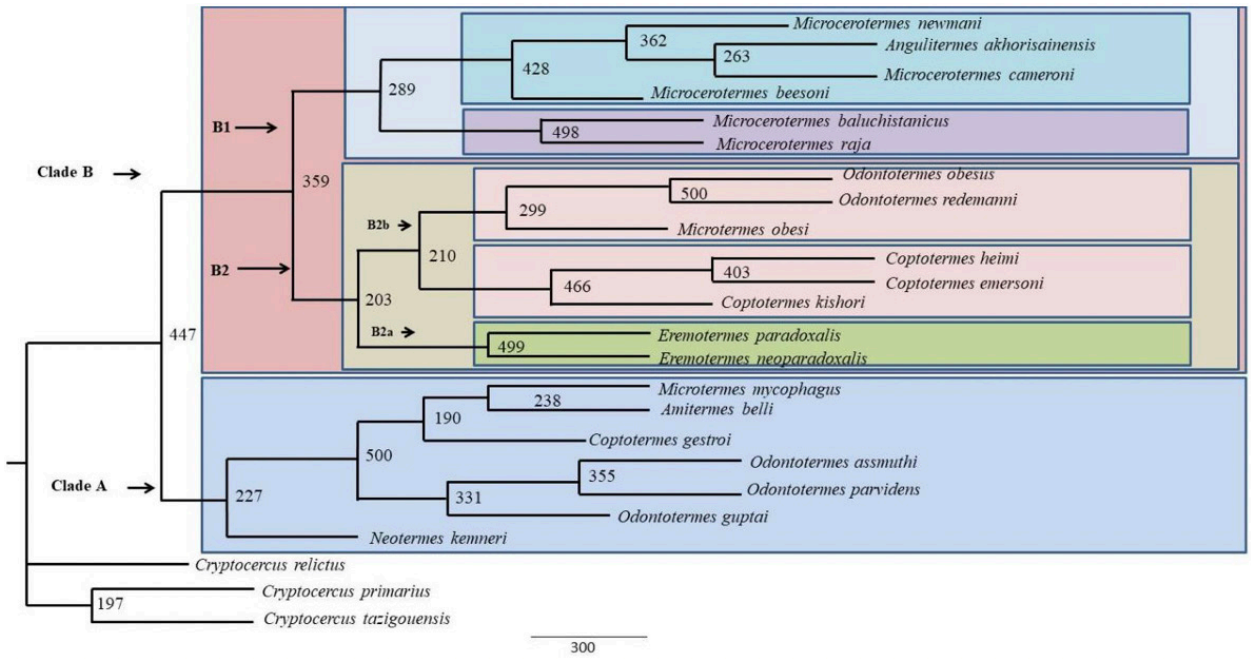

Figure S2. Phylogenetic tree built on maximum likelihood method to know the relatedness between isopterans; *Cryptocercus* sequences being taken as an outgroups. (*Bootstrap values obtained from 500 replicates datasets displayed at nodes; 500 = 100%)

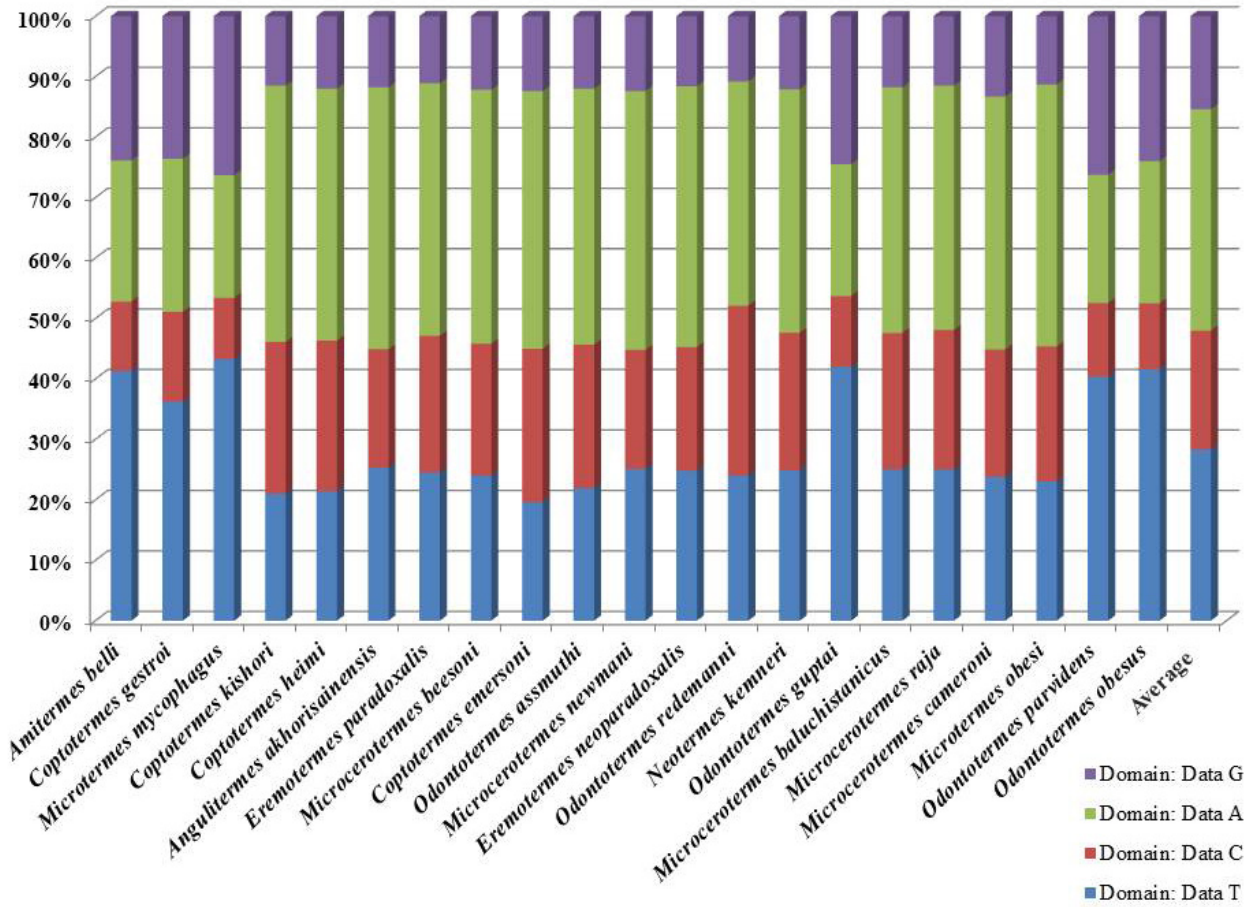

Figure S3. Graph displaying percentage of nucleotide composition in studied termite species.

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