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International Journal of Biodiversity and Conservation

Full Length Research Paper

Intra and inter specific genetic divergence of termites species based on ISSR markers and 28S rDNA sequences

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The comprehensive analysis of five identified and two unknown termite species with their binary scores of three markers *viz.,* morphological, ISSR scoring and 28s rDNA sequence has been made to reveal in unweighted pair group method with arithmetic mean (UPGMA) cluster that *Trinervitermes biformis* (Wasmann) is out grouped with 35.7% of dissimilarity with other species; *Odontotermes obesus* (Rambur) and *Odontotermes redemanni* (Wasmann) share same cluster with 32.7% of dissimilarity; similarly *Odontotermes ceylonicus* (Wasmann) and *Odontotermes horni* (Wasmann) share a maximum of 32.5% of dissimilarity. The UNKNOWN01 had more similarity with *O. obesus* (Rambur) and *O. obesus* (Rambur) in turn showed high similarity with that of *O. redemanni* (Wasmann). UNKNOWN02 was found along with *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann). The study imparts cumulative or comprehensive analysis using different markers such as morphological, ISSR and nuclear genes like 28s rDNA that depict accurate measures of genetic diversity among different species of termites.

Key words: Termite diversity, *Odontotermes, Trinervitermes*, inter simple sequence repeat (ISSR), 28s rDNA, unweighted pair group method with arithmetic mean (UPGMA), multiple sequence analysis.

INTRODUCTION

Termites are a well-known eusocial group of hemimetabolous insects classified under the taxonomic rank of infraorder Isoptera. In recent years, approximately 4000 living and many fossil, termite species are recognized and classified into 12 families. Termitidae being the largest family contains 14 subfamilies, 280 genera and over 2600 species (Kambhampati and Eggleton, 2000; Eggleton, 2001; Ohkuma et al., 2004; Krishna et al., 2013). Termites mostly feed on dead plant material for its cellulose content, generally in the form of wood, leaf litter on soil and animal dung. It is important to note that only about 10% of the estimated 4,000 species are considered as pests (Krishna et al., 2013; Bechly, 2007; Eggleton, 2010).

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Molecular markers are biological molecules of an organism, which can be an effective tool to measure genetic diversity, similarity within and among species, to identify and categorize species according to systematic classification, selectable trait for breeding programs, assess population dynamics and natural forces acting on specific alleles (Chauhan and Rajiv, 2010). Inter species analyses using a variety of markers, such as: Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), Sequence-Specific Amplification Polymorphism (S-SAP), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeats (SSR), have determined that high resolution melting (HRM) analysis to detect SNPs served best to detect accurate genetic diversity (Sorkheh et al., 2017).

ISSR markers are helpful in identifying closely related species instantly and are very useful in studying genetic diversity among closely related species which are generally difficult to identify by other means (Salhi-Hannachi et al., 2005; Okpul et al., 2005). ISSR is a multilocus marker similar to Randomly Amplified Polymorphic DNA (RAPD). They produce non-homologous similar sized fragments of DNA and pose difficulty in reproducibility (Sanchez, 1996). Although up to 99% of reproducibility has been reported by Fang and Roose (1997) in certain crop plants, which might be due to repeated inbreeding. These limitations can be overcomed if the markers are mapped on to the genome (Semagn, 2006). ISSR markers are also used to distinguish maternal and non-maternal aphids easily compared to single nucleotide polymorphism (SNPs) (Abott, 2001).

Termite genetic diversity can be analysed efficiently using ISSR markers, which can be confirmed by using nuclear genes or mitochondrial gene analyses (Long et al., 2009). Termites undergo moderate inbreeding, resulting in a wide range of genetic similarities studied by using ISSR markers (Husseneder and Grace, 2001). ISSR markers have been used in only a few animal models (Dusinsky et al., 2006); and when used, ISSR markers have efficiently discriminated both between and within species (Wang et al., 2009).

Mitochondrial DNA (mtDNA) can be used to ascertain the genetic relatedness among termites that are isolated geographically (Austin et al., 2002). The sympatric association between different species of *Reticulitermes* was analysed using mtDNA, 16s rDNA, NADH dehydrogenase and gas chromatography markers. The results pointed out certain clinical variations which do not support natural hybridization among the collected samples (Marini and Mantovani, 2002). Ribosomal molecular markers like 16s rDNA and hybridization based markers like RFLP, can be used for identification of termites, which lack distinguishable morphological characters among different species (Wang et al., 2009). Similarly, the 28s rDNA D2 region of the *Culex* mosquito revealed complex genotypic diversity (Shanmugavel et al., 2014).

In view of the aforementioned information, an attempt has been made to record the molecular diversity of termites belonging to Termitidae family based on ISSR markers and 28s rDNA sequences in order to delineate the extant of divergence among the collected termite samples belonging to the Termitidae family at Jnanabharathi campus, Bangalore University, Bengaluru which is an unexplored site with reference to Termite diversity.

MATERIALS AND METHODS

Study area

The present study is intended to yield new insights in understanding the genetic diversity of termite species in Bangalore. Jnanabharathi campus was selected for sample collection as the campus hosts a wide range of flora and fauna along with a variety of habitats *viz-a-viz* intensity of human environmental interference (Pranesh and Harini, 2015). Samples were collected during November, December and January in 2010, 2011 and 2012 to maintain uniformity in season.

Method of sample collection and its storage

Heavy-headed hammer, pickaxe, shovel and digging bar were used as appropriate to open termite nests and splitting wood to expose specimens. Based on habit and habitat, mount-building termites were collected by using a rubber-pump manual aspirator. Termites, which construct galleries on trees, and those which dwell underground, were collected by hand picking using feather-light forceps or a vacuum pump aspirator (Pranesh and Harini, 2014). The collected samples were stored in 70% ethanol at room temperature in air-tight vials for further analysis.

Identification of collected termite samples

The collected termite samples were preserved in 70% alcohol, categorized (based on casts) and counted. The samples were identified based on the morphological description given by Roonwal and Chhotani (1989) and Chhotani (1997). Morphological analyses were done with the aid of a Motic microscope with an attached 3MP camera. The measurements were made using the calibrated Motic Images obtained with Plus 2.0 Ver. software.

ISSR marker analysis

Isolation of DNA and quantification

The samples were removed from 70% ethanol and kept in double distilled sterile water for 2 min. Legs were separated from the worker termites of each species and collected in different micro centrifuge tubes (1.5 ml capacity). The collected voucher samples

Table 1. ISSR Oligonucleotide sequences.

Primer No.	Primer name	Primer sequence
ISSR1	$(GA)_8CG$	5'-GAGAGAGAGAGAGAGACG-3'
ISSR 2	$(GA)_8TG$	5'-GAGAGAGAGAGAGAGATG-3'
ISSR 3	$(AC)_7CGCG$	5'-ACACACACACACACCGCG-3'
ISSR 4	$(AC)_8TA$	5'-ACACACACACACACACTA-3'
ISSR 5	$(AT)_9GC$	5'-ATATATATATATATATATGC-3'
ISSR 6	$(AT)_9GAG$	5'-ATATATATATATATATATGAG-3'
ISSR 7	$(AGAC)_4GC$	5'-AGACAGACAGACAGACGC-3'
ISSR 8	$AC(GACA)_4$	5'-ACGACAGACAGACAGACA-3'
ISSR 9	$(GACA)_4GT$	5'-GACAGACAGACAGACAGT-3'
ISSR 10	$(GACA)_4CT$	5'-GACAGACAGACAGACACT-3'
ISSR 11	$(ATG)_5GA$	5'-ATGATGATGATGATGGA-3'
ISSR 12	$(TCC)_5GT$	5'-TCCTCCTCCTCCTCCGT-3'
ISSR 13	$(CTC)_5GT$	5'-CTCCTCCTCCTCCTCGT-3'
ISSR 14	$(AC)_8G$	5'-ACACACACACACACACG-3'
ISSR 15	$(CT)_8A$	5'-CTCTCTCTCTCTCTCTA-3'
ISSR 16	$(AC)_8AT$	5'-ACACACACACACACACAT-3'
ISSR 17	$(AC)_8T$	5'-ACACACACACACACACT-3'

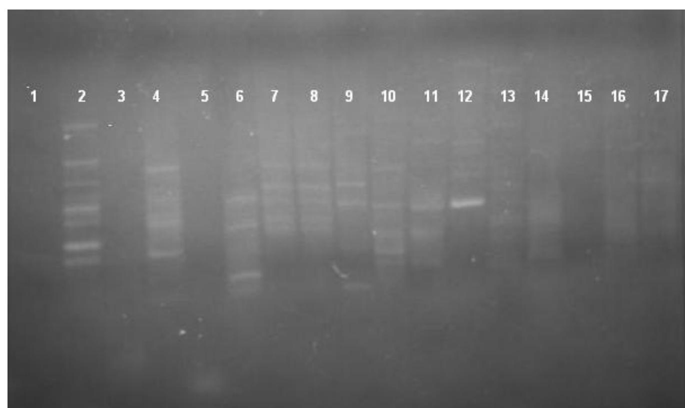

Figure 1. DNA profile of 17 ISSR primers with *O. horni* diploid genome for standardization.

were homogenized, DNA was isolated and column purified using kit procured from Aristogene biosciences Pvt. Ltd., according to manufacturer's protocol. The DNA was quantified using UV spectroscopy (Sambrook and Russell, 2001) and it was made sure that the purity of the DNA with absorbance at 260/280 ratio ranges from 1.8 to 2.0.

Amplification of DNA using ISSR markers

The amplification of DNA was done according to Al-Otaibi (2008). The amplification was done using 17 ISSR primers (Table 1). PCR reaction was done using a PCR master mix provided by Aristogene biosciences Pvt. Ltd. The final total volume of PCR reaction was 40 µl, containing 0.5 Units of Taq DNA polymerase, 2 mM $MgCl_2$, 200 µM of each dNTPs, 50 ng of DNA and 1000 pmol of primer, added to the reaction. Initially for standardization purposes, 2 µl of *O. horni* DNA were added for every 37 µl of master mix; 17 different aliquots of 39 µl of the master mix with DNA were divided in separate portions, and each portion kept in labeled PCR vials; to each vial, 1 µl of different ISSR primers were added and suitable PCR reaction temperature was set for 40 cycles according to Al-Otaibi (2008).

Electrophoresis of PCR products

The PCR products with tracking dye were loaded onto 2% agarose gel, mounted in an electrophoretic unit according to Sambrook and Russell (2001). The agarose gel used for standardization of primers is as shown in Figure 1. Among the total of 17 primers, 8 oligonucleiotides were successfully amplified. Further, among them, 6 ISSR primers (2, 4, 6, 10, 11 and 12) were used which gave visible scorable bands. ISSR primers 7 and 8 were not considered, because a similar banding pattern was obtained while standardizing the primers. Thus, the present genetic diversity study included 5 different known species and two unknown species of termites with 6 ISSR primers, which were amplified with the standardized conditions as stated earlier (Figure 2A to F).

Scoring and statistical calculations

The ISSR banding patterns obtained by electrophoresis were scored (1 for presence of a visible band and 0 for absence of a particular band), and analysed using an online statistical tool (http://genomes.urv.cat/UPGMA/index.php) for the calculation of the Similarity Matrix based on Jaccard's coefficient, UPGMA phenogram rooted tree and Cophenetic Correlation coefficient. The scoring was also used to calculate heterozygosity (Torre et al., 2012) using MS-excel with the Hardy-Weinberg formula as specified in the following:

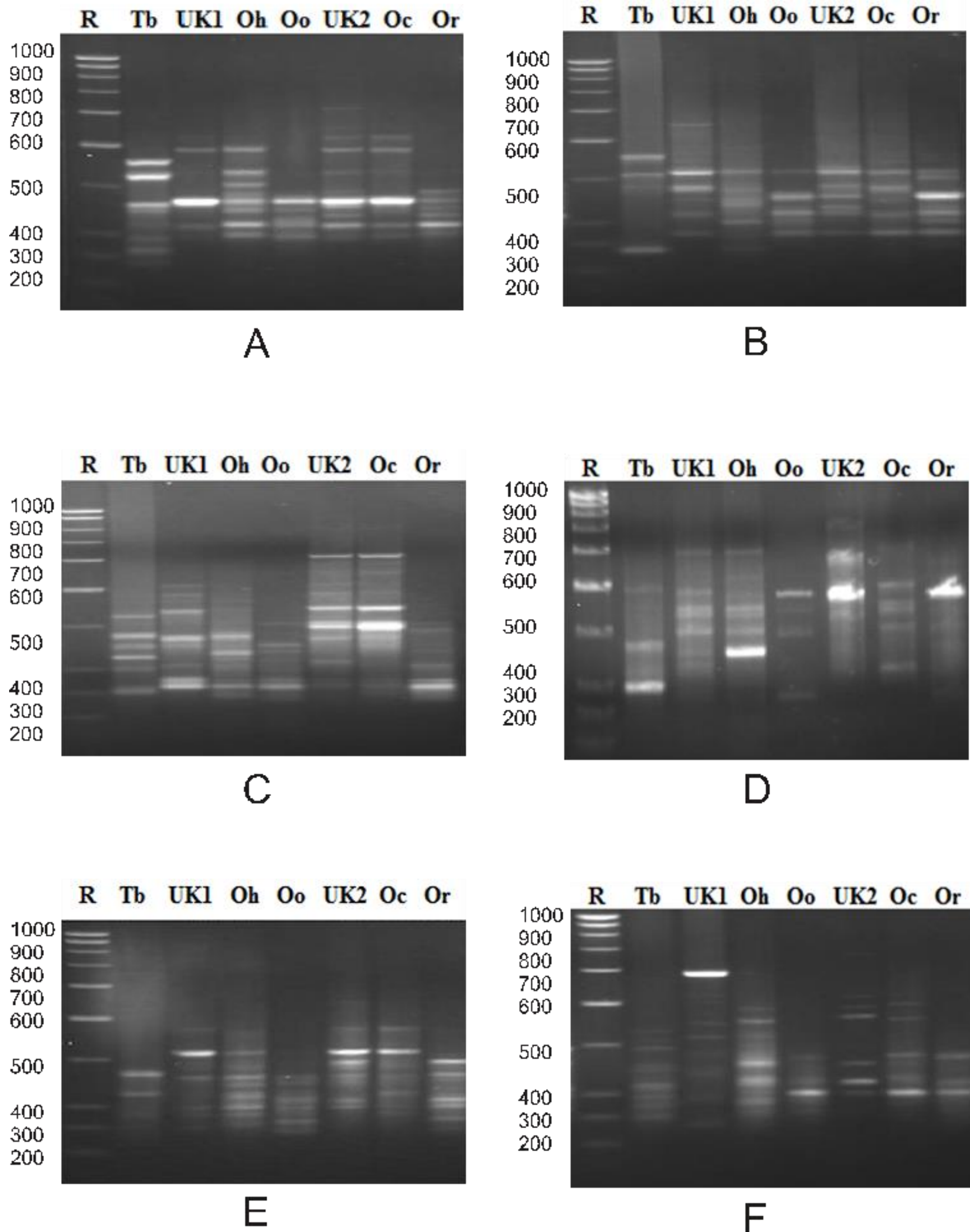

Figure 2. (A-F) DNA fragment profiles of the 7 genotypes amplified with 6 ISSR primers; namely, A - ISSR02; B - ISSR04; C - ISSR06; D - ISSR10; E - ISSR11; F - ISSR12. The order of PCR products loaded on the gel is as follows: (R) Molecular ruler, 1000 base-pair ladder; Tb, *T. biformis* (Wasmann); UK1, UNKNOWN01; Oh, *O. horni* (Wasmann); Oo, *O. obesus* (Rambur); UK2, UNKNOWN02; Oc, *O. ceylonicus* (Wasmann) and Or, *O. redemanni* (Wasmann).

Table 2. List of primers used for amplifying 28s rDNA regions.

S/N	Primer name	Primer sequence	Source
1	Rd1.2a	CCC SSG TAA TTT AAG CAT ATT A	
2	Rd3.2a	AGT ACG TGA AAC CGT TCA SGG	
3	Rd4.2b	CCT TGG TCC GTG TTT CAA GAC GG	
4	28SA	GAC CCG TCT TGA AGC ACG	Whiting (2002)
5	28SB	TCG GAA GGA ACC AGC TAC	
6	Rd4.5a	AAG TTT CCC TCA GGA TAG CTG	
7	Rd7.b1	GAC TTC CCT TAC CTA CAT	
8	Rd4b	CCT TGG TCC GTG TTT CAA GAC	Jarvis et al. (2004)
9	Rd3b	CCY TGA ACG GTT TCA CGT ACT	
	Second set of 28s rDNA primers for *O. redemanni*		
10	Rd 1a	CCCSCGTAAYTTAGGCATAT	
11	Rd 2a	TCATGCACTTTGGCAAGTCC	
12	Rd 3a	AGTACGTGAAACCGTTCAGG	
13	Rd 4a	GGAGTCTAGCATGTGYGCAAGTC	Jarvis et al. (2004)
14	Rd 6a	GGCGAAAGGGAATCYGGTTC	
15	Rd 6b	AACCRGATTCCCTTTCGCC	
16	Rd 4b	CCTTGGTCCGTGTTTCAAGAC	
17	Rd 3b	CCYTGAACGGTTTCACGTACT	

In the Hardy-Weinberg equation $p + q = 1$, implies $(p + q)2 = 1$ (since the organism under investigation is diploid). Therefore,

$$p2 + 2pq + q2 = 1.$$

where p2 is the frequency of individuals with homozygotic dominant alleles, q2 is the frequency of individuals with homozygotic recessive alleles and 2pq represents frequency of individuals with heterozygotic alleles.

But q2 is also $= \frac{\text{number of bands absent in a locus}}{\text{sample size}}$ (to study banding patterns according to Torre et al., 2012). Which implies that with the value of q, we can find the value of p; that is, $p = 1 - q$. Therefore, heterozygosity = 2pq (According to Hardy-Weinberg law).

Nuclear DNA analysis using 28s rDNA primers

DNA isolated for the ISSR procedure was used in 28s rDNA amplification and the primers applied are shown in Table 2. The procedures employed to amplify the 28s rDNA regions, including the reaction mixture and its volume, were as specified by Whiting (2002) and Jarvis et al. (2004). Further, the amplified product was sequenced and the obtained sequence was submitted to secondary structure prediction software, according to Reuter and Mathews (2010). A similarity matrix, multiple sequence alignment, diversity indices and Neighbor joining tree were constructed using MEGA (ver. 7.0.14) program.

RESULTS

A total of five taxa were analysed using PAST software, with a total termite sample as follows: 4214 workers and 3232 soldiers, obtained from the Jnanabharathi campus. The mean Dominance value was 0.2111 with a range of 0.2088 (lower limit) and 0.2134 (higher limit). This result alone cannot be useful to predict species richness in a given area. Thus, Simpson's index is calculated, which is 1-D. A mean value of 0.7889 was obtained, with 0.7866 at its lower limit and 0.7912 as its upper limit (Table 3a and b).

The five species in the sample, identified based on morphological traits, were *Trinervitermes biformis* (Wasmann), *Odontotermes horni* (Wasmann), *Odontotermes obesus* (Rambur), *Odontotermes ceylonicus* (Wasmann) and *Odontotermes redemanni* (Wasmann). But *T. biformis* (Wasmann) soldier termites are bimorphic, based on the size and shape of the head. The soldiers of *T. biformis* (Wasmann) are divided into soldier-major and soldier-minor groups. The head is ovoid and a rostrum protrudes at the anterior. At the posterior, the head is bulged. Whereas, the soldiers of *O. obesus* (Rambur) and *O. redemanni* (Wasmann) show a high level of morphological similarity, except in the mandibular index. Both have a convexly-curved head, or an oval-shaped head capsule, with weak convergence at the anterior. *O. horni* (Wasmann) is a larger species with total body length ranging from 7.37 to 10.05 mm. These are the most prevalent species found on tree galleries. They have a sub rectangular head with a thick, strong mandible. Similarly, *O. ceylonicus* (Wasmann) has a strong rectangular head with a strong mandible, but the

Table 3a. Distribution of different species of termites in various localities of Jnanabharathi.

Different types and No. of species collected	No. of nest samples collected	Number of individuals collected		
		Workers	Soldiers	Total
1. *O. ceylonicus* (Wasmann)	4	1091	61	1152
2. *O. horni* (Wasmann)	13	921	645	1566
3. *O. obesus* (Rambur)	10	1462	672	2134
4. *O. redemanni* (Wasmann)	6	727	621	1348
5. *T. biformis* (Wasmann)	16	13	1233	1246

Table 3b. Diversity indices.

Diversity	B	Lower	Upper
Taxa_S	5	5	5
Individuals	7446	7446	7446
Dominance_D	0.2111	0.2088	0.2134
Simpson_1-D	0.7889	0.7866	0.7912
Shannon_H	1.583	1.578	1.589

body size is much smaller than *O. horni* (Wasmann) and slightly bigger than the other three species.

ISSR marker analysis

All five species and two unknown samples were subjected to ISSR marker analysis. A total of 244 visible and scorable bands were obtained. All the visible bands were scored into one (‘1’ for presence of a particular band with a specific band size) and zero (‘0’ for absence of a band at a particular band size corresponding to the other samples). There was only one monomorphic band obtained from the amplification. Thus a total of 98.72% polymorphism was observed from the ISSR profile. The scoring was recorded according to the band size based on the one kb ladder, which was loaded along with each amplification product in a separate well. The scoring was also analysed to calculate heterozygosity (Table 4a to c), Jaccard’s coefficient (Table 4d) and to construct a UPGMA tree (Figure 3).

The amplification result reveals very little polymorphism between species. But with high resolution it is possible to differentiate even a 10 bp difference between the banding patterns. This led to observation of bands with more than 98% polymorphism. To get a clear idea of polymorphism, the gel was scored and tabulated (Table 4a). According to the scoring, a consolidated data (Table 4b) was generated to study the banding pattern of the ISSR markers. There is only one monomorphic band in the ISSR11 marker at 380 bp. Among seven species, a maximum of six species shows monomorphic bands for the three markers, that is, ISSR02, ISSR04 and ISSR10 at 370, 430 and 520 bp, respectively. There were five lanes of bands at 350, 320 and 440 bp length products amplified using ISSR02, ISSR04 and ISSR10, respectively. The other two lanes were produced with the primer ISSR12 alone at 330 and 300 bp length with monomorphic bands for a maximum of five species (Figure 2A to F).

There were about 14 lanes with a maximum of four species with monomorphic bands, whereas the other two did not match. Similarly, 42.86% of monomorphism (that is, a maximum of three species with monomorphic bands) were obtained at 16 different lanes; and two species with monomorphic bands were observed at 26 different lanes. This indicates that the level of monomorphism between the collected specimens is very low for the primers used in the amplification. A total of 38 different positions show single bands with varying sizes. The single bands of maximum size were obtained for the primer ISSR12.

Heterozygosity value plays a major role in finding the markers’ ability to detect polymorphism. Generally, for ISSR markers, the heterozygosity values range from 0.2 to 0.4. In the present study, heterozygosity values range from 0.2444 to 0.3366, where the highest value was scored by ISSR10 and the lowest value was obtained using the ISSR12 marker (Table 4c).

The UPGMA tree was constructed based on the Jaccard’s coefficient values (Figure 3). Analysis of the tree indicates that *O. redemanni* (Wasmann) and *O. obesus* (Rambur) share 25% dissimilarity between

Table 4a. ISSR markers banding pattern detected in 5 termite species and two unknown species.

Primer No.	PCR product molecular weights (bp)	Types of species investigated						
		T. biformis (Wasmann)	*UNKNOWN01*	*O. horni* (Wasmann)	*O. obesus* (Rambur)	*UNKNOWN02*	*O. ceylonicus* (Wasmann)	*O. redemanni* (Wasmann)
ISSR02	620	0	0	0	0	1	0	0
	550	0	1	0	0	1	1	0
	520	0	0	0	0	1	1	0
	510	0	1	1	0	1	1	0
	490	1	0	0	0	0	0	0
	460	0	1	0	1	0	0	0
	440	1	0	0	0	0	0	0
	400	0	0	1	0	0	0	0
	380	0	0	1	0	1	0	1
	370	0	1	1	1	1	1	1
	360	1	0	0	0	0	0	1
	350	1	0	1	1	1	0	1
	340	0	0	0	1	1	0	1
	330	0	0	1	0	1	1	1
	280	0	0	1	1	0	0	0
	220	1	0	0	0	0	0	0
	180	1	0	0	0	0	0	0
ISSR04	550	0	1	0	0	0	0	0
	460	1	0	0	0	0	0	0
	430	0	1	1	1	1	1	1
	420	0	1	0	0	0	0	0
	400	1	0	0	0	0	0	1
	380	0	1	0	0	1	0	0
	370	0	0	0	0	0	1	0
	360	0	0	0	1	1	0	1
	350	0	0	1	0	0	0	0
	340	0	0	0	0	1	0	1
	320	0	1	0	1	1	1	1
	250	0	0	1	0	0	0	1
	200	0	1	0	1	0	1	1
	180	1	0	0	0	0	0	0

Table 4a. Contd.

	610	0	0	0	0	1	1	0
	580	0	0	0	0	1	1	0
	570	0	0	0	0	0	1	0
	520	0	1	0	0	1	0	0
	490	0	1	0	0	1	0	0
	480	0	1	0	0	1	1	0
	450	1	0	0	0	0	0	0
	430	0	0	0	0	1	1	1
	420	0	0	0	0	1	1	1
	400	1	0	1	0	0	0	0
ISSR06	380	0	1	1	0	1	1	0
	370	1	1	1	1	0	0	0
	360	0	0	1	0	0	0	0
	350	1	0	0	0	0	0	0
	340	0	0	1	0	0	0	0
	330	0	0	0	0	1	0	0
	300	1	1	1	0	0	0	1
	250	0	1	0	0	0	0	0
	240	0	1	1	1	0	0	1
	230	1	0	0	0	0	0	0
	180	0	0	1	1	0	0	1
	700	0	0	0	0	1	0	0
	600	0	1	1	0	1	1	0
	580	0	1	1	0	1	1	0
	570	0	0	0	0	1	0	1
	520	1	1	1	1	1	0	1
	500	0	0	1	1	0	0	0
ISSR10	490	1	0	1	0	0	1	0
	450	0	0	0	1	1	0	1
	440	0	1	1	1	1	0	1
	400	0	0	1	0	0	0	0
	380	0	0	1	1	0	0	1
	250	0	0	0	1	0	0	1
ISSR11	540	0	0	0	0	1	0	0

Table 4a. Contd.

	470	0	1	0	0	1	1	0
	420	0	1	1	0	1	1	0
	400	0	0	0	0	1	0	1
	390	1	0	0	0	0	0	0
	380	1	1	1	1	1	1	1
	350	0	0	1	1	1	0	0
	330	1	0	1	0	0	1	0
	310	0	0	0	1	1	0	1
	300	0	0	1	1	1	0	1
	270	0	0	1	0	0	0	0
	200	0	0	1	1	0	0	1
	180	0	0	0	1	0	0	0
	650	0	0	0	1	0	0	0
	580	0	1	1	0	0	0	0
	560	0	1	0	0	0	0	0
	540	0	1	0	0	0	0	0
	520	0	1	0	0	1	0	0
	500	0	1	0	0	0	1	0
	480	0	0	1	0	1	0	0
	450	0	0	0	0	1	0	0
	440	0	0	1	0	0	1	0
	430	0	1	0	0	0	0	0
	420	1	0	0	0	0	0	0
ISSR12	410	0	1	1	0	0	0	0
	405	1	0	0	0	0	0	0
	390	1	0	0	0	0	0	0
	370	0	0	1	1	0	1	1
	360	0	0	1	0	1	1	1
	350	1	0	0	0	0	0	0
	340	0	1	0	0	0	0	0
	330	0	0	1	1	1	1	1
	320	1	0	0	0	0	0	0
	300	0	0	1	1	1	1	1
	290	1	0	1	0	0	0	0
	250	1	1	1	1	0	0	0

Table 4a. Contd.

230	0	0	1	1	0	0	0
200	1	0	1	0	0	0	0
180	0	1	0	0	0	0	0

them. It is also observed that *T. biformis* (Wasmann) is in a separate cluster with a maximum of 44.8% dissimilarity among all of the identified and unidentified species (Table 4d).

Nuclear DNA analysis using 28s rDNA primers

Multiple sequence alignment

The forward and reverse sequences were combined to make single contig sequence for each species using the DNA Baser program. The number of nucleotides, Multiple Sequence Alignment (MSA), and nucleotide analysis was done by using MEGA software. *O. ceylonicus* (Wasmann), *O. redemanni* (Wasmann), *O. horni* (Wasmann), *O. obesus* (Rambur), *T. biformis* (Wasmann), UNKNOWN01 and UNKNOWN02 are the samples sequenced for both forward and reverse nucleotide sequences to create a single contig sequence. The final length of 849, 890, 753, 643, 728, 861 and 531 bp were obtained, respectively. These sequences were subjected to MSA using MEGA software, which revealed 1124 sites with a total of 74 conserved regions and a sum of 2618 regions of gaps among all the seven samples of termites. The results of MSA is tabulated in Table 5 where homology is marked with an asterisk (*) and the gaps are mentioned with a dash (-).

The nucleotide analysis (Table 6a) revealed that the average frequency of GC rich regions is 30 and those with AT is 33. The R value for the fraction of transitional (132) and transvertional (263) pairs were found to be 0.5, which is a moderate value. The identical pair number was found to be 207, which is the sum of MSA analysis of identical pair numbers at the first position (73); second position (74) and at third position (60). The highest sum value of 61 was obtained for the TT sequence frequency, followed by AA of about 55; CC of about 48, and GG of about 43. These ranges of frequencies are clearly represented in a pi-chart for detailed speculation in Figure 4.

Nucleotide frequency of all the seven partially amplified 28s rDNA sequences discloses how much a perticular nucleotide appears in every species for a perticular gene. It is clear that A+T is always higher than G+C in all the partially amplified 28s rDNA sequences, exccept in *O. horni* (Wasmann) and *T. biformis* (Wasmann) with a higher amount of G+C residues than A+T residues (Table 6b).

The MSA of the entire seven termite samples also revealed 74 conserved regions (with only single nucleotide sites and few base stretches with many gaps) and 39 regions of variable sites. The variable sites are used to analyze the variable secondary structures of 28s rDNA. The bases beyond 50 nucleotides were considered for studying 28s rDNA structural variations and are highlighted in green colour (Table 6c). A maximum of 111 nucleotide variations and a minimum of single base variations were recorded. There were up to six variable sites beyond 50 bases and nine single nucleotide variations.

28s rDNA nucleotide sequence structure and its analysis

The nucleotide sequence of the 28s rDNA sequence was submitted to the Mathew online lab portal and the secondary structure of the partially amplified sequence of 28s rDNA gene was constructed.

There are six different types of rDNA structure identified in the study (Figure 5). Primary structure consists of single stranded nucleotide bases; a hairpin loop is the most common type of secondary structure with pseudo double stranded forms with unpaired loops; zigzag conformers are tertiary structures with three dimensional orientation of the molecule; and lastly pseudo knots are similar to that of pseudo double-stranded structure, but the bonding is between the far located bases oriented spatially in a three dimensional way. The number of variable sites according to MSA, their relevant divergent regions on the amplified 28s rDNA sequence and the observed secondary structure are summarized in Table 7.

O. ceylonicus (Wasmann) possesses nine hairpin loops, 10 single-stranded loops and eight

Table 4b. Consolidated analysis of ISSR marker scoring.

Marker name	Length of PCR product in base pairs	Number of species having monomorphic bands at specified length	Number of lanes with same bands
ISSR11	380	7	1
ISSR02	370	6	
ISSR04	430	6	3
ISSR10	520	6	
ISSR02	350	5	
ISSR04	320	5	5
ISSR10	440	5	
ISSR12	330, 300	5 (each)	
ISSR02	510, 330	4 (each)	
ISSR04	200	4	
ISSR06	380, 370, 300, 240	4 (each)	14
ISSR10	600, 580	4 (each)	
ISSR11	440, 300	4 (each)	
ISSR12	370, 360, 250	4 (each)	
ISSR02	550, 380, 340	3 (each)	
ISSR04	360	3	
ISSR06	480, 430, 420, 180	3 (each)	16
ISSR10	490, 450, 380	3 (each)	
ISSR11	470, 350, 330, 310, 200	3 (each)	
ISSR02	520, 460, 360, 280	2 (each)	
ISSR04	400, 380, 340, 250	2 (each)	
ISSR06	610, 580, 520, 490, 400	2 (each)	26
ISSR10	570, 500, 250	2 (each)	
ISSR11	400	2	
ISSR12	580, 520, 500, 480, 440, 410, 290, 230, 200	2 (each)	
ISSR02	620, 490, 440, 400, 220, 180	1 (each)	
ISSR04	550, 460, 420, 370, 350, 180	1 (each)	
ISSR06	570, 450, 360, 350, 340, 330, 250, 230	1 (each)	38
ISSR10	700, 400	1 (each)	
ISSR11	540, 390, 270, 180	1 (each)	
ISSR12	650, 560, 540, 450, 430, 420, 405, 390, 350, 340, 320, 180	1 (each)	

pseudo knots (Figure 6A). Among the 39 variable sites, five variable sites were selected which were beyond 50 bases long. VS1 spans 71st base to 132nd base in *O. ceylonicus* (Wasmann) which had one single-stranded loop, one hairpin loop, two pseudo knot and pseudo double helical structure that was formed from the 97th base to 132nd base with 836th base to 811th base. The marked VS2 ranged from 137th base to 189th base, which formed a pseudo double-stranded structure along with the 803rd base to 751st base. Similarly, the VS3 stretches from the 248th base to 363rd base with two hairpin loops and two pseudo knots; VS4 spans from 406th base to 483rd base where it is clear that there is a complex tertiary structure in this position with overlapping hairpin loops, unpaired strands and unpaired loops; VS5 started from the 562nd base to 646th base with a

Table 4c. ISSR marker polymorphism data of seven termite colonies.

Primer number	Primer sequence	Total number of fragments	Number of polymorphic fragments	Percentage of polymorphism	Range of fragment size (bp)	Heterozygosity
ISSR 2	$(GA)_8TG$	17	17	100	180-620	0.2853
ISSR 4	$(AC)_8TA$	14	14	100	180-550	0.2614
ISSR 6	$(AT)_9GAG$	21	21	100	180-610	0.2711
ISSR 10	$(GACA)_4CT$	12	12	100	250-700	0.3366
ISSR 11	$(ATG)_5GA$	13	12	92.31	180-540	0.2739
ISSR 12	$(TCC)_5GT$	26	26	100	180-650	0.2444

Table 4d. Similarity Matrix of seven termite colonies based on Jaccard's coefficient obtained from 103 ISSR fragments.

Colony	*T. biformis* (Wasmann)	UK1	*O. horni* (Wasmann)	*O. obesus* (Rambur)	UK2	*O. ceylonicus* (Wasmann)	*O. redemanni* (Wasmann)
T. biformis (Wasmann)	1						
UK1	0.086	1					
O. horni (Wasmann)	0.177	0.25	1				
O. obesus (Rambur)	0.094	0.204	0.364	1			
UK2	0.043	0.295	0.271	0.254	1		
O. ceylonicus (Wasmann)	0.056	0.28	0.276	0.157	0.404	1	
O. redemanni (Wasmann)	0.109	0.153	0.322	0.5	0.4	0.24	1

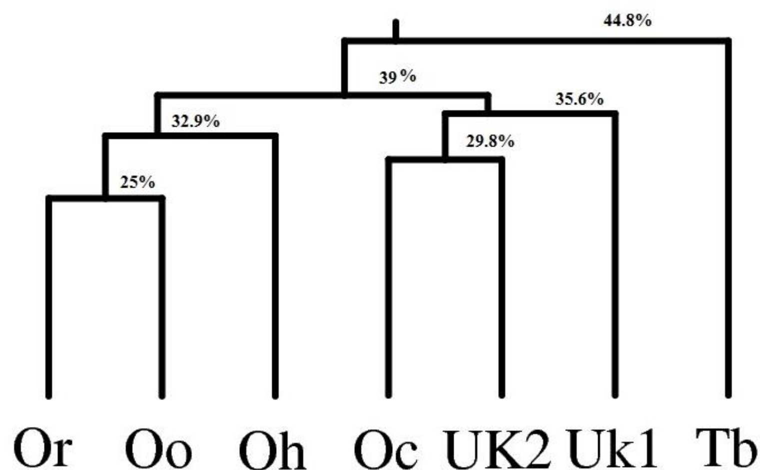

Figure 3. Dendogram generated by UPGMA cluster analysis based on Jaccard's coefficient using 103 ISSR fragments.

complex of a hairpin loop, single-stranded primary structure, pseudo double strand and unpaired loops; the last, VS6, which spans from the 679th base to 747th base as already mentioned formed a pseudo double-stranded structure with 258th base to 194th base.

The 28s rDNA sequence secondary structure (Figure 6B) of *O. redemanni* (Wasmann) also showed divergence in the marked variable sites (according to MSA). At position VS1 from 74th base to 135th base there are two pseudo knots at the terminal position and two hairpin loops along with a long unpaired strand; position VS2, ranging from 140th base to 192nd base, where the initial

Table 5. Multiple Sequence Alignment of all the five identified species and the two Unknown species.

O. redemanni	-	-	-	-	-	-	C	C	G	A	A	A	C	G	A	C	C	T	C	A	A	C	C	T	A	T	T	G	G	G
O. horni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK01	-	-	-	-	-	-	C	A	A	T	G	T	-	-	-	C	C	T	T	A	A	C	C	T	A	T	-	-	-	T
T. biformis	-	-	-	-	-	-	-	-	-	-	-	-	C	A	C	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-
O. cylonicus	C	A	T	G	G	A	T	A	G	T	G	T	C	A	T	C	T	G	T	G	G	C	T	C	A	T	T	C	T	C
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	A	A	A	C	T	G	C	C	C	G	A	T	G	G	G	T	G	A	G	A	T	C	T	A	C	G	T	T	T	T
O. horni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK01	C	T	C	A	A	A	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	A	A	A	T	G	T	G
T. biformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. cylonicus	A	A	T	C	T	A	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	A	A	A	T	G	G	A
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	T	G	A	C	G	T	T	G	A	G	T	T	G	A	T	G	C	A	T	C	G	T	A	C	A	A	G	A	C	C
O. horni	-	-	-	-	-	-	-	-	-	-	-	G	C	T	G	C	G	G	T	T	A	T	G	G	G	A	A	C	A	A
UK01	T	A	A	G	A	C	G	T	C	G	T	T	G	C	T	A	C	T	T	T	G	T	T	G	A	-	G	C	C	T
T. biformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	G
O. cylonicus	C	G	T	G	A	T	T	A	A	G	T	G	A	T	T	A	C	T	T	T	A	T	T	G	A	-	A	T	T	G
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	T	G	C	A	T	G	C	T	G	G	-	-	-	-	-	-	-	-	-	T	G	C	C	A	G	G	T	G	T	G
O. horni	C	C	T	T	G	C	G	T	G	A	C	A	C	C	T	A	T	T	C	T	T	T	C	C	C	G	C	A	A	A
UK01	C	G	A	C	A	C	T	C	G	T	A	T	G	C	A	T	G	T	G	T	T	C	T	T	A	G	T	G	G	G
T. biformis	C	G	T	A	G	C	C	C	T	C	T	C	C	C	G	G	A	-	-	T	-	-	-	-	-	-	-	-	-	-
O. cylonicus	C	T	T	A	G	A	T	T	A	A	T	A	A	G	A	A	C	-	-	T	T	T	T	A	G	T	G	G	G	C
O. obesus	-	-	-	-	T	G	T	A	A	A	T	A	A	C	A	A	A	-	-	T	T	T	T	C	C	C	T	C	T	T
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	C	T	A	A	T	T	T	T	G	G	T	A	A	G	C	T	A	G	A	A	C	T	A	G	A	G	-	C	T	G
O. horni	C	T	T	T	T	A	A	T	A	G	T	C	G	T	C	G	G	G	A	G	C	G	C	A	C	C	G	G	A	C
UK01	C	C	A	T	T	T	T	T	G	G	T	A	A	G	C	A	G	A	A	C	T	G	G	C	G	-	-	A	T	G
T. biformis	-	-	T	T	T	C	A	A	G	G	T	C	C	G	A	G	G	G	G	A	C	G	A	C	C	C	G	G	A	C
O. cylonicus	C	-	A	T	T	T	T	T	G	G	T	A	A	G	C	A	G	A	A	C	T	G	G	C	G	-	-	A	T	G
O. obesus	C	T	T	T	T	C	A	T	G	G	A	C	A	G	T	C	A	A	A	A	A	T	G	C	A	-	-	T	C	G
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	T	G	G	G	A	C	G	A	A	C	C	A	T	A	C	G	A	C	G	G	G	T	T	G	A	C	G	C	G	C
O. horni	-	G	C	T	G	T	A	A	A	A	A	A	T	C	C	T	T	G	T	T	A	C	T	C	C	G	G	T	A	A
UK01	C	G	G	G	A	T	G	A	A	C	C	G	G	A	A	G	G	A	G	G	G	T	T	A	A	G	G	T	G	C
T. biformis	A	C	C	G	C	C	G	C	A	A	C	T	G	C	G	G	T	G	C	T	C	T	T	C	G	C	G	T	T	C
O. cylonicus	A	G	G	G	A	T	G	C	T	C	C	T	A	A	C	G	T	T	T	A	G	C	T	A	A	G	G	T	G	C
O. obesus	A	A	A	A	C	C	-	-	T	-	T	G	G	A	T	A	T	C	A	A	G	G	T	T	C	T	A	T	T	C
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	C	T	A	A	A	C	C	G	T	G	A	G	C	-	-	-	T	A	C	A	T	G	G	C	T	G	A	C	C	A
O. horni	T	T	G	A	A	C	G	T	G	G	A	-	-	C	C	T	T	T	G	A	A	T	G	T	A	T	C	G	T	T
UK01	C	G	G	A	A	T	A	C	A	C	G	-	-	-	-	-	C	T	C	A	T	C	A	G	A	T	A	C	C	A
T. biformis	C	A	A	A	C	C	C	T	A	C	C	T	C	C	C	T	G	C	T	A	G	A	G	G	A	T	T	C	C	A
O. cylonicus	C	T	A	A	A	T	G	C	T	C	G	-	-	-	-	-	C	T	C	A	T	C	A	G	A	T	A	C	C	A
O. obesus	C	A	A	A	A	A	T	T	T	A	-	-	-	-	-	-	A	C	C	G	G	G	T	G	C	T	A	T	C	T

Table 5. Contd.

UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	A	G	A	C	T	T	G	C	T	T	T	G	G	G	T	G	C	T	T	G	T	A	C	A	C	T	G	C	A	G
O. horni	A	C	T	A	G	T	G	G	G	C	C	A	G	A	T	T	T	T	T	G	G	T	A	A	G	C	A	A	A	G
UK01	G	A	A	-	A	A	G	G	T	G	T	T	G	G	T	T	C	A	T	C	T	A	G	A	C	A	G	C	A	G
T. biformis	G	G	G	A	A	C	T	C	G	A	A	C	G	C	T	T	A	T	G	C	G	A	G	A	G	A	A	C	A	G
O. cylonicus	G	A	A	-	A	A	G	G	T	G	T	T	G	G	T	T	C	A	T	T	T	T	A	A	C	A	G	C	A	G
O. obesus	C	C	A	-	A	A	T	A	A	A	T	T	-	-	-	-	-	-	-	-	-	-	G	A	T	T	C	C	A	G
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	T	C	A	C	G	C	T	G	G	T	C	T	T	G	G	G	A	G	T	C	G	G	A	A	T	G	C	G	C	A
O. horni	A	-	G	A	A	C	T	C	T	T	C	C	C	G	G	G	G	C	T	C	C	C	G	C	C	A	A	C	G	T
UK01	G	-	A	C	G	G	T	G	G	C	C	A	T	G	G	A	A	G	T	C	G	G	A	A	T	C	C	G	C	T
T. biformis	A	-	A	A	A	C	T	C	T	G	C	C	C	G	G	G	T	C	T	C	C	C	G	A	C	-	-	-	-	-
O. cylonicus	G	-	A	C	G	G	T	G	G	T	C	A	T	G	G	A	A	G	T	A	G	A	A	A	T	C	C	G	C	T
O. obesus	G	-	G	T	A	A	T	A	T	T	-	-	-	-	T	T	G	T	T	A	A	A	A	A	G	C	A	A	A	T
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	A	A	A	T	G	G	A	G	T	G	T	G	T	A	A	T	C	A	A	T	T	C	A	C	C	T	G	-	-	C
O. horni	C	T	C	C	G	C	T	T	T	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
UK01	A	A	G	G	A	G	T	G	T	G	T	A	A	C	A	A	C	T	C	A	C	C	T	G	C	C	G	A	-	A
T. biformis	-	-	G	G	C	G	T	C	T	C	C	G	G	G	T	C	C	C	T	T	T	G	G	G	T	T	A	C	C	C
O. cylonicus	A	A	G	G	A	G	T	G	T	G	T	A	A	C	A	-	A	C	T	C	A	C	C	T	G	C	C	G	A	A
O. obesus	G	A	C	A	A	C	G	C	T	T	T	T	C	G	A	G	A	T	T	T	T	T	G	C	C	T	G	T	G	T
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	C	G	A	C	G	G	C	T	A	A	A	T	A	T	A	A	C	C	-	-	-	-	-	-	-	-	-	-	-	-
O. horni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	G	G	C	G	-	A	T	G
UK01	T	G	A	A	C	T	A	G	C	C	C	T	G	A	A	A	A	T	G	G	A	T	G	G	C	G	C	T	T	A
T. biformis	C	G	A	C	G	A	G	C	T	T	C	T	C	T	T	G	C	G	-	-	-	A	G	G	G	C	C	C	G	A
O. cylonicus	T	G	A	A	C	C	A	G	C	C	C	T	G	A	A	A	A	T	-	-	-	G	-	-	-	-	-	-	A	A	T
O. obesus	T	G	A	A	G	A	G	T	A	T	C	A	T	T	T	G	C	G	-	-	-	T	-	-	-	-	-	-	T	A	C
UK02	-	-	-	-	-	-	-	-	-	G	T	G	A	T	T	A	C	G	-	-	-	T	-	-	-	-	-	-	T	A	C
O. redemanni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	G	-	-	-	-	-	-	-	-	-	-
O. horni	C	G	G	G	A	T	G	A	A	C	-	-	C	G	A	A	C	G	A	G	G	G	G	T	T	A	A	G	G	T
UK01	A	G	C	G	T	G	T	T	A	C	C	C	A	C	A	C	C	C	T	C	C	C	G	C	C	A	G	G	G	T
T. biformis	C	G	G	T	T	C	G	G	T	T	C	C	G	C	T	G	C	C	G	G	-	-	-	-	-	-	-	-	G	T
O. cylonicus	G	G	C	G	C	T	C	C	A	G	C	G	A	G	T	T	G	C	T	G	-	-	-	-	-	-	-	-	G	T
O. obesus	C	G	C	A	T	A	G	T	A	T	C	C	A	A	C	A	G	C	T	G	-	-	-	-	-	-	-	-	G	T
UK02	C	G	C	A	T	A	G	T	A	T	C	C	A	A	C	A	G	C	T	G	-	-	-	-	-	-	-	-	G	T
O. redemanni	-	A	G	-	A	G	A	T	C	G	A	T	A	G	C	G	A	A	A	C	A	A	G	C	T	A	C	C	G	T
O. horni	G	C	C	G	G	A	A	-	T	A	T	A	C	G	C	T	C	A	T	T	T	C	C	C	T	T	T	C	G	A
UK01	A	A	T	C	G	G	A	T	T	T	A	A	A	G	A	T	T	G	A	T	G	C	T	C	T	G	G	C	G	T
T. biformis	T	C	C	G	G	A	A	T	T	G	G	A	A	C	C	G	G	A	G	T	T	C	C	C	T	T	T	C	G	C
O. cylonicus	T	T	A	A	G	A	A	T	A	T	T	A	-	A	C	T	C	A	A	T	T	C	C	C	T	T	T	C	G	T
O. obesus	T	T	A	A	G	A	A	T	A	T	T	A	-	A	C	T	C	A	A	T	T	C	C	C	T	T	T	C	G	T
UK02	T	T	A	A	G	A	A	T	A	T	T	A	-	A	C	T	C	A	A	T	T	C	C	C	T	T	T	C	G	T
O. redemanni	G	A	G	G	G	A	A	A	G	T	T	G	A	A	A	A	G	C	A	C	T	T	C	G	A	A	A	G	G	C
O. horni	G	G	G	G	G	G	G	G	G	G	C	A	A	A	C	A	C	C	G	-	-	-	-	-	-	A	A	C	T	-
UK01	G	T	A	G	G	C	A	G	-	-	G	C	G	T	G	G	C	G	G	T	C	T	C	T	G	A	T	T	A	C
T. biformis	C	C	T	C	G	A	C	G	G	G	C	G	T	T	G	G	T	A	G	C	C	T	A	A	G	A	C	C	C	C

Table 5. Contd.

O. cylonicus	T	G	A	-	-	-	-	-	-	-	T	T	A	T	G	A	C	G	A	A	A	T	A	A	A	A	T	G	T	C
O. obesus	T	G	A	-	-	-	-	-	-	-	T	T	A	T	G	A	C	A	A	A	A	T	A	A	A	A	T	G	T	C
UK02	T	G	A	C	C	A	T	-	-	-	T	T	A	T	G	A	C	A	A	A	A	T	A	A	A	A	T	G	T	C
O. redemanni	A	-	-	-	-	-	G	A	G	T	C	A	A	A	A	T	A	C	C	T	G	A	A	G	C	A	C	C	G	T
O. horni	-	-	-	-	-	T	A	A	A	C	A	A	A	A	G	G	T	G	T	T	T	G	T	T	-	-	-	C	A	T
UK01	A	A	C	A	G	T	T	T	A	T	A	A	G	C	C	T	A	G	G	T	G	G	T	A	-	-	-	A	C	A
T. biformis	G	C	C	T	C	A	T	C	G	G	C	A	T	C	G	G	A	T	T	T	C	T	C	C	T	A	G	G	G	C
O. cylonicus	A	A	A	A	A	T	A	T	A	A	A	A	A	A	G	C	A	G	T	T	A	A	G	C	C	A	C	A	A	C
O. obesus	A	A	A	A	A	T	A	T	A	A	A	A	A	A	G	C	A	G	T	T	A	A	G	C	C	A	C	A	A	C
UK02	A	A	A	A	A	T	A	T	A	A	A	A	A	A	G	C	A	G	T	T	A	A	G	C	C	A	C	A	A	C
O. redemanni	C	G	-	A	G	T	A	G	G	A	G	G	G	A	A	G	C	G	C	C	T	T	C	T	T	G	T	G	C	T
O. horni	C	T	A	G	A	C	A	G	C	A	T	T	G	A	A	C	C	G	A	G	A	C	C	C	C	A	C	T	G	G	T
UK01	C	C	G	G	G	T	G	G	A	A	C	G	G	C	C	G	C	T	A	G	T	G	C	A	G	A	T	C	T	T
T. biformis	T	T	A	G	G	A	T	C	G	A	C	T	G	A	C	T	C	G	T	G	T	G	C	A	A	C	G	G	C	T
O. cylonicus	T	T	A	G	G	A	G	C	G	A	C	T	A	A	C	C	C	A	T	G	T	C	C	A	A	T	T	G	C	T
O. obesus	T	T	A	G	G	A	G	C	G	A	C	T	A	A	C	C	C	A	T	G	T	C	C	A	A	T	T	G	C	T
UK02	T	T	A	G	G	A	G	C	G	A	C	T	A	A	C	C	C	A	T	G	T	C	C	A	A	T	T	G	C	T
O. redemanni	T	T	T	G	A	T	G	G	A	T	A	A	C	T	G	G	A	C	C	T	G	T	T	T	C	T	G	T	C	T
O. horni	G	G	C	C	A	T	G	G	A	A	G	T	C	G	G	A	A	C	C	C	T	T	T	C	C	G	C	T	A	A
UK01	G	-	-	-	-	-	-	G	T	G	G	T	A	G	T	A	G	C	G	A	A	T	A	T	T	C	A	A	A	T
T. biformis	G	-	-	-	-	-	-	T	T	C	A	C	A	C	G	A	A	A	C	C	C	T	-	-	-	-	-	-	-	-
O. cylonicus	G	-	-	-	-	-	-	G	T	G	G	T	A	G	T	A	G	C	A	A	T	T	A	T	T	C	A	C	A	T
O. obesus	G	-	-	-	-	-	-	T	T	C	A	C	A	T	G	G	A	A	A	C	T	T	T	C	T	C	C	-	-	-
UK02	G	-	-	-	-	-	-	T	T	C	A	C	A	T	G	G	A	A	A	C	T	T	T	C	T	C	C	-	-	-
O. redemanni	-	-	-	-	T	T	T	G	T	G	A	T	G	A	C	A	C	-	-	A	-	-	C	G	C	A	G	A	T	G
O. horni	G	G	A	G	T	G	T	G	T	A	A	C	A	A	C	T	C	A	C	C	T	G	C	C	G	A	A	T	T	G
UK01	G	A	G	A	T	C	T	T	T	G	A	A	G	A	C	T	G	-	-	-	-	-	A	A	G	T	G	G	G	G
T. biformis	-	-	-	-	-	-	-	-	T	C	T	C	C	G	C	A	T	-	-	-	-	-	C	A	G	C	C	C	T	C
O. cylonicus	G	G	A	A	A	C	T	T	T	C	T	C	C	A	C	A	T	-	-	-	-	-	C	A	G	C	C	T	T	C
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	A	T	-	-	-	-	-	C	A	G	C	C	T	T	C
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	A	T	-	-	-	-	-	C	A	G	C	C	T	T	C
O. redemanni	C	A	G	A	T	A	G	T	G	G	T	C	C	C	T	A	G	T	A	C	C	T	C	A	-	-	-	-	-	-
O. horni	G	A	A	C	T	A	G	C	C	C	T	G	A	A	A	A	C	T	G	C	A	C	T	A	A	A	A	G	-	-
UK01	A	A	G	G	G	T	T	C	C	G	T	G	T	G	A	A	C	A	G	C	G	G	T	T	G	G	A	C	A	C
T. biformis	A	A	A	A	T	G	G	C	C	C	T	C	G	C	T	T	G	A	G	T	A	T	T	T	G	-	-	-	-	-
O. cylonicus	A	A	A	G	T	T	C	T	C	A	T	T	T	G	A	A	T	A	A	T	T	G	C	T	A	C	T	-	-	-
O. obesus	A	A	A	G	T	T	C	T	C	A	T	T	T	G	A	A	T	A	A	T	T	G	C	T	A	C	T	A	-	-
UK02	A	A	A	A	A	A	G	G	T	G	T	T	G	G	C	T	C	A	T	T	T	G	A	A	T	A	A	T	T	G
O. redemanni	-	-	-	-	-	-	-	-	T	G	C	T	C	A	A	G	C	T	A	G	T	G	C	T	G	C	A	C	T	C
O. horni	-	-	-	-	-	-	C	C	G	T	T	C	G	A	A	C	C	G	G	G	G	T	C	C	A	C	C	C	C	C
UK01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	T	C	A	G	T	C	G	A	T	C	C	T	A
T. biformis	-	-	C	T	A	C	T	A	C	C	-	-	-	-	A	C	C	A	A	G	A	T	C	T	G	C	A	C	C	G
O. cylonicus	-	-	-	-	-	-	-	-	-	-	-	A	C	C	A	C	C	A	A	G	A	T	C	T	G	C	A	C	T	A
O. obesus	-	-	-	-	-	-	-	-	C	C	A	C	C	-	-	-	-	A	A	G	A	T	C	T	G	C	A	C	T	A
UK02	G	A	C	T	A	C	T	A	C	C	A	C	C	A	A	G	A	T	A	C	A	A	C	T	G	C	A	C	T	A
O. redemanni	A	C	G	A	T	A	A	C	T	G	A	T	G	C	C	G	A	G	C	A	G	G	T	T	T	T	G	G	T	T
O. horni	A	G	G	G	T	T	C	C	T	C	A	C	G	G	A	C	C	T	C	C	C	C	G	G	C	C	G	C	C	T

Table 5. Contd.

UK01	A	G	G	G	T	C	A	G	G	C	A	A	-	-	-	-	-	-	-	-	-	-	-	-	A	C	G	C	T	T
T. biformis	G	C	G	G	T	G	G	C	T	C	C	A	G	G	A	A	T	T	G	G	G	C	T	C	A	C	G	C	C	C
O. cylonicus	A	A	G	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. obesus	A	A	G	A	T	A	A	T	T	C	A	A	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
UK02	A	A	G	A	T	A	A	T	T	C	A	A	C	A	A	C	T	C	-	C	C	C	T	G	C	C	G	A	A	T
O. redemanni	T	G	G	A	C	A	G	C	C	G	T	T	T	G	C	A	C	A	A	G	-	A	G	A	A	T	G	C	A	G
O. horni	A	-	-	-	-	-	A	C	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK01	A	-	-	-	-	-	T	G	T	G	T	T	G	T	A	A	G	T	A	C	A	C	G	-	-	-	C	T	T	G
T. biformis	A	-	-	-	-	-	G	G	C	C	C	T	T	C	C	G	C	G	C	T	-	C	A	-	-	-	C	C	G	C
O. cylonicus	-	-	-	-	-	-	A	A	T	T	C	A	A	C	A	T	A	G	A	T	T	T	A	C	A	T	C	A	A	A
O. obesus	A	-	-	-	-	-	G	A	T	T	-	-	-	-	-	-	-	-	-	-	-	T	A	C	A	T	C	A	A	A
UK02	A	-	-	-	-	-	G	A	T	T	T	A	C	A	T	C	A	A	A	A	A	T	G	G	A	T	G	G	T	G
O. redemanni	T	C	G	T	T	C	G	T	-	A	A	G	C	C	C	T	T	G	G	A	G	A	A	A	T	C	T	G	A	T
O. horni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
UK01	T	T	C	G	T	G	T	C	A	A	T	A	C	C	C	G	A	A	A	G	G	G	A	A	G	-	-	-	-	C
T. biformis	C	G	C	G	C	C	C	T	C	C	T	A	C	T	C	G	C	C	G	G	G	G	C	T	T	C	A	G	C	C
O. cylonicus	T	G	C	T	T	C	T	T	A	A	T	T	A	T	C	T	T	C	A	T	G	T	C	T	T	C	C	T	A	C
O. obesus	T	G	C	T	T	C	T	T	A	A	T	T	A	T	C	T	T	C	A	T	G	T	C	T	T	C	C	T	A	C
UK02	C	T	T	C	A	G	T	T	A	G	T	T	A	T	C	T	T	C	A	T	G	T	C	T	T	C	C	T	A	C
O. redemanni	G	C	G	C	A	A	G	T	G	A	G	G	C	G	A	C	C	C	G	T	C	T	T	G	-	-	-	-	-	-
O. horni	G	G	G	G	C	C	A	T	A	A	T	T	G	T	G	-	-	C	C	C	C	A	G	C	G	G	A	G	G	A
UK01	C	G	G	T	T	A	A	T	A	T	T	C	C	G	G	-	-	C	A	C	C	A	G	A	A	T	G	T	G	G
T. biformis	C	T	A	T	T	-	-	-	-	-	T	T	T	G	C	A	T	T	G	C	C	T	T	C	C	G	G	C	G	G
O. cylonicus	T	C	A	T	T	A	A	T	A	T	A	T	A	T	T	A	C	A	T	T	A	T	A	T	T	A	A	T	G	G
O. obesus	T	C	A	T	T	A	A	T	A	T	A	T	A	T	T	A	-	A	T	T	A	T	A	T	T	A	A	T	G	G
UK02	T	C	A	T	T	A	A	T	A	T	A	T	A	T	T	A	A	T	A	T	A	T	A	T	T	A	A	T	G	G
O. redemanni	-	-	-	A	A	A	A	G	G	G	G	G	A	A	C	A	A	A	A	G	C	G	A	A	A	G	G	A	A	A
O. horni	G	T	A	T	T	A	G	T	G	A	C	T	A	A	C	T	T	G	G	G	G	G	C	C	C	T	G	G	A	A
UK01	A	T	T	C	A	C	A	A	C	G	G	C	A	A	C	-	-	-	-	-	G	T	T	A	A	A	C	G	-	-
T. biformis	C	C	G	G	G	T	A	T	A	G	G	C	A	C	G	G	A	G	C	T	T	T	A	G	C	G	C	C	A	T
O. cylonicus	T	T	T	A	G	C	A	T	C	G	G	T	A	A	C	T	C	G	C	T	G	G	A	G	C	G	C	C	A	T
O. obesus	T	T	T	A	G	T	A	T	C	G	G	T	A	A	C	T	C	G	C	T	G	G	A	G	C	G	C	C	A	T
UK02	T	T	T	A	G	T	A	T	C	G	G	T	A	A	C	T	C	G	C	T	G	G	A	G	C	G	C	C	A	T
O. redemanni	T	C	T	T	T	T	T	T	T	T	A	A	A	T	C	C	G	G	A	C	C	C	C	T	C	C	A	A	G	A
O. horni	C	C	A	T	T	T	T	-	-	-	C	A	G	G	G	G	T	A	A	T	T	T	A	T	T	C	G	G	G	C
UK01	-	-	A	A	C	C	T	-	-	-	G	G	A	G	A	C	G	C	C	G	G	C	A	T	T	T	G	C	C	C
T. biformis	C	C	A	T	T	T	T	-	-	-	C	A	G	G	G	C	T	A	G	T	T	G	C	T	T	C	G	G	C	A
O. cylonicus	C	C	A	T	T	T	T	-	-	-	C	A	G	G	G	C	T	G	G	T	T	C	A	T	T	C	G	G	C	A
O. obesus	C	C	A	G	T	T	T	-	-	-	C	A	G	G	G	C	T	G	G	T	T	C	A	T	T	C	G	G	C	A
UK02	C	C	A	T	T	T	T	-	-	-	C	A	G	G	G	C	T	G	G	T	T	C	A	T	T	C	G	G	C	A
O. redemanni	G	G	G	A	A	C	C	G	A	A	C	C	G	T	T	C	T	A	T	G	T	G	C	C	C	T	C	G	C	T
O. horni	G	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	G	G	C	C	T	T	T
UK01	C	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	G	G	A	G	T
T. biformis	G	G	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	G	T	T	-	-	-
O. cylonicus	G	G	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	G	T	T	-	-	-
O. obesus	G	G	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	A	G	T	T	G	T
UK02	G	G	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	G	T	C	T	A	T
O. redemanni	T	T	T	C	G	A	A	G	C	T	C	C	T	C	G	G	A	G	C	C	C	G	T	A	A	C	T	G	T	T
O. horni	G	G	T	A	C	A	C	A	C	T	C	C	T	T	A	A	T	G	G	T	A	G	T	A	A	T	T	C	C	C
UK01	T	C	T	C	T	C	C	T	C	T	C	C	T	T	G	A	C	A	G	C	-	-	-	-	C	C	T	G	C	G
T. biformis	G	T	T	A	C	A	C	A	C	T	C	C	T	T	A	G	C	G	G	-	-	-	-	-	A	T	T	C	C	G
O. cylonicus	G	T	T	A	C	A	C	A	C	T	C	C	T	T	A	G	C	G	G	-	-	-	-	-	A	T	T	T	C	T

Table 5. Contd.

O. obesus	T	A	C	A	C	T	G	A	C	T	C	C	T	T	A	G	C	G	G	-	-	-	-	-	A	T	T	T	C	T
UK02	G	T	T	A	C	A	C	A	C	T	C	C	T	T	A	G	C	G	G	-	-	-	-	-	A	T	T	T	C	T
O. redemanni	A	G	G	G	A	C	T	C	G	G	A	G	A	C	G	-	-	C	C	T	T	C	G	G	T	A	G	A	A	C
O. horni	A	C	T	T	C	C	A	T	G	G	G	C	C	G	C	C	T	C	C	G	A	G	G	A	C	T	G	A	A	G
UK01	A	G	A	G	C	G	G	A	G	G	C	C	C	A	C	G	G	A	A	T	C	G	C	G	T	C	A	A	G	C
T. biformis	A	C	T	T	C	C	A	T	G	G	C	C	A	C	C	G	T	C	C	T	G	C	T	G	T	C	T	T	A	A
O. cylonicus	A	C	T	T	C	C	A	T	G	A	C	C	A	C	C	G	T	C	C	T	G	C	T	G	T	T	A	A	A	A
O. obesus	A	C	T	T	C	C	A	T	G	A	C	C	A	C	C	G	T	C	C	T	G	C	T	G	T	T	A	A	A	A
UK02	A	C	T	T	C	C	A	T	G	A	C	C	A	C	C	G	T	C	C	T	G	C	T	G	T	T	A	A	A	A
O. redemanni	C	G	G	G	C	T	G	-	-	-	-	-	A	G	T	C	T	A	C	T	G	T	G	A	C	C	C	C	-	-
O. horni	T	G	G	G	G	A	A	A	G	G	A	C	T	T	T	C	C	G	T	G	T	G	A	A	C	A	A	C	A	G
UK01	G	G	A	G	A	A	G	T	G	G	C	C	T	T	T	C	G	G	C	T	G	G	T	A	G	A	G	C	A	T
T. biformis	G	C	A	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	A	C	G	C	C	T	-
O. cylonicus	T	G	A	A	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	T	T	C	A	A	C	A	C	C	T	-
O. obesus	T	G	A	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	A	C	A	C	C	T	-
UK02	T	G	A	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	A	C	A	C	C	T	-
O. redemanni	-	-	-	-	A	T	A	C	A	G	A	T	T	T	C	T	G	A	G	T	C	C	C	C	C	G	-	-	-	-
O. horni	T	T	G	G	A	C	A	C	G	G	G	T	T	A	C	T	C	G	A	T	C	C	T	A	A	G	A	C	A	T
UK01	C	G	C	A	A	C	T	A	T	G	C	G	G	T	G	T	C	C	G	G	A	G	C	G	C	A	G	T	T	G
T. biformis	-	-	-	-	T	T	C	A	T	G	G	T	C	T	C	C	C	A	T	G	A	G	C	T	C	C	G	G	T	T
O. cylonicus	-	-	-	-	T	T	T	C	T	G	G	T	A	T	C	T	G	A	T	G	A	G	C	G	A	G	C	A	T	T
O. obesus	-	-	-	-	T	T	T	C	T	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	-	-	-	-	T	T	T	C	T	G	G	T	A	T	C	T	G	A	T	G	A	G	C	G	A	G	C	A	T	T
O. redemanni	-	-	-	-	-	-	-	-	-	-	C	G	A	G	T	C	C	T	C	T	A	G	A	A	C	A	A	G	T	A
O. horni	A	G	G	G	A	A	A	C	T	C	C	G	T	T	T	T	A	A	A	G	T	G	C	G	C	T	C	T	T	G
UK01	A	C	G	G	T	C	C	T	T	G	A	A	A	A	T	C	C	A	G	G	G	G	A	G	A	G	A	T	C	C
T. biformis	T	A	G	G	C	G	C	C	T	T	A	A	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. cylonicus	T	A	G	G	C	A	C	C	T	T	A	G	C	T	A	A	A	C	G	T	T	A	G	G	A	G	C	A	T	C
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	T	A	G	G	C	A	C	C	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	G	C	G	T	T	T	G	G	A	C	G	C	A	A	A	A	A	C	T	T	C	G	C	A	G	A	A	A	G	C
O. horni	C	G	C	C	T	C	G	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK01	C	G	G	C	T	T	A	C	C	G	G	G	G	G	A	T	T	C	T	C	A	C	G	T	-	-	-	-	C	T
T. biformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. cylonicus	C	C	T	C	A	T	C	G	C	C	A	G	T	T	C	T	G	C	T	T	A	C	C	A	A	A	A	A	A	T
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. redemanni	G	C	G	T	T	T	C	A	A	A	T	A	C	C	C	C	C	T	C	T	C	G	A	C	T	G	A	A	A	
O. horni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
UK01	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
T. biformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
O. cylonicus	G	G	G	G	T	T	A	A	A	T	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
O. obesus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
UK02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

length formed a part of pseudo knot along with position one. There are three hairpin loops and a majority of the bases form single-stranded primary structure; VS3 starts from 206th base and ends at 324th base with five hairpin loops, two unpaired loops, one pseudo knot and a wide range of primary structure spanning between the loops; VS4 stretches from 369th nucleotide base to 450th base of the sequence, which starts as a part of a hairpin loop, two unpaired loops, four pseudo double-stranded structures, three stretches of primary structures in between them and ends by forming a part of a hairpin loop. Similarly, VS5 initiates with 526th base and ends with 616th base, which starts with a pseudo double-stranded structure with three dimensional orientation at

Table 6a. Nucleotide pair frequencies of all the seven termite samples.

Domain info	Data	1st position data	2nd position data	3rd position data
ii	207	73	74	60
si	132	41	42	48
sv	263	87	84	92
R	0.5	0.5	0.5	0.5
Total	602.2	200.8	200.7	200.7
TT	61	21	24	17
TC	32	11	9	12
TA	34	12	11	10
TG	31	10	9	12
CT	34	11	10	13
CC	48	17	15	16
CA	38	12	13	12
CG	31	12	9	11
AT	33	11	11	11
AC	34	11	12	12
AA	55	19	21	15
AG	31	10	10	11
GT	31	9	10	13
GC	30	10	9	11
GA	35	9	13	12
GG	43	16	15	13

All frequencies are averages (rounded) over all taxa. ii = Identical Pairs; si = Transitionsal Pairs; sv = Transversional Pairs; R = si/sv.

Table 6b. Nucleotide frequency of all the seven partially amplified 28s rDNA sequences.

Sequence	T	C	A	G	Total
O. ceylonicus (Wasmann)	30.7	20.6	29.4	19.2	849
O. redemanni (Wasmann)	23.5	23.7	26	26.8	885
O. horni (Wasmann)	23.1	26.6	23.5	26.8	753
O. obesus (Rambur)	32.5	20.8	31.4	15.2	643
T. biformis (Wasmann)	22.9	34.5	16.6	26	728
UK1	23.1	22.5	24.3	30.1	861
UK2	30.5	22.2	30.9	16.4	531
Average	26.3	24.4	25.8	23.5	750

531st base with 539th base; it possesses three hairpin loops and two unpaired loops along with a pseudo double-stranded structure; and lastly the VS6 position extends from 684th base to 753rd base, which starts with a pseudo double-stranded structure for only a 2 base pair length, the sequence possesses three hairpin loops, and one more pseudo double-stranded structure interrupted by single stranded nucleotide sequences.

The secondary structure of *O. horni* (Wasmann) partially amplified 28s rDNA (Figure 6C) study shows variable structure at marked variable sites where the VS1 polymorphic site ranges from the 155th nucleotide base to 216th base with a single base pair structure with seven unpaired loops, two pseudo knots at 176th base and 193rd base; at VS2 stretching from 221st base to 273rd base of the structure with an unpaired base in a loop followed by a hairpin loop and a pseudo knot at the 220 to 240 range, along with single stranded stretch with two hairpin loops.

At VS3 spanning from 274th base to 345th base with two base pair span of pseudo double stranded structure followed by a stretch of unpaired length of DNA with two

Table 6c. Variable regions of the 28s rDNA MSA.

Number of variable sites	Range of variable region	Number of bases in the sites
1	71-97	27
2	99-126	28
3	139-141	3
4	144-153	10
5	155-216*	61
6	218-219	2
7	221-273*	53
8	289	1
9	291-292	2
10	308	1
11	318	1
12	320-330	11
13	332-450*	111
14	454-492	39
15	499-502	4
16	504-585*	82
17	619	1
18	621-662	42
19	664-673	10
20	675-700	26
21	702-707	6
22	709-802*	94
23	804-811	8
24	815	1
25	817-819	3
26	821	1
27	823	1
28	825-852	28
29	865	1
30	867-936*	70
31	938-948	11
32	979	1
33	981-982	2
34	984-998	15
35	1000-1033	34
36	1035-1047	13
37	1049-1068	20
38	1070-1071	2
39	1123	1

hairpin loop stacked one above the other, the single stranded structure extended until 333 bases, and this is followed by a zigzag of tertiary structure, which extended beyond the 354th base. Similarly, the VS4, which stretches from 399th base to 474th base, is initiated with a two-base pair pseudo double helical structure followed by an unpaired loop until the 318th base, this is followed by two hairpin loops with unpaired loop within and single stranded span of DNA until the 471st base. The VS5 extends from the 517th base to 595th base in *O. horni* (Wasmann), and has three pseudo knots, five pseudo double-stranded structures, three unpaired loops and two hairpin loops. At the end, the VS6 site starting from the 628th position to 690th base has six pseudo double-

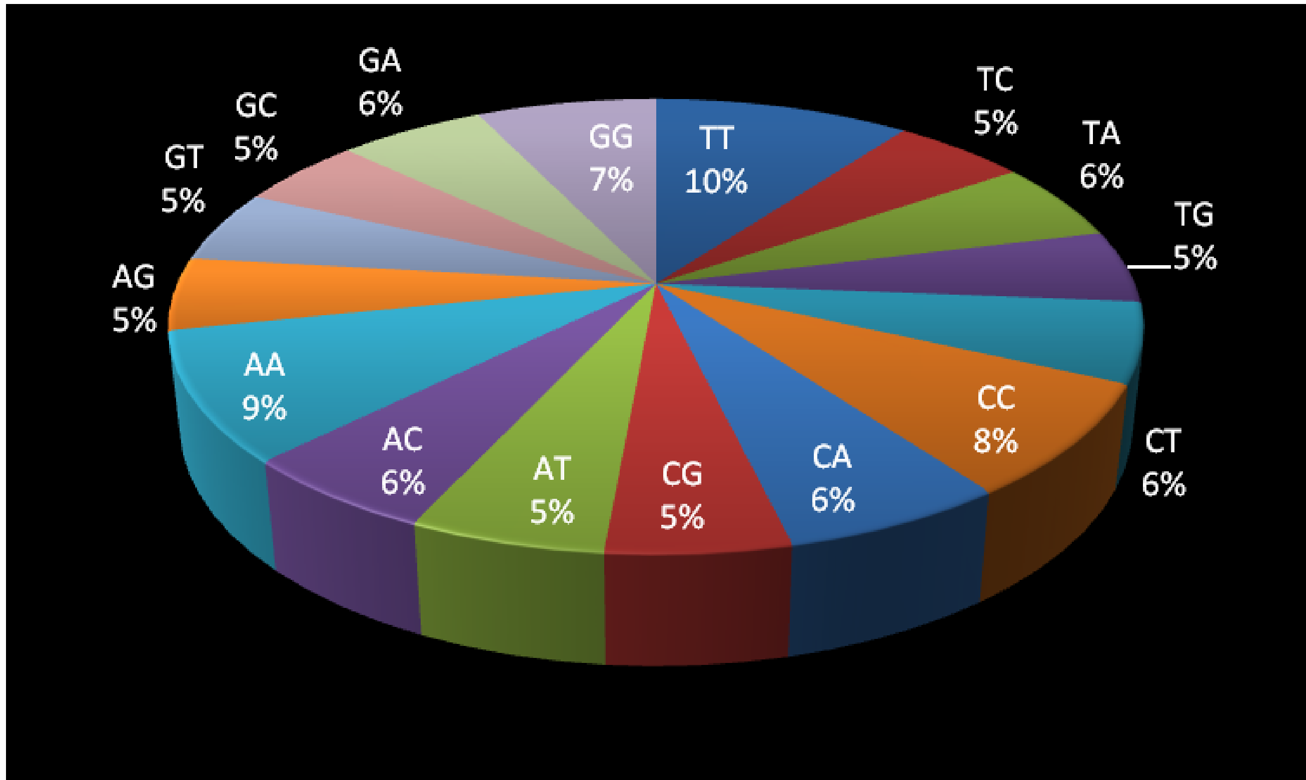

Figure 4. Pi-chart of sum of Nucleotide pair frequencies for all three positions.

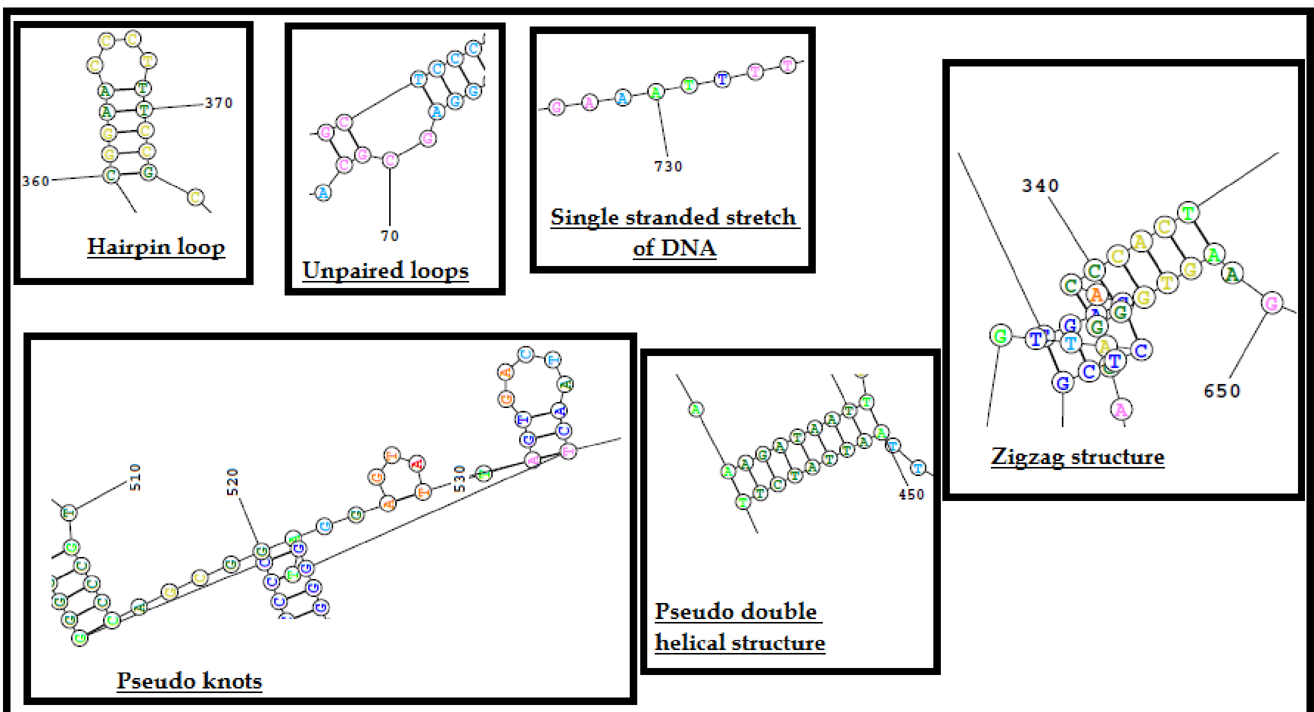

Figure 5. Types of rDNA secondary structure.

Table 7. Variable sites of MSA and their observed secondary structures.

Species	Observed secondary structures	VS 1	VS 2	VS 3	VS 4	VS 5	VS 6
1. *O. ceylonicus* (Wasmann)	Variable site range in bases	71 to 132	137 to 189	248 to 363	406 to 483	562 to 646	679 to 747
	1. SS-loop	1	0	0	1	1	0
	2. DS	Present	Present	Absent	Absent	Present	Present
	3. H-loop	1	0	2	2	1	0
	4. 3D	Present	Absent	Present	Present	Present	Absent
	5. Pknot	Present	Absent	Present	Absent	Absent	Absent
	6. zigzag	Absent	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Present	Present	Present	Present	Present	Present
2. *O. redemanni* (Wasmann)	Variable site range in bases	74 to 135	140 to 192	206 to 324	369 to 450	526 to 619	684 to 753
	1. SS-loop	0	0	0	2	2	0
	2. DS	Absent	Absent	Absent	Present	Present	Present
	3. H-loop	2	3	5	2	3	3
	4. 3D	Present	Present	Present	Absent	Present	Absent
	5. Pknot	Present	Present	Present	Absent	Absent	Absent
	6. zigzag	Absent	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Present	Present	Present	Present	Absent	Present
3. *O. horni* (Wasmann)	Variable site range in bases	115 to 216	221 to 273	274 to 354	399 to 474	517 to 595	628 to 690
	1. SS-loop	7	1	1	0	3	4
	2. DS	Absent	Absent	Present	Present	Present	Present
	3. H-loop	0	3	2	1	2	0
	4. 3D	Present	Present	Present	Present	Present	Present
	5. Pknot	Present	Present	Absent	Absent	Present	Absent
	6. zigzag	Absent	Absent	Present	Absent	Absent	Present
	7. SS-length	Present	Present	Present	Present	Absent	Present
4. *O. obesus* (Rambur)	Variable site range in bases	37 to 96	101 to 153	164 to 267	310 to 390	433 to 511	564 to 633
	1. SS-loop	0	1	1	2	1	1
	2. DS	Absent	Absent	Present	Present	Present	Present
	3. H-loop	2	1	4	3	2	2
	4. 3D	Absent	Absent	Absent	Absent	Absent	Present
	5. Pknot	Absent	Absent	Absent	Absent	Absent	Present
	6. zigzag	Absent	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Present	Present	Present	Present	Present	Absent

Table 7. Contd.

5. *T. biformis* (Wasmann)	Variable site range in bases	55 to 114	119 to 171	179 to 297	348 to 429	520 to 586	619 to 678
	1. SS-loop	3	3	6	2	3	4
	2. DS	Present	Present	Present	Present	Present	Present
	3. H-loop	6	6	6	2	4	1
	4. 3D	Present	Present	Present	Present	Present	Present
	5. Pknot	Present	Present	Present	Present	Present	Present
	6. zigzag	Absent	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Absent	Absent	Present	Present	Absent	Absent
6. UK1	Variable site range in bases	64 to 123	128 to 180	239 to 357	403 to 483	526 to 607	642 to 711
	1. SS-loop	2	3	11	6	2	5
	2. DS	Present	Present	Present	Present	Present	Present
	3. H-loop	2	0	4	3	5	4
	4. 3D	Absent	Present	Present	Present	Present	Present
	5. Pknot	Absent	Present	Present	Present	Present	Present
	6. zigzag	Absent	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Absent	Present	Present	Present	Present	Present
7. UK2	Variable site range in bases	Nil	1 to 30	31 to 84	127 to 189	307 to 391	426 to 486
	1. SS-loop	Nil	0	1	3	0	7
	2. DS	Nil	Present	Absent	Present	Present	Present
	3. H-loop	Nil	0	2	0	2	2
	4. 3D	Nil	Absent	Absent	Absent	Absent	Absent
	5. Pknot	Nil	Absent	Absent	Absent	Absent	Absent
	6. zigzag	Nil	Absent	Absent	Absent	Absent	Absent
	7. SS-length	Nil	Present	Present	Absent	Present	Present

VS: Variable site; UK: unknown; SS: single stranded; DS: double stranded; H: hairpin; 3D: three dimension; Pknot: pseudo knot; SS: single stranded.

stranded structures, a zigzag structure along with variable position three, four unpaired loops and a very short stretch of single stranded DNA.

O. obesus (Rambur) genomic 28s rDNA structure (Figure 6D) reveals that the VS1, whose length is from 37th base to 96th base, possesses two hairpin loops and stretches of unpaired nucleotides; VS2 from 101st base to 153rd base possesses one hairpin loop and one unpaired loop along with single-stranded stretches; VS3 starts with 164th base and ends with 267th base possesses a pseudo double-stranded structure, which starts at the 175th base through 179th base, and is complimented by base sequences from 211 to 214th, the sequence in between is

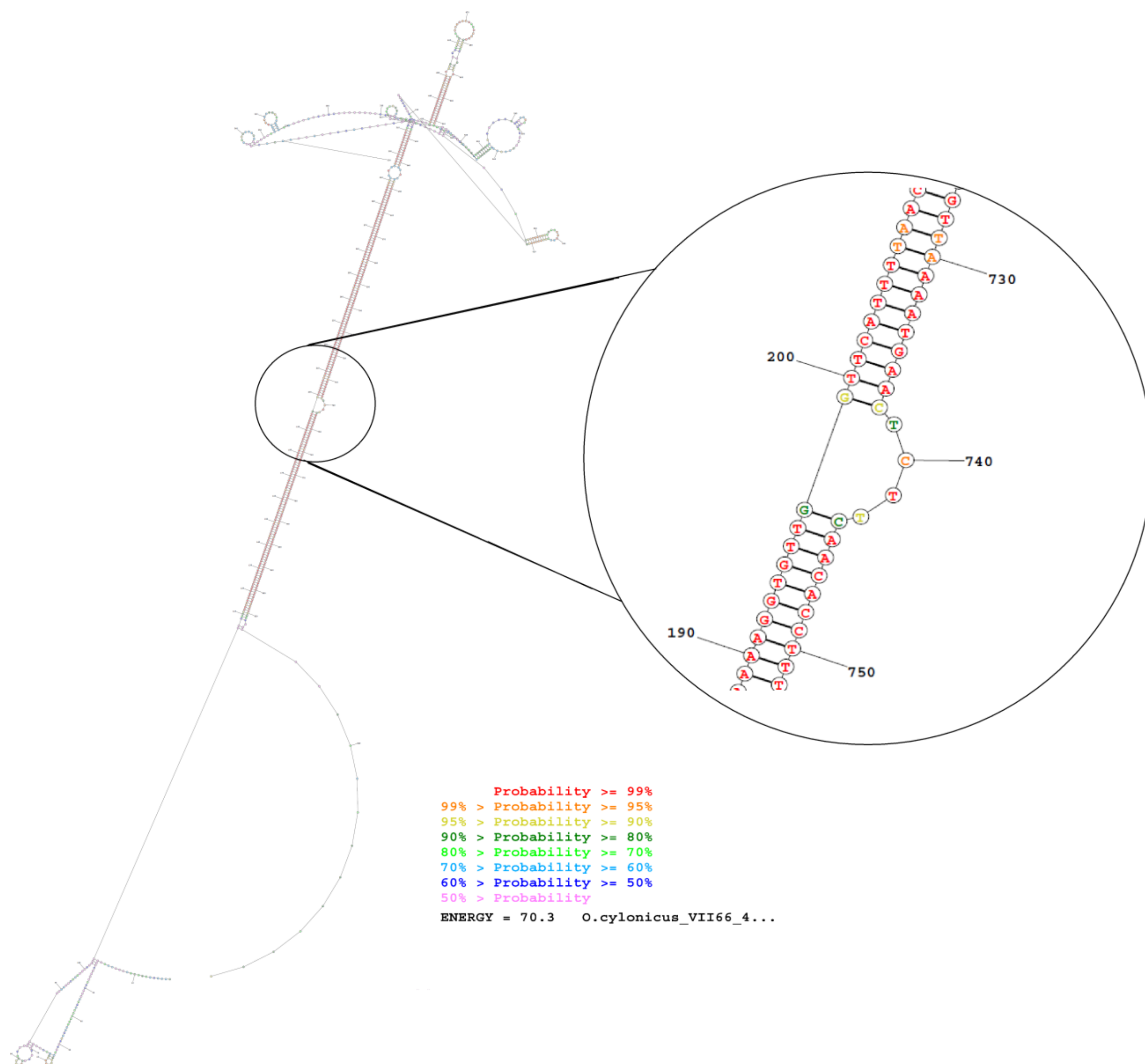

Figure 6A. 28s rDNA structure of *O. ceylonicus* (Wasmann) species.

folded into two hairpin loops and an unpaired loop. This structure is followed by single-stranded stretches and two hairpin loops. Position VS4 starts from the 310th base to 390th base, and there is a hairpin loop followed by a single-stranded structure; in the stretch between the 326th base to 388th base, one can see two hairpin loops, two unpaired loops and one pseudo double-stranded structure. Position VS5 starts from the 433rd base to 511th base, where the first base is a part of a hairpin loop. This is brought about by the unpaired loop and a pseudo double-stranded structure starting from the 450th base to 459th base, which is followed by a single-stranded structure and a hairpin loop. The VS6 site starting from 564th base to 633rd base possesses a complex pseudo knot, along with two hairpin loop double-stranded structures and an unpaired loop; this is brought about by one more pseudo double-stranded structure.

The secondary structure of *T. biformis* (Wasmann) partially amplified 28s rDNA (Figure 6E) is similar to that of the other species. The VS1 spans from the 55th base to 114th base, which is part of a hairpin loop in a complex of five hairpin loops, three unpaired loops entangled with a few double-stranded structures oriented three dimensionally. The VS2, with a length stretching from the 119th base to 171th base, is also a part of a variable site one complex structure. VS3 spans from the 179th base to 297th nucleotide base, which shows a very complex spatial orientation with six unpaired loops, six hairpin

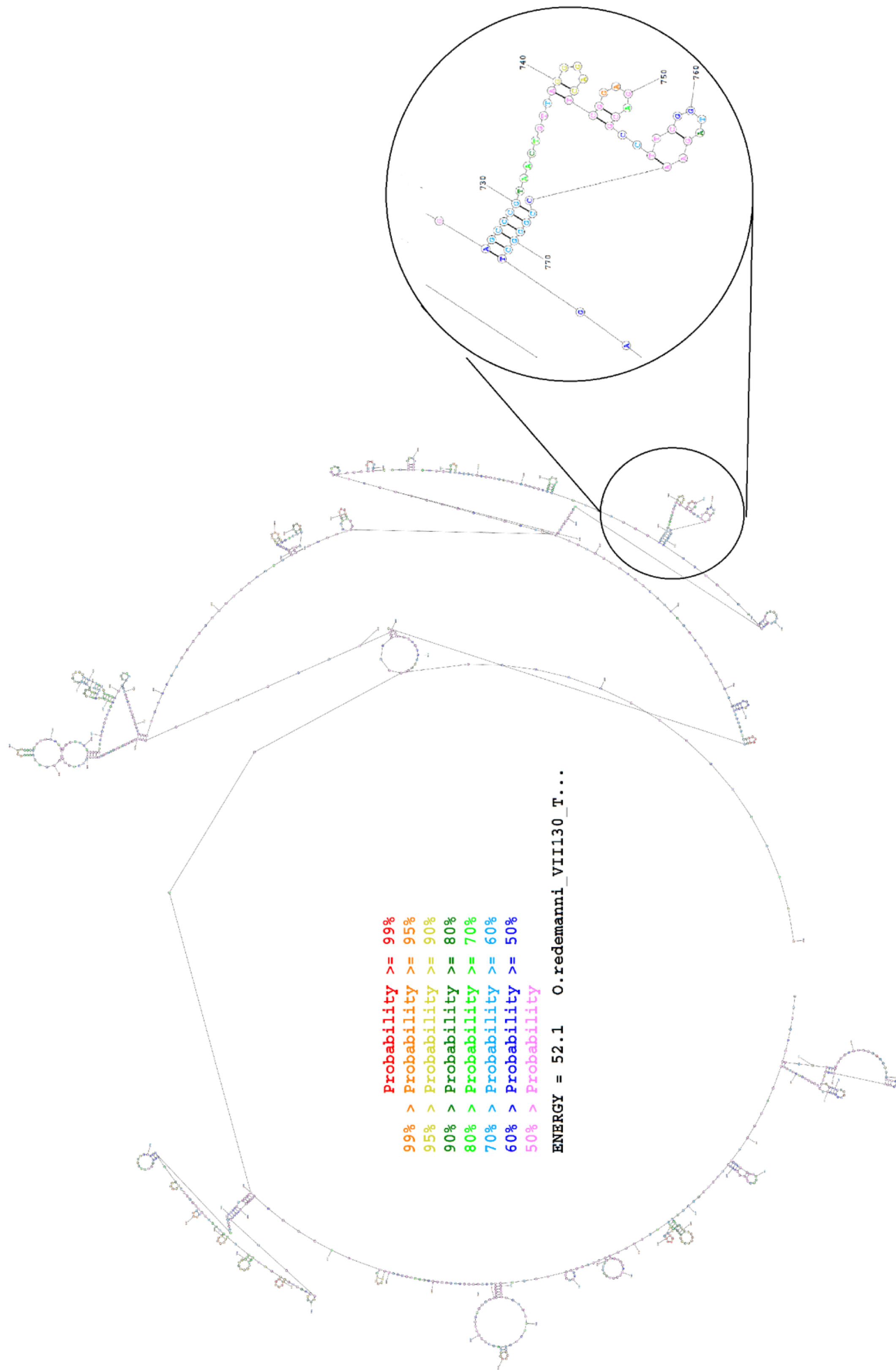

Figure 6B. 28s rDNA structure of *O. redemanni* (Wasmann) species.

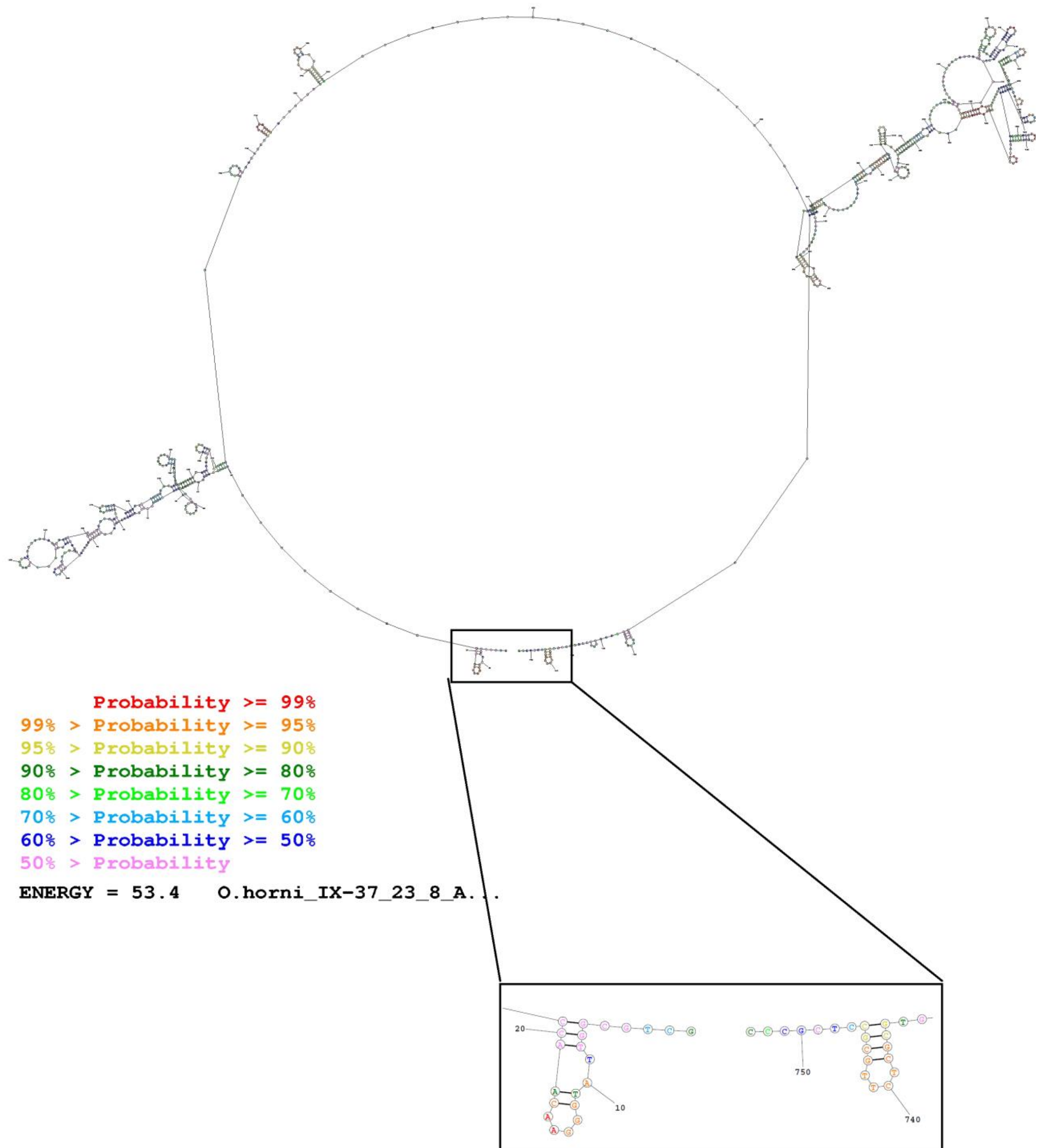

Figure 6C. 28s rDNA structure of *O. horni* (Wasmann) species.

loops, five pseudo double-stranded positions, and a few single stranded stretches in between the structures. Similarly the VS4, spanning from 348th base to 429th base, is part of a complex structure with simpler forms, it possesses two unpaired loops, two hairpin loops, four pseudo double-stranded structures and scattered single-stranded structures. VS5 stretching between the 520th base to 586th base possesses four hairpin loops, three

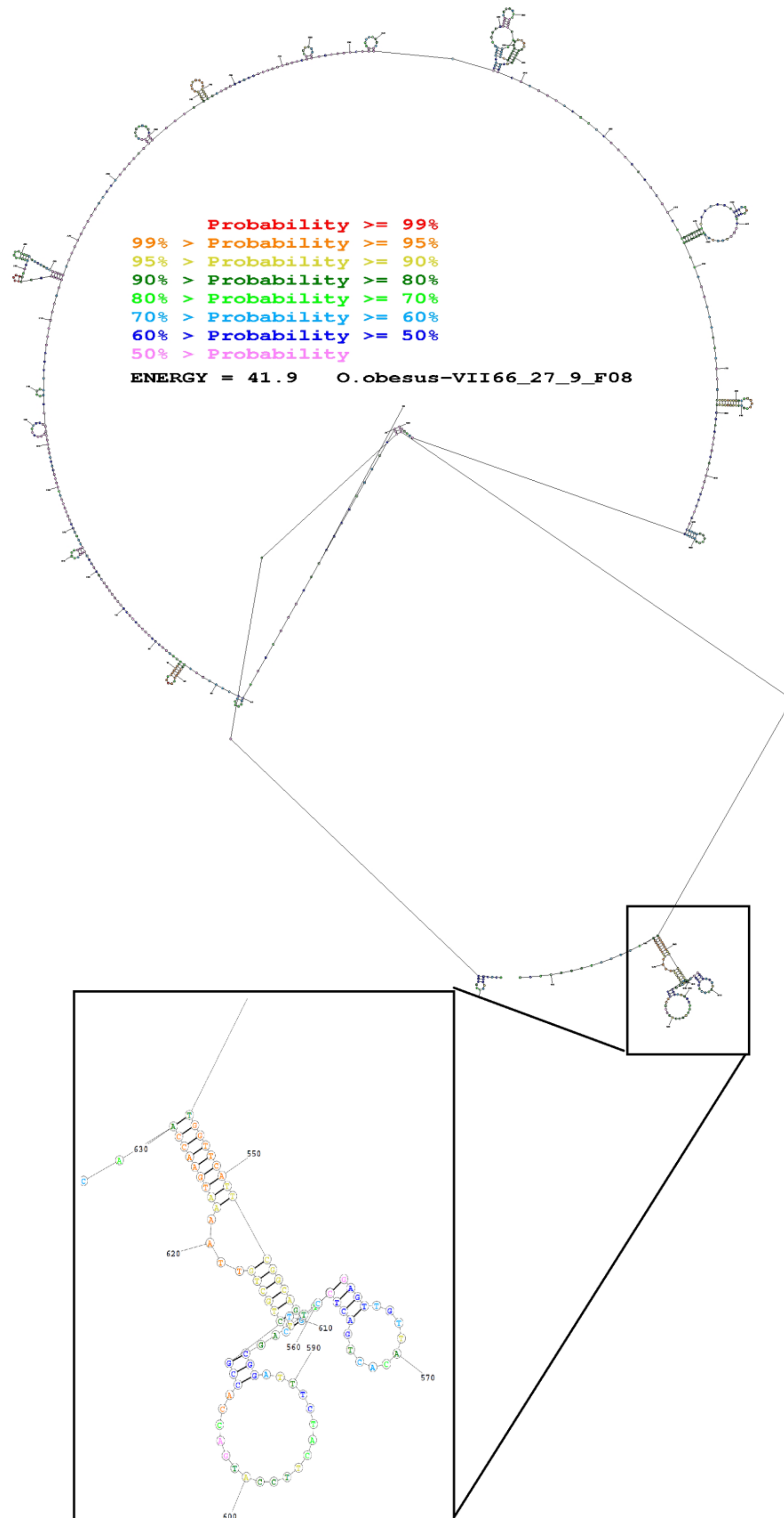

Figure 6D. 28s rDNA structure of *O. obesus* (Rambur) species.

Figure 6E. 28s rDNA structure of *T. biformis* (Wasmann) species.

unpaired loops and a few pseudo double-stranded structures along with complex pseudo knots connected with VS4. VS6 spreading from the 619th base to 678th base also forms a complex with VS4 and possesses one hairpin loop, four unpaired loops and interspersed with pseudo double-stranded structures.

Figure 6F, depicting the secondary structure of UNKNOWN01 termite species' partially amplified 28s rDNA structure, was also compared to the rest of the species in this study. The VS1 region spanning from the

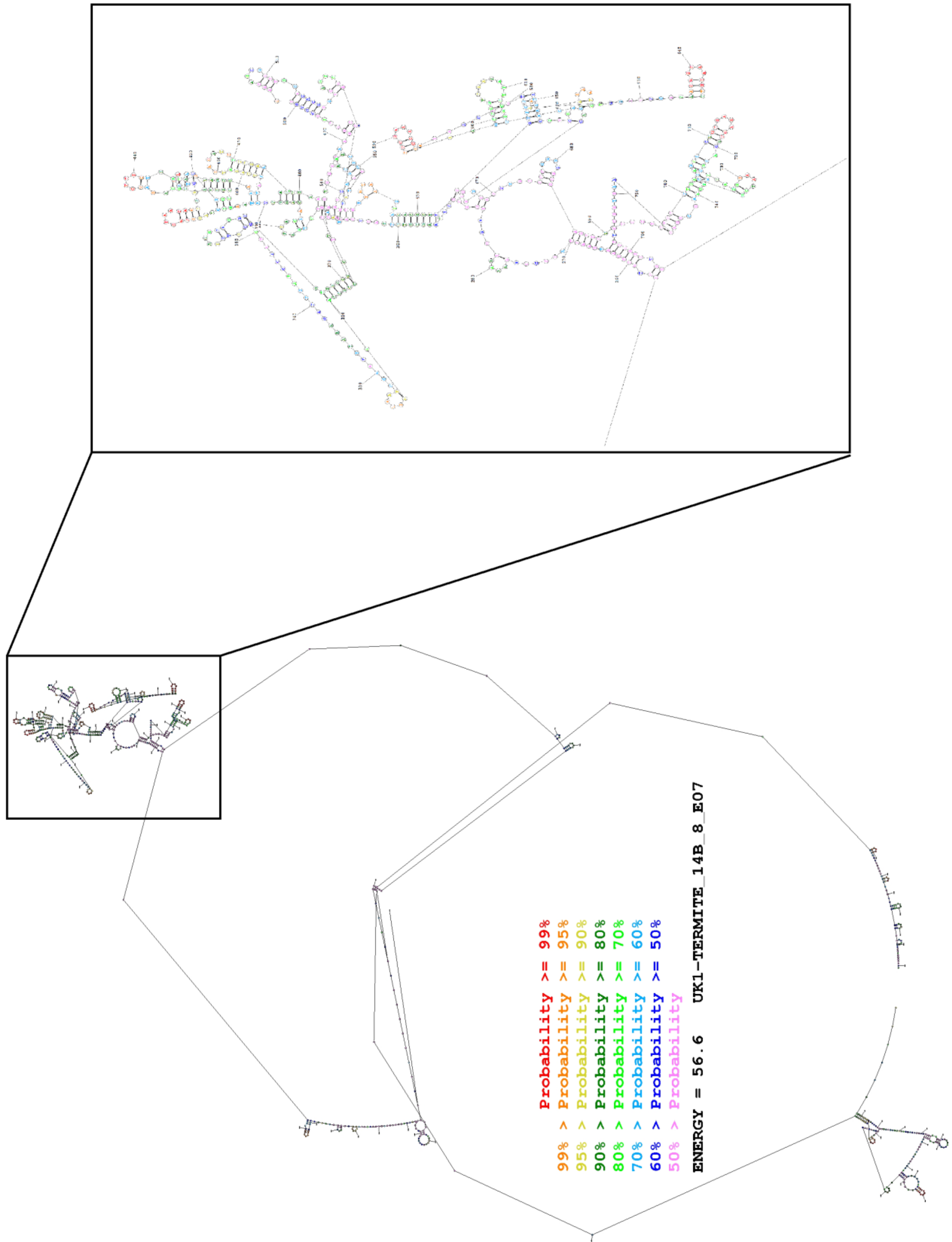

Figure 6F. 28s rDNA structure of UNKNOWN01 species.

64th base to 123rd base is part of an unpaired loop, followed by two pseudo double-stranded structures, two hairpin loops and one unpaired loop. VS2 ranges from the 128th base to 180th base, where the 129th base binds with the 21st base. This is followed by a hair pin loop, a pseudo knot, pseudo double-stranded structure, single-stranded stretches and a complex three dimensional orientation of a pseudo double-stranded structure, two unpaired loops and a pseudo double-stranded structure. VS3 ranges from the 239th base to 357th base, which starts with a pseudo double-stranded structure, an unpaired loop and a hairpin loop; this is followed by wide stretches of single-stranded DNA, ten unpaired loops (few short and few long), three hairpin loops and three dimensionally oriented structures.

The VS4 of UNKNOWN1 28s rDNA extending from base 403 to base 483 possesses an extremely complex three-dimensional structure with three hairpin loops, six unpaired loops, five pseudo double-stranded structures and a few pseudo knots. VS5 extends from the 526th base to 607th base, which starts from a hairpin loop followed by five pseudo knots, four hairpin loops, two unpaired loops, three pseudo double-stranded structures, and wide stretches of single stranded lengths oriented three dimensionally. Similarly, VS6, which begins from the 642nd base to 711th base, starts from a hairpin loop, followed by three more hairpin loops, five unpaired loops and some three-dimensionally oriented single-stranded structures with pseudo double-stranded structures.

The partially amplified 28s rDNA secondary structure of UNKNOWN02 (Figure 6G) was comparatively simpler as there was no three dimensional folding. The VS1 divergent site was not observed in this species. However, the VS2 divergent site started from base one to nucleotide base 30, which has a linear primary structure until the 30th base. The 30th base complementarily binds with the 149th base. The divergent site VS3 extends from base 31 to base 84; and it is clear that there are two hairpin loops, one unpaired loop and a long stretch of DNA in its primary structure. The divergent site VS4, extending from base 127 to base 189, possesses three unpaired loops, complementary pairing with the 149th base to 30th base, and two pseudo double-stranded structures. The divergent site VS5, extending from base 307 to base 391, possesses one long and one short hairpin loop, along with a single-stranded and a double-stranded structure. The VS6 site, extending from the 426th base to 486th base, possesses seven unpaired loops, two hairpin loops, some spatially oriented, single-stranded structures, and pseudo double-stranded structures. It is to be noted that the description of the two dimensional structure of rDNA does not coincide with tabulated results, because the number of pseudo double helical structures and hairpin loops can mislead the count. Similarly, the region of single-stranded loops to that of primary structure can be misinterpreted. Thus, in Table 7, certain characters are represented as only present or absent, and not by numbers as described in the text.

Phylogenetic analysis based on 28s rDNA sequence

The 28s rDNA sequences of all the collected termite species were submitted to MEGA software analyses for constructing a Neighbor-Joining tree according to Saitou and Nei (1987). The optimal tree with a sum of branch length = 3.74020557 is shown (Figure 7). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 (Kumar et al., 2016). According to the cluster results (Figure 7) there are two main clusters: (1) *O. redemanni* (Wasmann), *O. horni* (Wasmann) and UNKNOWN01 forming one cluster; and (2) the rest, that is, *T. biformis* (Wasmann), UNKNOWN02, *O. obesus* (Rambur) and *O. ceylonicus* (Wasmann) in another group, accounting for 90% of evolutionary divergence. There is a maximum of 10% divergence between UNKNOWN02 and cluster one, which includes *O. obesus* (Rambur) and *O. ceylonicus* (Wasmann). The divergence between *O. obesus* (Rambur) and *O. ceylonicus* (Wasmann) is about 50%. The divergence between *O. redemanni* (Wasmann) and cluster two, which includes UNKNOWN01 and *O. horni* (Wasmann) is about 80%. Similarly, the divergence between the *O. horni* (Wasmann) and UNKNOWN01 is 60%. Estimation of average evolutionary divergence of overall sequence pairs yielded a value of 1.293, and the number of base substitutions per site from estimation of coefficient of evolutionary differentiation is 1.73291×10^8.

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, was judged from the extent of differences in base composition biases between sequences according to the Disparity Index test (Kumar and Gadagkar, 2001). A Monte Carlo test (500 replicates) was used to estimate the P-values, which are shown below the diagonal (Table 8). P-values which are smaller than 0.05 are considered significant (marked with yellow highlights). The estimates of the disparity index per site are shown for each sequence pair above the diagonal.

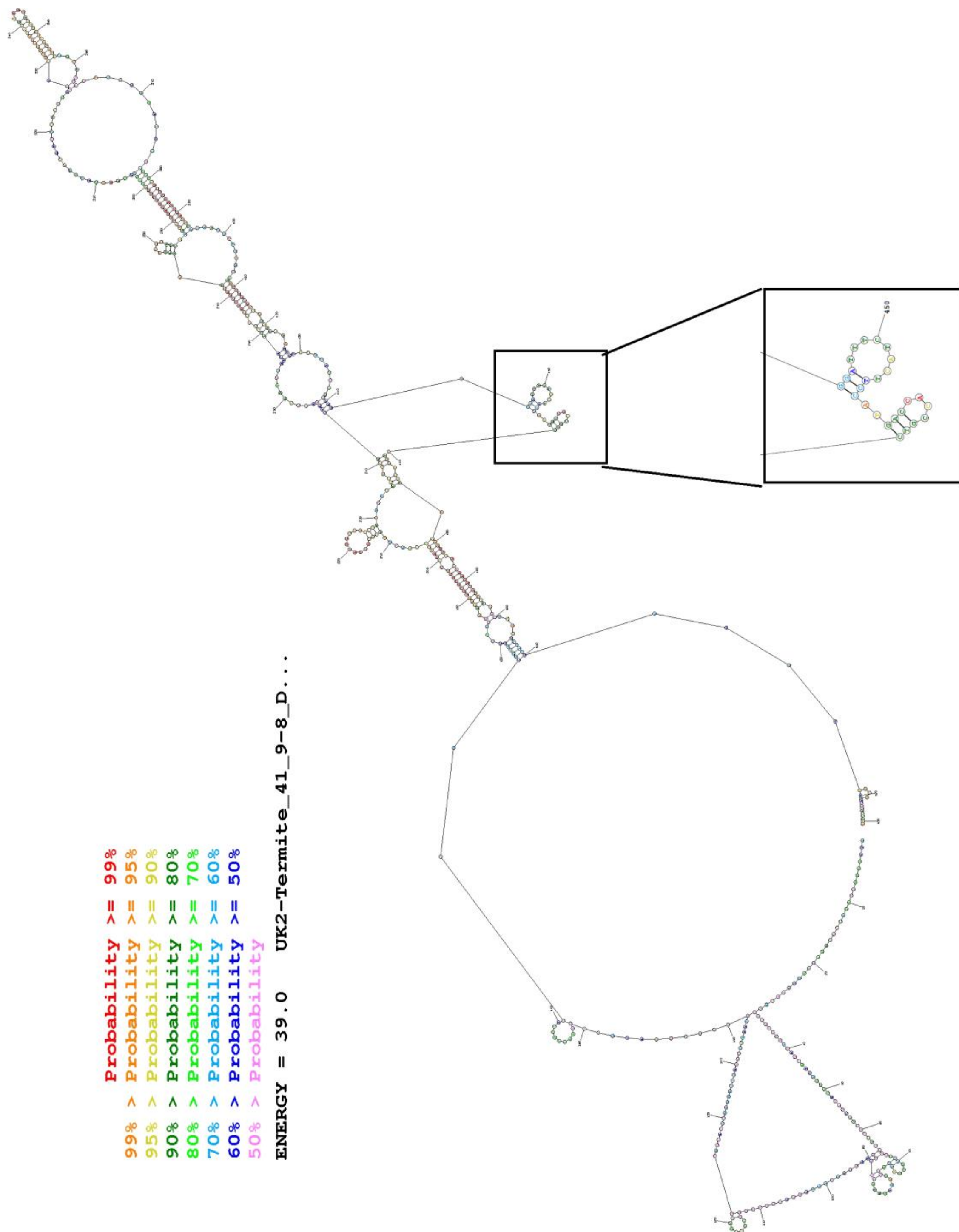

Figure 6G. 28s rDNA structure of UNKNOWN02 species.

Cumulative phylogenetic analysis

In the present study, three markers *viz.* morphological, ISSR banding pattern and 28s rDNA sequences are used for analyzing the genetic divergence between the identified and unidentified termite samples. As there were

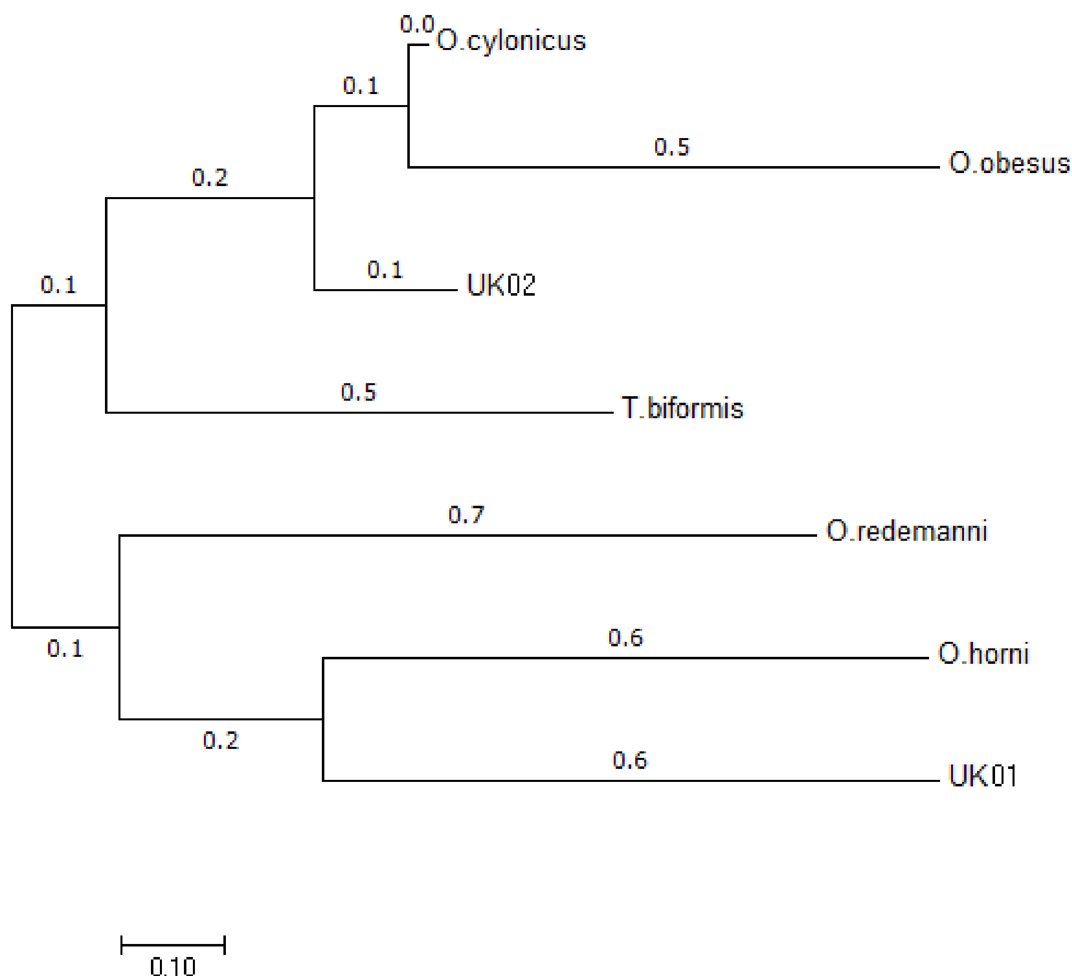

Figure 7. Evolutionary relationships of taxa.

no soldiers among the unidentified termite samples, they were not identified morphologically and there is no morphological data for the same. Furthermore, the investigation of these samples was carried out in an integrated approach. All of the three marker data were combined by converting the data into zeros and ones. The morphological data was converted into binary codes by using Tukey Kramer test results, ISSR banding pattern was scored in binary fashion and the sequences of all the species (both identified and unidentified) were coded into binary digits by using an algorithmic logic. Here A (Adenine) bases were coded as 10, G residues (Guanine) as 11, C bases (Cytosine) as 00 and lastly T (Thymine) was coded as 01. Latter from this generalized data the common similarity matrix using Jaccard coefficient followed by construction of UPGMA dendrogram using Jaccard similarity matrix along with bootstrap values and Cophenetic Correlation Coefficient values were calculated using online statistical software (http://genomes.urv.cat/UPGMA/index.php).

The online package generated 100 bootstrap replicates from 1205 variable in each of five rows and among them the best dendrogram was selected with highest Cophenetic Correlation Coefficient value. The obtained dendrogram was strikingly similar to that of the morphological analysis, which involved binary scoring using a Tukey-Kramer test. The termite species *T. biformis* (Wasmann) formed the first cluster with 35.7% of dissimilarity between the other species. *O. obesus* (Rambur) and *O. redemanni* (Wasmann) shared 1.3% dissimilarity and *O. horni* (Wasmann) shared 1.5% dissimilarity with *O. ceylonicus* (Wasmann). These two groups shared 1.7% of dissimilarity. The Cophenetic Correlation Coefficient value was 0.85, which is the highest among the 100 bootstrap replicates generated by the online program.

Table 8. Test of the homogeneity of substitution patterns between sequences.

Species	*O. ceylonicus* (Wasmann)	*O. redemanni* (Wasmann)	*O. horni* (Wasmann)	*O. obesus* (Rambur)	*T. biformis* (Wasmann)	UK1	UK2
O. ceylonicus (Wasmann)		0.940	2.345	0.574	5.214	3.351	0.000
O. redemanni (Wasmann)	0.070		0.000	3.491	1.637	0.000	1.125
O. horni (Wasmann)	0.010	1.000		5.836	0.667	0.000	2.565
O. obesus (Rambur)	0.080	0.002	0.000		10.354	6.342	0.387
T. biformis (Wasmann)	0.000	0.024	0.116	0.000		1.533	5.387
UK1	0.000	1.000	1.000	0.000	0.026		3.524
UK2	1.000	0.050	0.000	0.128	0.000	0.000	

Table 9a. Similarity Matrix computed with Jaccard coefficient for all the identified samples based on three markers.

Correlation	Oc	Or	Oh	Oo	Tb
Oc	1	0.325	0.349	0.323	0.284
Or		1	0.334	0.345	0.317
Oh			1	0.298	0.275
Oo				1	0.265
Tb					1

Table 9b. Similarity Matrix computed with Jaccard coefficient for all the identified and unidentified samples based on two markers.

Correlation	Oc	Or	Oh	Oo	Tb	UK1	UK2
Oc	1	0.32	0.35	0.32	0.29	0.33	0.34
Or		1	0.34	0.33	0.33	0.33	0.31
Oh			1	0.3	0.28	0.33	0.31
Oo				1	0.28	0.34	0.31
Tb					1	0.32	0.27
UK1						1	0.34
UK2							1

The Jaccard similarity matrix (Table 9a) specifies a highest similarity value of 0.349 between *O. horni* (Wasmann) and *O. ceylonicus* (Wasmann). Similarly, the lowest value was recorded between *T. biformis* (Wasmann) and *O. obesus* (Rambur) with 0.265. Thus *T. biformis* (Wasmann) is an out group in the cluster and *O. redemanni* (Wasmann) shows maximum similarity with *O. obesus* (Rambur) with about 0.345.

The same online package was utilized to generate the similarity matrix using a Jaccard coefficient analysis. The dataset of 7 rows with 1165 variables in each row was analysed, which included only the binary codes of sequence and the binary scoring of ISSR markers. The similarity matrix (Table 9b) suggests a maximum of 33% similarity between *T. biformis* (Wasmann) and *O. redemanni* (Wasmann). *O. obesus* (Rambur) shows 33% similarity between *O. redemanni* (Wasmann) and 34% similarity between UNKNOWN01. *O. redemanni* (Wasmann) shows a maximum of 34% similarity with *O. horni* (Wasmann). A maximum similarity value was recorded between *O. horni* (Wasmann) and *O. ceylonicus* (Wasmann); and the least value was recorded by UNKNOWN02 with *T. biformis* (Wasmann), with about 27% similarity.

The similarity matrix calculated, based on Jaccard's coefficients, was used to construct a UPGMA tree (Figure 9) with the highest Cophenetic Correlation Coefficient value of about 0.72 among 100 bootstrap replicates. The

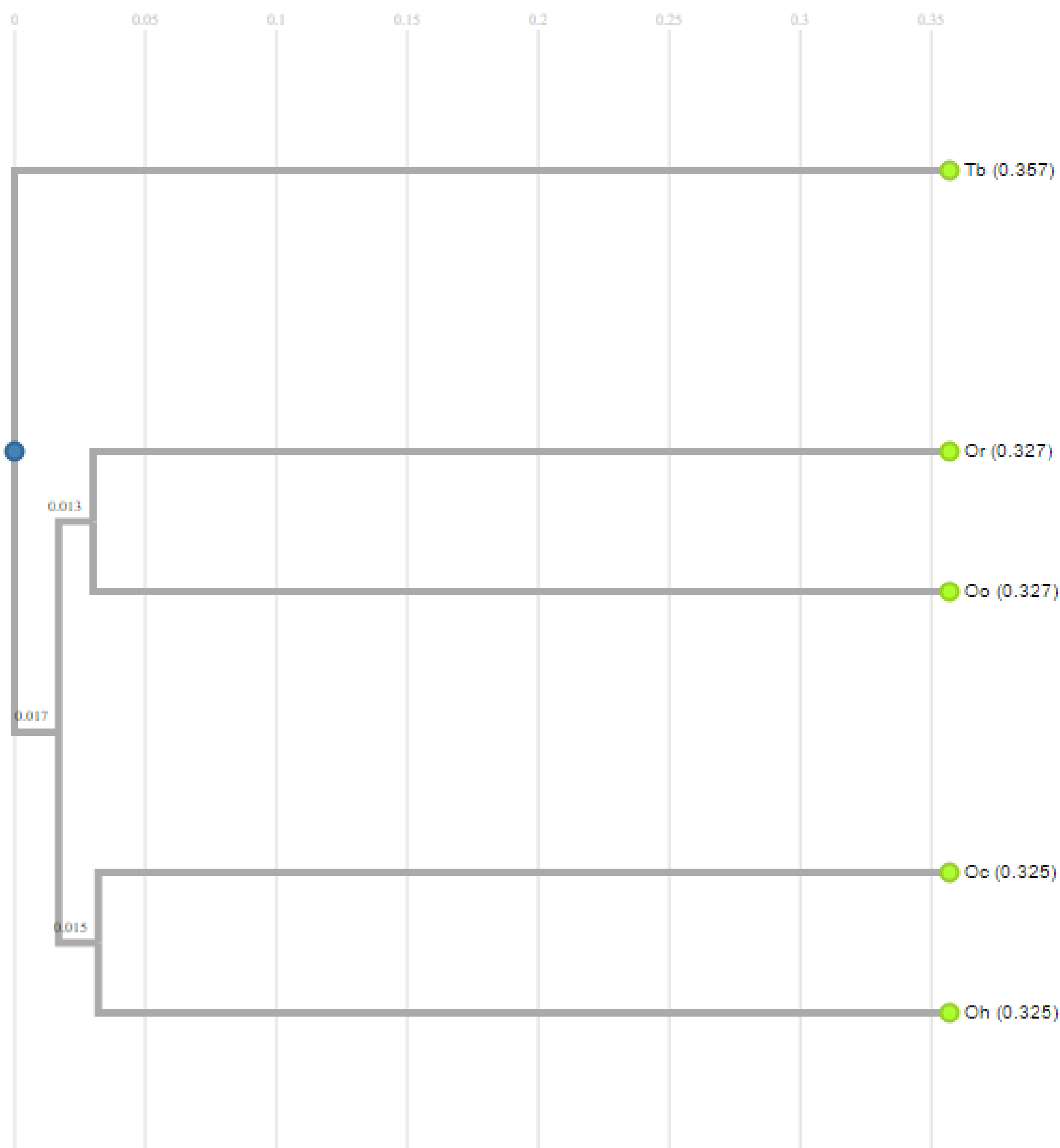

Figure 8. UPGMA tree based on Similarity Matrix computed with Jaccard's coefficient for all the three markers.

guided-tree construction reveals that there are three clusters, among which *T. biformis* (Wasmann) forms the first out group with a maximum of about 35.3% similarity with the rest of the species. The next group includes *O. redemanni* (Wasmann), *O. obesus* (Rambur) and UNKNOWN01. *O. redemanni* (Wasmann) shows about 33.4% of similarity with *O. obesus* (Rambur) and UNKNOWN01 together. And lastly, *O. obesus* (Rambur) shows 32.8% of similarity with UNKNOWN01. Similarly, the third group which includes UNKNOWN02, *O. horni* (Wasmann) and *O. ceylonicus* (Wasmann) shows 34% of similarity with the second group (Figure 8). UNKNOWN02 recorded 33.6% of similarity with *O. horni* (Wasmann) and *O. ceylonicus* (Wasmann); and *O. horni* (Wasmann) shows a maximum of 32.6% of similarity with *O. ceylonicus* (Wasmann).

DISCUSSION

The diversity index values reveal that termite species richness is quite high, or extending to about average;

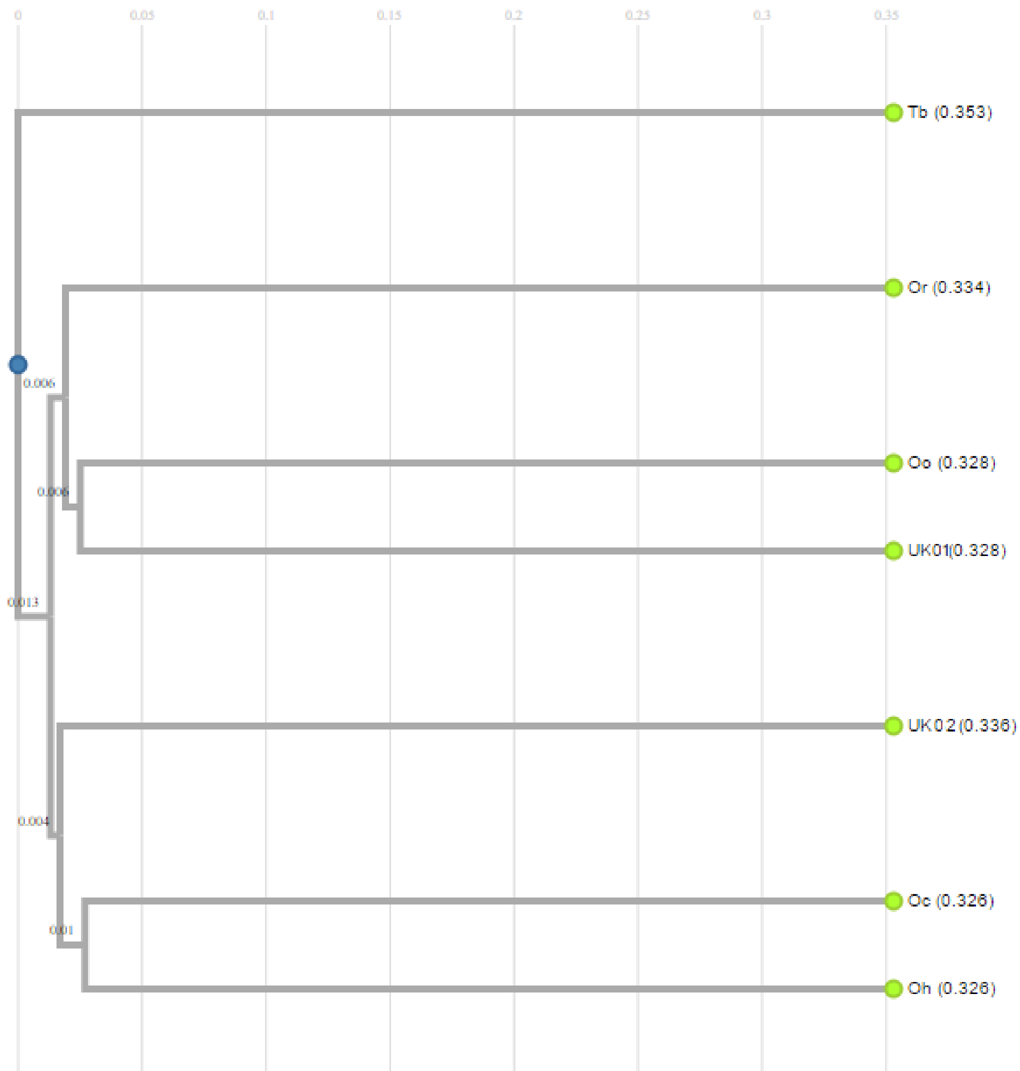

Figure 9. UPGMA tree based on Similarity Matrix computed with Jaccard's coefficient for all the seven samples with two markers.

whereas, Shannon-H index with a mean value of 1.583 (1.578 lower limit and 1.589 upper limit) indicated an average species richness in the given locality. Shannon-H indices are weaker as it is difficult to compare communities which differ greatly in richness (Magurran, 2010). Thus, accordingly, the use of Simpson's 1-D value is critical in this study making it more accurate for the present data (Table 3a and b).

Among the 57 termite nests where samples were collected, five genotypes each of different species along with two unidentified samples were considered for the DNA fingerprinting study. The study includes 17 ISSR primers among which only six primers were considered since they produced visible scorable bands. The six primers produced 103 bands, which were 99.03% polymorphic. Morphologically, *O. obesus* (Rambur) and *O. redemanni* (Wasmann) share more than 50% of similarity. Generally, they pose difficulty in differentiating them morphologically (Pranesh and Harini, 2014). Similar results were obtained when these two species were subjected to DNA fingerprinting using ISSR markers. The ISSR analysis has many advantages over other markers such as: it targets microsatellite DNA sequences that are abundant throughout the eukaryotic genome that evolve rapidly, and it also includes amplification of specific repeatable fragments (Godwin et al., 1997; Sheppard and Smith, 2000). Similar results were obtained in this study between unknown samples and known species.

The amplification using ISSR markers revealed a total of 103 visible bands among which 102 were polymorphic. The basic analysis of the banding pattern (Table 4a to d) revealed that of 103 amplified loci, ISSR11 produced one monomorphic band of size 380 bp, apart from which, all of the amplified loci were polymorphic between the species. This implies that ISSR is an excellent marker for studying genetic variations between and among the species.

The banding pattern shows 12 monomorphic bands between UNKNOWN01, UNKNOWN02 and *O. cylonicus* (Wasmann) and 35 commonly missing bands, which are reported to be present in other species of the present study. This suggests that there is a high similarity between the three samples and also that they might be the same species; but because the nests are located far apart, there is some inbreeding, which shows polymorphism between the three samples. This theory has to be supported further by sequence similarity or by bar coding. A similar kind of banding pattern was observed between same species of honey bees that showed different levels of tolerance to mites (Al-Otaibi, 2008).

Each polymorphic fragment was scored as a locus with two allelic classes (either as absence or presence of a band or dominant allele); therefore, the maximum heterozygosity value of an ISSR locus was 0.5. The average heterozygosity recorded is 0.2788. The heterozygosity ranges from 0.244 to 0.3366. The low heterozygosity value indicates that there is likely high inbreeding within the population, and also the species observed in this study are relatively diverse for the markers used in this study. Similar results were observed in honey bees, which are also inbreeding species like that of termites (Al-Otaibi, 2008).

The obtained DNA profile indicates that the ISSR technique is a useful method for detecting genetic diversity among the termite species. The wide distribution of microsatellite DNA across the genome, combined with the easiness of detecting polymorphic loci using ISSR markers, gave the system great potential in studying termite genetic diversity. The genotyping of 6 shortlisted ISSR markers was used to discriminate among the colonies for the above purpose.

Since five samples were collected from each species for the study, it is difficult to identify species specific bands; but from the result it can be observed that the two unknown samples share a common banding pattern to that of *O. ceylonicus* (Wasmann). The ISSR primer ISSR02 produced bands of size 550, 510, and 370 bp. ISSR04 produced bands of size 430 and 320 bp. ISSR06 produced 480 and 380 bp products. ISSR10 produced 600 and 580 bp products. And ISSR11 produced 470, 420 and 380 bp products, which showed 100% monomorphism between the two unknown samples and *O. ceylonicus* (Wasmann). ISSR12 recorded zero monomorphic bands with these three samples. ISSR12 shows maximum dissimilarity between these three samples and also with other species observed in this study. Similar results were obtained in many plant cultivars and other species, which are used for breeding purposes as reported in the next paragraph with details. ISSRs serve as potential markers for identifying both inter species variance and intra species variance.

Genetic polymorphism has been reported in a broad range of taxa including: *Corchorus* spp. (Javan et al., 2012), *Silva* genus (Garriga et al., 2013), for indexing blueberry cultivars (Berezovskaia et al., 2003), *Ipomoea* species (Moulin et al., 2012), *Bombinae* species (Liu and Wendel, 2001), *Apis mellifera* species and their breeding colonies (Al-Otaibi, 2008), Cotton cultivars (Bornet, 2002), and *Brassica oleracea* species (Korman, 1991). These, and many other species whose inter- and intra-specific banding pattern can be easily examined using ISSR markers, can be effectively used for research on breeding, diversity study, detecting polymorphism and identifying similarities between and within the species.

The similarity matrix in Table 9a and b was computed with Jaccard's coefficients, using the scored data obtained from the ISSR marker banding pattern. The average similarity coefficient value is 0.235; the lowest similarity value of 0.043 was recorded between UNKNOWN02 and *T. biformis* (Wasmann). Highest similarity coefficient value (0.404) was recorded between UNKNOWN02 and *O. ceylonicus* (Wasmann). These values specify that genetic similarity between the samples is very low and also the diversity is very high with respect to the repetitive sequences in the genome. A UPGMA rooted phenogram (Figure 8) also was constructed using Jaccard's similarity coefficients based on scored data obtained from six ISSR primers for seven genotypes. The cophenetic correlation value was 0.90, which makes the UPGMA cluster highly reliable (Carr, 1999; Lander and Botstein, 1989). The first two groups in the dendrogram share 44.8% of similarity with *T. biformis* (Wasmann). This specifies that *T. biformis* (Wasmann) belongs to a separate genus and supports the morphological identification methodology. The cluster group one composed of *O. redemanni* (Wasmann), *O. obesus* (Rambur) and *O. horni* (Wasmann) species, shares 39% of similarity with cluster group two, which includes *O. ceylonicus* (Wasmann), UNKNOWN02 and UNKNOWN01. In group one, *O. redemanni* (Wasmann) and *O. obesus* (Rambur) share 32.9% of similarity with *O. horni* (Wasmann), Moreover, for *O. redemanni* (Wasmann) and *O. obesus* (Rambur) there is 25% of similarity. For the similarly in group two, *O. ceylonicus* (Wasmann) and UNKNOWN02 share 35.6% of similarity with UNKNOWN01; and for *O. ceylonicus* (Wasmann) and UNKNOWN02 there is 29.8% similarity.

In this study, very high percentage of polymorphic bands was obtained (99.03%), suggesting that there is a high diversity in Inter Sample Sequence Repeats among the termite species. The repetitive DNA sequences can be used to produce species specific markers, which aids in robust detection of termite species. The percentage of polymorphism indicates that the marker is efficient enough to detect polymorphism; but due to its average reproducibility, the marker has less application in molecular biology compared to other molecular markers. The study suggests that these polymorphic bands are not only useful to study the genetic relatedness between the termite samples but also serves as useful tools for genetic marker analysis.

Nucleotide analysis of partially amplified 28s rDNA sequence of seven termite samples

Generally mtDNA serves as a very good tool for studying population genetics, due to variability in intraspecificity (Hassouna et al., 1984). But, 28s rRNA sequences can also be used to analyse the same since they have a wide range of structural polymorphism (Yeap et al., 2010).

Various researchers around the world used both mitochondrial and nuclear markers to confirm phylogenetic relationships between termite species, which are likely to be synonymous. In this study, *O. obesus* (Rambur) and *O. redemanni* (Wasmann) show strikingly similar morphological features, but they are not synonymous species, such as the known synonyms: *Reticulitermes flavipes* and *Reticulitermes santonensis*. Thus, we need a strong basis to prove this statement. In this regard, there are 3 markers *viz.,* morphological, ISSR and 28s rDNA sequences that may prove to be useful. Apart from this, the study also provides an initiative to understand nuclear genes in finding out variations between known and unknown species. Even though it is well understood that the mitochondrial COI gene is useful to identify insect species, the 28s rDNA is used in this study to determine the genetic difference between species based on nuclear gene polymorphism and not just to identify possible species differences based on DNA Bar coding.

The nucleotide pair analysis (Table 6a) revealed that the average frequency of GC rich regions is 30, and that of AT is 33. The R value for the fraction of transitional (132) and transvertional (263) pairs was found to be 0.5, which is a moderate value. The identical pair number was found to be 207, which is the sum of MSA analysis of identical pair numbers at first position (73); second position (74) and position three (60). The highest sum value of 61 was obtained for the TT sequence frequency followed by AA of about 55; CC of about 48 and GG of about 43. These ranges of frequencies are clearly represented in a pi-chart for detailed inspection (Figure 4). In Table 6b, nucleotide frequencies of all seven sequences are tabulated.

The average nucleotide frequency of all the 7 samples yielded A+T of about 52.1% and G+C of about 47.9%, which specifies that A+T content is more than that of G+C. Similar results were obtained between three genera, *viz., Microcerotermes, Microtermes* and *Odontotermes* by Singla et al. (2013) using 12s rRNA and mtDNA genes. Likewise, A+T contents were found to be higher than G+C content (Austin et al., 2005a).

In the present study, MSA led to the identification of 39 variable sites, among which 6 sites were beyond 50 bases long and 9 single nucleotide variable regions were identified. The results are in accordance with Austin et al. (2005b) and Pei et al. (2010), where mtDNA for 47 haplotypes were observed, which belonged to *Reticulitermes flavipes* (Kollar) of North America. They also noted an ITS2 region, which was helpful to resolve phyletic relationships between 10 *Reticulitermes* haplotypes that were not fully resolved by using nuclear markers.

Secondary structural analysis of partially amplified 28s rDNA sequences of all seven termite samples

The 28s rDNA is a nuclear gene of eukaryotes, which can be used to study the genetic makeup and relatedness between and within species. The intra and inter species variation can be documented with a much better accuracy when comparisons of secondary structure models are taken into account than by sole sequence alignment. The sequence regions within interrupted sequence alignments, a number of conserved secondary structure features can however be identified in all species which improves the mapping of the size-variable segments (Yeap et al., 2010).

In the present study, MSA of the entire seven termite sample analysis revealed 74 conservative regions (with only single nucleotide sites and a few base stretches with many gaps) and 39 regions of variable sites. These 39 variable sites were considered for the identification of the 28s rDNA secondary structure variations. The scenario is completely different in the Pei et al. (2010) study related to secondary structure of 16s rRNA gene in prokaryotes of both Archaea and Eubacteria. The study reported here identified 6.7% of conserved regions among the species and these conserved regions were considered to construct secondary structure of the ribosomal subunit 16s. By studying the conserved regions one can identify the species but by considering the variable regions genetic diversity can be acknowledged (Chakravorty et al., 2007; Yu et al., 2013). Termite 28s rDNA structural variation studies have not taken place until now,

because the tool has wider applications in microorganisms and fungi identification than in insects. Thus, although one can find literature for 28s rDNA sequences on termites, it is very rare to find literature on its structure. The present study focuses on the sequence variation and its structural diversity among termites.

Yu et al. (2013) have used length variable regions (LVRs) for comparing the secondary structural variations among *Eurydema maracandica* species for both 18s and 28s rRNA. The LVRs based on the domain amplicons of both 18s and 28s rRNA sequences of the specific monophyletic species served as a good tool for studying morpho-molecular structures (Ouvrard et al., 2000; Scharf et al., 2005). Similarly, in this study as the domain markers are used for amplification of the variable sites, MSA suffices as a good tool for identifying morpho-molecular structures between the species. From a total of 39 variable sites among all the seven samples, six variable sites were considered as the lengths were more than 50 bases, while six types of secondary structures were observed. These structures are present in variable number at variable positions across six variable sites. For example, *O. redemanni* (Wasmann) is morphologically very much similar to that of *O. obesus* (Rambur); the secondary structure of these two species at VS1 shows 3-D structures and Pknot structures in the former, but absent in the latter. Also, one can observe in both species that single-stranded loops and pseudo double-stranded structures are absent, and hairpin loops are two in number though the length varies at the VS1 position. Similarly, in species *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann) (which are morphologically similar), the first three components vary and last four components are similar between each other at VS1. The pattern of structural similarity between *T. biformis* (Wasmann) and the rest of the species is strikingly different, justifying that *T. biformis* (Wasmann) is an out group. Interestingly, the two unknown species are also different from one another in the pattern of secondary structures. UNKNOWN01 is more similar to that of *O. ceylonicus* (Wasmann) and UNKNOWN02 is more similar to that of *O. obesus* (Rambur), though with variable sequence length.

Phylogenetics and evolutionary divergence

The evolutionary relatedness between/within termite species based on mitochondrial genes (16s, 18s, COI, etc.) is a well-studied area (Kambhampati and Eggleton, 2000). But evolutionary relatedness using ribosomal nuclear markers is less, because these markers are not used for identification of insects or studied for their expression sites (Jenkins, 2001). But in the present study, aiming at genetic diversity between termite species, nuclear sequence comparison plays a vital role, and the 28s rDNA sequence was easily available for this purpose. The obtained sequence was analysed using MEGA7 software to construct a Neighbor-Joining tree based on the Maximum Composite Likelihood Method (Tamura et al., 2004). The cluster (Figure 7) shows *T. biformis* (Wasmann) with some similarity with *O. ceyloncus* (Wasmann) and *O. obesus* (Rambur). Similarly, *O. redemanni* (Wasmann) shows similarity with *O. horni* (Wasmann) by forming a group. The result, however, is not in accordance with either ISSR marker analysis or Morphological analysis.

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences, was established according to a Disparity Index test (Kumar and Gadagkar, 2001). A Monte Carlo test (500 replicates) was used to estimate the P-values. P-values, which are smaller than 0.05 are considered significant (marked with yellow highlights) (Table 8). The estimates of the disparity index per site are shown for each sequence pair above the diagonal. This analysis allows us to check the significance between a pair of sequences and compare the phylogenetic result obtained from Maximum Likelihood Method (Aanen et al., 2002; Inward et al., 2007).

Cumulative phylogenetic analysis of the five identified termite species

Cumulative or comprehensive analysis is the most effective tool to identify genetic divergence. This comprehensive analysis generally includes morphological, anatomical, biochemical and nucleic acid variations to generate comparatively a more accurate consolidated phylogenetic tree than cladistic trees generated by individual markers (Inward et al., 2007). The present cumulative analysis includes binary morphological scorings, ISSR scorings and nucleotide sequences in binary codes. These three markers can be applied to only the five identified termite species as the two unknown species was devoid of any soldier termites during sampling.

The binary codes of morphological, ISSR and 28s rDNA sequences of five identified species were subjected to online phylogenetic analysis. The online package generated 100 bootstrap replicates and the best phylogenetic tree was selected based on the maximum Cophenetic Correlation Coefficient value (Carr et al., 1999; Lander and Botstein, 1989). The phylogenetic tree, generated by using a Jaccard similarity matrix (Table 9a), is in accordance with morphological analysis using a Tukey-Kramer test. *T. biformis* (Wasmann) forms an out group with 35.7% of dissimilarity compared to the other

species. *O. obesus* (Rambur) and *O. redemanni* (Wasmann) share the same cluster with 32.7% of dissimilarity; similarly, *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann) share a maximum of 32.5% of dissimilarity (Figure 9). In Inward et al. (2007), the largest taxa Termitidae of Isoptera was clearly segregated phyletically with wide acceptance by studying its comprehensive characteristics like termite worker gut morphology, mandible morphology and COII gene sequence analysis. Similar work by Jarvis et al. (2004) on earwigs indicates that the epizoic *Hemimerus* is not sister to the remaining Dermaptera, but rather nested as sister to Forficulidae and Chelisochilisochidae. The study included large subunit ribosomal (28S), small subunit ribosomal (18S), histone-3 (H3) nuclear DNA sequences, and forty-three morphological characters.

Cumulative phylogenetic analysis of the five identified and two unidentified termite species

During the genetic diversity study, there were two unidentified termite samples due to non-availability of soldiers in the samples. These two samples were also considered for generating cumulative/comprehensive analysis while excluding morphological data (because the morphological data was generated based on the soldiers external morphology). Similarity matrix was generated according to Jaccard coefficients to generate 100 bootstrap replicates and the one with maximum Cophenetic Correlation Coefficient value was selected to construct a UPGMA tree. The guided tree was very much similar to the results obtained by ISSR marker analysis, secondary structural pattern of 28s rDNA and its sequence analysis. *T. biformis* (Wasmann) was noted as an out group in the guided tree, UNKNOWN01 had more similarity with *O. obesus* (Rambur) and *O. obesus* (Rambur) in turn showed high similarity with that of *O. redemanni* (Wasmann). UNKNOWN02 was found to cluster along with *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann). Similarly, Forschler and Jenkins (1999) resolved inconsistencies and published a taxonomic revision of subterranean termites and along with its complicated taxonomic keys by using a multidisciplinary approach based on behavioral, ecological, chemo-taxonomical and genetic data.

Conclusion

This study included 54 termite nest samples with five identified species and two unknown species. The study at Jnanabharathi campus gives insight into the termite distribution pattern. The genetic diversity, using 6 ISSR markers on five morphologically identified species and two unidentified species, revealed a total of 103 visible bands among which 102 were polymorphic. The banding pattern shows 12 monomorphic bands between UNKNOWN01, UNKNOWN02 and *O. cylonicus*. The heterozygosity values ranged from 0.244 to 0.3366. The average similarity coefficient value was 0.235. The similarity coefficient and heterozygosity values specify that genetic similarity between the samples is very low and also the diversity is very high with respect to the repetitive sequences in the genome. The cophenetic correlation value was found out to be 0.90 for the constructed UPGMA cluster. The highlights of the UPGMA tree are 25% of similarity between *O. redimanii* and *O. obesus*. Likewise, the similarity between *O. cylonicus* and UNKNOWN02 is 35.6%. The similarity between UNKNOWN01 and *O. cylonicus* along with UNKNOWN02 is 29.8%. The study reveals a very high percentile (99.03%) of the polymorphic bands and suggests that there is a high diversity in inter simple sequence repeats among the termites. These repetitive DNA sequences can be used to produce species-specific DNA markers, which aids in robust detection of termite species.

The nucleotide analysis of five known and two unknown species for the 28s rDNA gene revealed comparatively more A+T content than that of G+C. except in *O. horni* and *T. biformis*. A total of 39 variable sites, 74 conservative sites and a sum of 2618 gap regions among 1124 sequence length were obtained after MSA. The sequence was analysed to generate secondary structure with least energy, which generated a total of six types of secondary structures that varied for each species. The variable sites of MSA were used to analyse the pattern of secondary structures. The pattern of secondary structure was similar between *O. obesusi* and *O. redemanni*; as well as between *O. ceylonicus* and *O. horni*. Likewise, the two unknown species UNKNOWN01 and UNKNOWN02 showed similarity towards *O. obesus* and *O. ceylonicus,* respectively; whereas, *T. biformis* exhibited very little structural similarity between the species at variable sites. The sequence was also analysed to generate a phylogenetic tree, which was not in accordance with morphological or ISSR marker analysis. The cluster analysis (Figure 7) shows *T. biformis* (Wasmann) with some similarity with *O. ceyloncus* (Wasmann) and *O. obesus* (Rambur). Similarly, *O. redemanni* (Wasmann) shows similarity with *O. horni* (Wasmann) by forming a group.

All the data obtained were analysed for genetic diversity by pooling the coded binary digits. This data was submitted to generate two guided trees one with all the three markers, *viz.,* morphological, ISSR and nucleotide sequence, and the other without morphological data, as two unknown species should also be considered for analyzing its genetic divergence and similarity between

the other identified samples. In the first guided tree, *T. biformis* (Wasmann) is out grouped with 35.7% of dissimilarity with other species; *O. obesus* (Rambur) and *O. redemanni* (Wasmann) share the same cluster with 32.7% of dissimilarity. Similarly, *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann) share a maximum of 32.5% of dissimilarity. Similarly, in the second guided tree *T. biformis* (Wasmann) was noticed as an out group in the guided tree; UNKNOWN01 had more similarity with *O. obesus* (Rambur), and *O. obesus* (Rambur) in turn showed high similarity with that of *O. redemanni* (Wasmann). UNKNOWN02 was found to cluster along with *O. ceylonicus* (Wasmann) and *O. horni* (Wasmann). These results were in accordance with morphological, ISSR and secondary structural patterns found between the identified and unidentified species.

Thus, the cumulative or comprehensive analysis using different markers, like morphological, ISSR and nuclear genes like 28s rDNA, depicts an accurate measure of genetic diversity between different species of termites. The results of this research also suggest to use different markers for the analysis of different species, and the impact of change in environment on the genetic diversity or even genetic structure of the species, because termites as used in this study are an excellent bio indicator.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Ethnobotany and population structure of *Balanites aegyptiaca* (L.) Delile in Sahelian zone of Cameroon

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Indigenous tree species have multiple functions and the strong pressure exerted on them threatens their populations. The study aims to investigate the main uses of *Balanites aegyptiaca* and assess its population structure in the Sahelian zone of Cameroon. The study was carried out using ethnobotanical and quantitative ecological methods. The results showed that the species is multipurpose and produces six different products and services. The first three most mentioned were food, firewood and handicraft. The fruits and leaves were the most appreciated parts of the species. In all, 1026 individuals were recorded and it occurred at a density of 114 individuals.ha^{-1}. Individuals belonging to 5-15 cm dbh were most important (49.55%) and the adult trees (>75 cm dbh) represented only 3%. An analysis of population structure showed that the population was essentially young and presented an "L" shape. The absence of old individuals in the area showed that the regeneration and the management of this species were unsustainable. Studies on the forestry of the species are necessary to satisfy the local population needs because the vulgarization of knowledge on the species can lead to its exploitation at a large scale.

Key words: *Balanites aegyptiaca*, regeneration, medicinal plant, Sahelian zone, Cameroon.

INTRODUCTION

Balanites aegyptiaca (L.) Delile, commonly known as the desert date (Matig et al., 2000) is a woody plant belonging to the family, Balanitaceae. This species originates from tropical Africa and grows in the Sahel region where it faces multiple challenges such as desertification and poor agricultural practices. *B. aegyptiaca* plays a multifunctional role in the lives of local people. In previous ethno-botanical studies (Ouédraogo

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et al., 2000; Chevallier et al., 2003; Malaisse, 2010), its importance as food and medicine for local people throughout Africa was illustrated (Arbonnier, 2000).

Ecologically, *B. aegyptiaca* is considered as a resilient species and highly versatile towards Sahelian soil and climatic conditions (Parkan, 1993; Hiernaux et al., 2006). *B. aegyptiaca* is widely found in the Sahelian zone, a region situated in the Far North region of Cameroon. Unfortunately, the population of *B. aegyptiaca* has decreased continuously. Because of the rising prices of commodities, local communities fall back on the multipurpose species or non-timber forest products (NTFPs) to resolve their problems. NTFPs fulfill different roles in their sustenance and make them to live with less cash (Vedeld et al., 2007). This recourse to NTFPs exercises a strong pressure on the species and has resulted in the decline of the species throughout the region among which is *B. aegyptyaca*. Demographic explosion was cited as one of the factors leading to the loss of the species (Bitariho et al., 2006) and to reduction in the availability of products (Birkett and Stevens-Wood, 2005). Many studies were carried out on *B. aegyptiaca* in the Sudan-Sahelian zone of Cameroon. The most important is the vegetative propagation of some selected germplasm (Noubissie et al., 2011). However, nothing has been reported or published on the ethnobotanical studies. This will provide important information for its large scale production or its domestication. The objectives of this work were to investigate the uses and population structure of *B. aegyptiaca* in the Sahelian zone of Cameroon.

MATERIALS AND METHODS

Study area and species description

This work was conducted in the Sahelian region of Cameroon. The study area is situated between latitudes 10.29181 and 10.95590°N and longitude 13.61175 and 14.93973°E. The relief is made of plains around the Mandara Mountains and a massif at the border with Nigeria (Figure 1). The average altitude is 1000 m in mountainous areas and about 300 m in plains. The climate is the Sahelian type with two seasons: a long dry season between November and June and a short rainy season from July to October of about 800 mm per year. At the peak of the dry season, temperature is about 35°C. The vegetation is dominated by the thorny Sahel steppes. A distinction can be made between the thorny Steppe and the periodically flooded meadows. The spiny steppe forms a brush of thorns which colonize the calcareous soils whose main constituent species are *Acacia seyal*, *B. aegyptiaca*, *Capparis* spp., *Combretum aculeatum* and *Ziziphus abyssinica*. Periodically flooded meadows destabilized by intensive grazing, bush fires and industrial agriculture present a wooded landscape whose main colonizers of its black clay soils are *A. seyal* and sometimes *Acacia nilotica* var. *adansonii* (Letouzey and Combrouz, 1959; Donfact,1998). The presence of some species such as *Anogeissus leocarpa* on loose and uncleared soil and *Boswellia dalzielii* on stony soil is most noted (Boutrais, 1978).

B. aegyptiaca is an Afro Asiatic tree. It has a vast geographical distribution (Berhaut, 1979; Lebrun et al., 1992; Arbonnier, 2000; Sands, 2003). On the Asian continent, the tree is found in the Middle East from south to north as far as latitude 35° 25’ N, in Arabia, Burma, India and Pakistan and all along the Persian Gulf. In Africa, its range extends west to east, in the Sahelian band from the Atlantic Ocean (Senegal, Mauritania) as far as Eritrea. It developed in the Sahelian to Sudano-Sahelian zones. It has few soil requirements but prefers sandy, stony or heavy soils. *B. aegyptiaca* is an indicator of overgrazing.

Methods

The study was carried out using both ethnobotanical and ecological methods. The ethnobotanical study was conducted through interview of elderly persons resident in the region. The latter was based on their availability and willingness to participate. Semi-structured interviews were conducted using questionnaires in four villages (Petté, Mindif, Moutourwa and Kalfou). A total of 300 persons were interviewed at the rate of 75 persons per village. Questions were asked with respect to habitat of the species, methods of harvest, different parts harvested and their uses and the availability of the species.

The ecological assessment was carried out during the dry season because of the inaccessibility of the area due to bad roads in the rainy season. Plots of 30 x 30 m were installed along a transect of 20 km in the savannah separated by a distance of at least 200 m. The number of plots in each site varied depending on the frequency of the searched species. A total of 100 plots at the rate of 25 plots per village were installed and all individuals of *B. aegyptiaca* were marked. Dendrometric parameters such as diameter at breast height (dbh) and height of the *B. aegyptiaca* trees were measured. *B. aegyptiaca* saplings were counted within plots whereas seedlings were counted beneath adult trees. According to Gouwakinnou et al. (2009), any young plant with basal diameter (at ground level) less than 1 cm were considered as seedlings and those with stem greater than 1 cm basal diameter or more than 1 m height but less than 5 cm dbh and/or less than 1.5 m height was considered as saplings.

Data analysis

The ethnobotanical data were coded and summarized as diagrams. From the quantitative inventory, the density of *B. aegyptiaca* was calculated. A size class frequency distribution plot (SCD) was drawn by plotting the density against size class.

RESULTS AND DISCUSSION

Used parts and services

The results of the survey show that the uses of *B. aegyptiaca* are numerous. Respondents indicated that all parts of the species are used and Figure 2 presents various uses of *B. aegyptiaca* plant parts harvested by the local communities. Six different services were noted in the Sahelian zone of Cameroon. Generally, all uses were cited by more than 50% of respondents. Food and firewood were cited by all the local communities (100%). Handicraft came second in 89% of people interviewed. Fifty eight percents of the respondents used *B.*

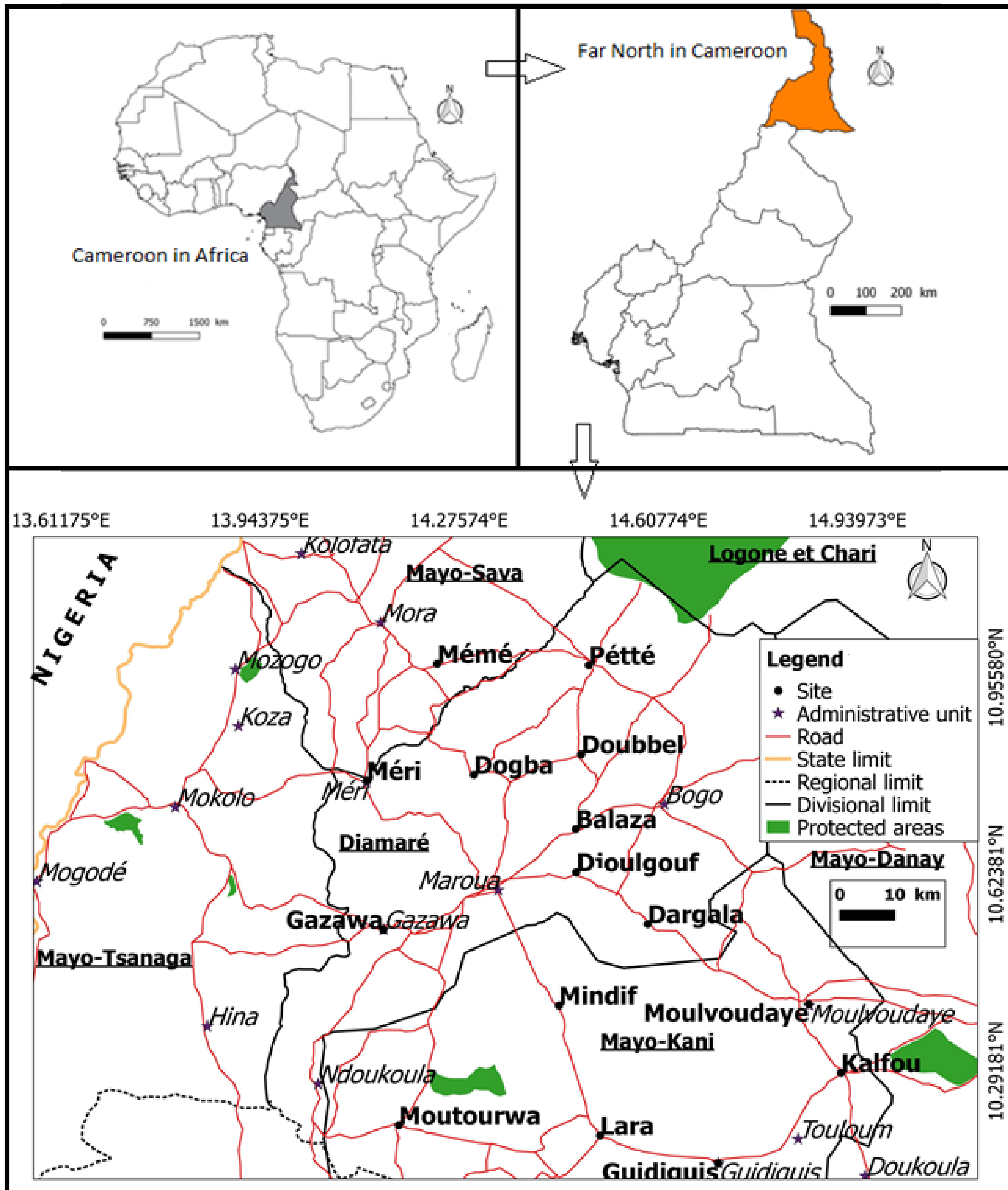

Figure 1. Localization of the study area.

aegyptiaca in the fishing domain contrary to Sagna et al. (2014) in Senegal where this usage was not reported.

Figure 3 shows the percentage of usage of different parts of *B. aegyptiaca* in the Sahelian zone of Cameroon. Fruit and leaves were the most used parts by local people and cited by 100% of respondents, followed by wood and bark (Figure 3). In Senegal, wood came in second position on the list of different used parts (Sagna et al., 2014). Contrary to the latter authors, the third position of wood in the present study could be justified by

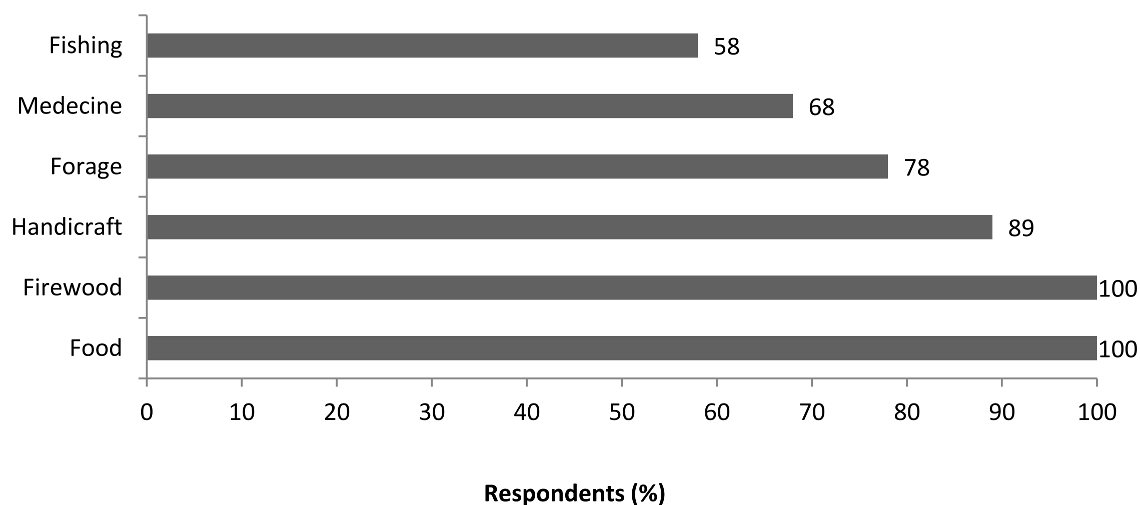

Figure 2. Services of *B. aegyptiaca* in Sahelian zone of Cameroon.

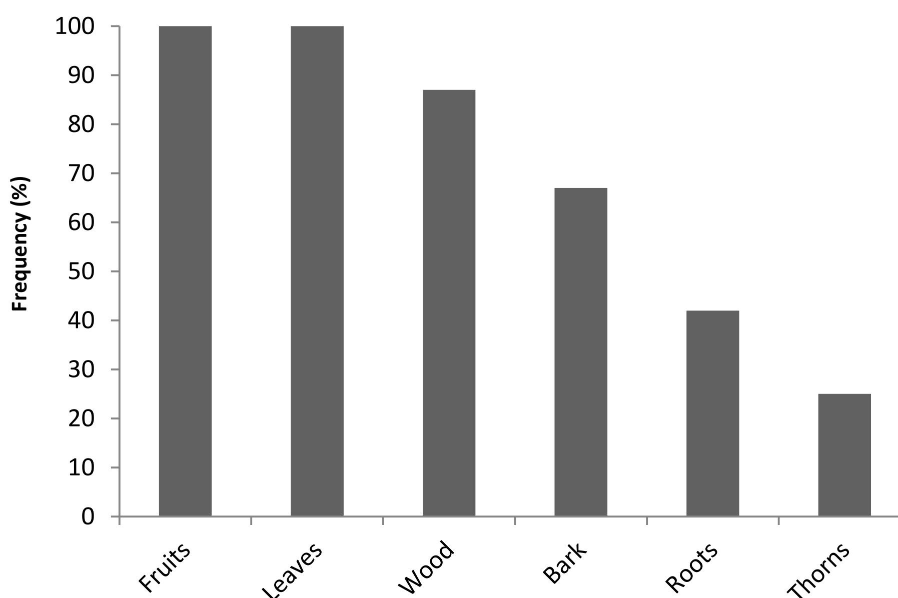

Figure 3. Different parts of *B. aegyptiaca* (L.) Delile used by local people in the Sahelian zone of Cameroon.

the fact that in the Sahelian zone of Cameroon people have other possibilities to get wood for their needs. The results corroborate with those of Folefack and Abou (2009) in Sahelian zone of Cameroon.

Uses

Medicinal uses

The use of *B. aegyptiaca* in traditional medicine was multiple. In the study area, different parts of the plant were cited by local communities for the treatment of various human ailments (Table 1). In Senegal, Sagna et al. (2014) reported ten ailments treated by different parts of *B. aegyptiaca*. The most cited ailments in the study area are: high blood pressure (31.6%), constipation (28.4%) and gastric ulcer (25.5%). Its use to treat other ailments (hemorrhoids, eye infections, stomach-ache, and anthrax) is less reported. The medicinal uses of *B. aegyptiaca* reported by local communities in the Sahelian zone were cited by other local communities of other

Table 1. Frequency and parts of *B. aegyptiaca* (L.) Delile used.

Diseases	Frequency (%)	Treatments (parts used)
Gastric ulcer	25.5	Squeezed pulp
Hypertension	31.6	Squeezed pulp
Constipation	28.4	Squeezed pulp
Abdominal pains	20.3	Macerated Young plants
Anthrax	20.1	Macerated Young plants
Cold	17.3	Calcinated bark
Burns	10.2	Calcinated bark
Hemorrhoids	8.8	Macerated bark
Tooth decay	8.4	Twigs

countries throughout the various regions of Africa (Saboo et al., 2014). A comparison of the data on the medicinal uses of this plant shows that there are several similarities within the regions. For example, this species is used for the treatment of several sorts of illnesses and symptoms which are most reported are infectious diseases (smallpox, anthrax and yellow fever), digestive tract ailments (gastric ulcer, constipation and hemorrhoids), sexually transmissible disease (syphilis), and chronic illnesses (hypertension and diabetes). Similarities of medicinal uses of this species in its distribution range are proof that it has some medicinal and pharmacological potential. However, there are uses that appear specific to certain regions. For instance, both roots and leaves were not reported for medicinal use in the Sahelian region of Cameroon, whereas macerated leaves are used to treat nose bleeding in Algeria, and jaundice in Sudan and Egypt, root powder is used as laxative for treatment of stomach and constipation in Chad. Like Chothani et al. (2011) and Sagna et al. (2014), the current results illustrate the widespread use of *B. aegyptiaca* for medicinal purposes.

In addition, these authors reported that all parts of the plant (bark, fruit, kernels and leaves) possess a wide array of active biomolecules justifying the diversity of it uses in medicine. Adamu et al. (2005) further asserts that the bark of *B. aegyptiaca* has antimicrobial proprieties against *Escherichia coli*, *Staphylococcus auraeus* and *Pseudomonas aeruginosa*. In the study area, thorns were used to solve the problem of luxation. In the other region, the use of *B. aegyptiaca* in veterinary medicine is well known. In Burkina Faso for example, decoction of the bark and roots is used for treating animal anthrax; the leaves and bark are ground and used as poultice on wounds (Chevallier et al., 2003).

Food uses

B. aegyptiaca leaves as well as the, fruit pulp and the kernel are used for food. Despite the bitter taste, it is sucked and greatly enjoyed by people. Using the pulp for making juice was sometimes reported (42.5%). The juice is used for preparing traditional dish called “gari” most appreciated by the Muslim community during the Ramadan periods. The use of kernels was indicated by 75% of respondents. After breaking the seeds, kernels are extracted and prepared. The resulting product can be dried and eaten like peanut and it is most appreciated by local people. According to some respondents (20%), oil extracted from the kernels is used sporadically in the region. Unfortunately, the extraction technique was not known in the region. Consequently, the use of its oil was not widespread contrary to the findings of Sagna et al. (2014) in the region of Ferlo (Senegal) where local Wolof people use it frequently. With regards to flowers and leaves, their use for food seems to be widespread. However, report of the use, control of these different parts and their harvesting methods remained primitive. Women use sickles to cut the leaves and branches. But to avoid destruction of the entire branch, they climb trees to harvest steak leaves. In all cases, women put the different plant parts harvested in their scarves or calabashes. To facilitate the collection of great quantities of *B. eagyptiaca* leaves, the branches are cut and staked. Once the heap is dry, women give a few blows on the heaps and pick up the leaves after removing the thorny branches. The leaves are used in the preparation of a sauce eaten with millet, maize or rice. Leaves were cited for culinary purposes by 87% of respondents. The cooking techniques varied according to cultures and tastes. Nevertheless, in the region, almost all ethnic groups knew these different cooking techniques of the sauce at *B. aegyptiaca* leaves. Very often, it was cooked with the groundnut paste. Somewhere else, it was mixed with *Vigna unguiculata* grain and *Ceratheca sesamoides* leaves making it sticky.

Use of wood

A high consumption of *B. aegyptiaca* wood was reported

by local people in the Sahelian zone (Figure 2). The wood is widely used for building huts (78.8%), fencing (48%) and animal pens (51.6%). Other uses of wood reported by the local communities included cooking (85.2%), charcoal (68.4%), making stands for the Koran (40%) and pestles (52%). For cooking, *B. aegyptiaca* supplies local people with combustible wood commonly used in households. According to respondents, the *B. aegyptiaca* wood was excellent firewood and most appreciated by local people. Contrary to other species, *B. aegyptiaca* was mostly used despite the irritating effect of the smoke to the eyes as reported by some households. This irritation problem was noted in Senegal (Sagna et al., 2014). *B. aegyptiaca* wood was cited among the most used in the handicraft. The trunk was used for making mortar, pestle, etc. The thorns were used by Muslim women to beautify the lip of young girls.

Use as forage

The tree is particularly appreciated for the fodder that it supplies to animals for a large part of the year. The aerial parts of the plant were most palatable and cited in descending order: leaves (100%), fruits (85.3%) and young plants (23%). Fruits are consumed exclusively by small ruminants that eat them when they fall on the ground and very often animals spend the whole day under *B. aegyptiaca* trees to wait fruits fall. In Burkina Faso, *B. aegyptiaca* is also considered as one of the important species due to its quasi-permanent feed availability (leaves, branches and fruit); consumption of its fodder particularly increases in the dry season when pasture is scarce (Chevallier et al., 2003).

Population structure

The analysis of ligneous populations according to Kemeuzé et al. (2009) is centred on three elements which are:

1. The distribution of individuals in height or diameter classes, as an indirect indicator of balance between the age class and phases lived by the population in terms of disturbance or regeneration;
2. The bad state of the population;
3. The intensity of regeneration, as a symbol of population renewal.

Population distribution

A total of 1026 *B. aegyptiaca* individuals were recorded from the study area. The species occurred at a density of 114 individuals/ha. It was noted that the diameter of the population of *B. aegyptiaca* varied from 5 to less than 90 cm. Individuals belonging to the diameter class of 5 to 15 cm were the most abundant (49.65%). They were followed by those belonging to the 15 to 30 cm class (36.16% of the total population). Adult trees represented approximately 3% of the population of *B. aegyptiaca*. Contrary to the results of Geerling (1987) in West Africa, the population in the Sahelian zone presented a weak diameter class.

The size class distribution showed that the *B. aegyptiaca* population was made up mostly of juveniles and that there was a remarkable absence of adult individuals. The population distribution presented the "L" shape which suggests that the population of *B.* a*egyptiaca*a regenerates well in the Sahelian zone but that the older individuals were few and did not maintain themselves. Seedling mortality was high (about 89.5%) (Figure 4). Results of the quantitative inventory equally revealed that the population density of this Balanitaceae was estimated at 114 individuals/ha of which the majority (66 individuals/ha) were young belonging to the 5 to 30 cm diameter class. This result implied that the species was overexploited by the local population in the region.

The size class diameter (SCD) plots (Figure 4) showed a higher density of young individuals of *B. aegyptiaca* but these died out before they developed into mature individuals. This loss of young individuals thus weakened the population. For a population to maintain itself, it needs to have abundant juveniles which will grow into adult size classes (Bationo et al., 2001). Similarly, the weak density of adult individuals affects regeneration in the population by lack of seeds (Ky-Dembele et al., 2007). The high mortally of seedlings may be explained by the constant disturbance of overexploitation for different uses. Most respondents related the precarious status of young individual of *B. aegyptiaca* to overexploitation and land clearance. Grazing has also an important negative effect on this category of individuals of the species. Indeed, in the dry season, animals graze the leaves and the tender branches of young individuals causing their disappearance in nature (Tabuti and Mugula, 2007).

Regeneration

In the study area, few individuals were in flowering or fruiting, indicating that the majority of the populations were young. Old individuals considered as seed producers were almost absent (Figure 4). This suggests that these young individuals were derived from seeds that were dispersed from elsewhere. This explains the gap between generations. This same observation was reported in Burkina Faso by Ouédraogo et al. (2006). This situation causes the species to be in a regressive dynamic characterized by the rarefaction or absence of

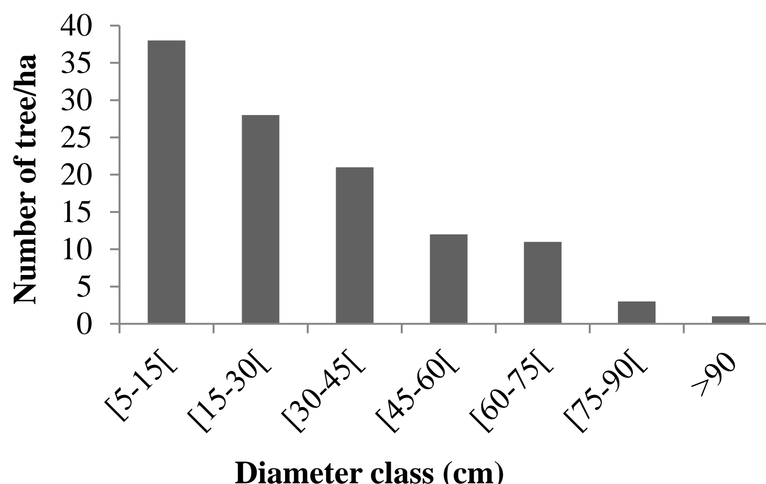

Figure 4. Population distribution by diameter class.

individuals of intermediate diameter classes able to ensure a bridge between the young individuals (seedlings, suckers, stump sprouts or woody tubers) and ageing individuals. Young individuals rarely reach adult age because of overexploitation and overgrazing. To solve this problem of seed production, artificial regeneration seems to be the most appropriate. Unfortunately, local communities in the region lack knowledge of artificial regeneration techniques. Moreover, when the local community is asked whether they are willing to regenerate this species, the majority do not find any interest in this practice. According to them, *B. aegyptiaca* is a "gift from God" and like most other local species, it will always be available.

Conclusion

B. aegyptiaca (L.) Delile occupies an important place in the ethnobotanical patrimony of the people of the Sahelian region of Cameroon. The multiplicity of usage of its different parts and the solicitation frequency classify it among the most appreciated species in the region. The analysis of the population structure show a juvenile population characterized by a quasi-rarity of old individuals. These young individuals could not all reach maturity because of overexploitation and overgrazing arising from absence of seed trees that denote a poor regeneration of the species in area. In the study area, the leaves, wood and fruits which constitute the used biological materials are harvested constantly and in great quantities. The harvest techniques of these different parts do not respect the norm for a sustainable management. Though the exerted pressure on the species could not put it in danger in the area, the vulgarization of the indigenous knowledge on the species can expose it to measures to be taken such as the creation of national parks and vulgarisation of vegetative propagation to improve its regeneration and increase its density in the area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Hunting of Preuss's red colobus (*Procolobus preussi*) in Korup National Park, Cameroon

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This study was carried out in Korup Region to investigate the reasons for hunting preuss's red colobus (PRC) in Korup National Park (KNP) Cameroon in order to contribute to its sustainable management. Questionnaires (open and closed end) were used to gather personal information from hunters on different hunting tactics, reasons for hunting PRC and threats to this species in KNP for a period of one month (January 2014). One hundred and eighty-seven hunters responded to questionnaires that were distributed in all 5 villages in the park and 5 other villages randomly selected from the 23 villages surrounding KNP. Excel software was used and all variables were used to calculate relative proportions. Results showed that 53.5% of hunters use shotguns and 46.5% use both shotgun and wire snares in hunting. Majority of the hunters (46%) practice both hunting and farming, 32% practice solely hunting, 18% practice hunting and fishing, and 4% practice hunting, farming and fishing. A large proportion (73.3%) of hunters does hunt PRC for protein and income. However, 45.3% of them do not eat PRC because of its bad odour, and 22% do not eat it because the meat is hard. Hunters in KNP depend on hunting for their survival. Therefore, this calls for conservation action, such as introducing an alternative source of livelihood and protein to hunters, which will help to improve their standard of living and supply protein for their healthy growth, thereby discouraging hunting.

Key words: Hunting, Korup National Park, anthropogenic activity, survival, *Procolobus preussi*.

INTRODUCTION

In Africa, primates are threatened with extinction (Fa et al., 2002). Hunting has been a greater threat to primates than habitat degradation in west and central Africa because local communities depend on bush meat as a food source (Milner-Gulland et al., 2003). Primates are particularly vulnerable to overexploitation due to the fact

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that they live at relatively low densities and have slow life-histories and also tend to be social, active and therefore highly visible by day (Cowlishaw and Dunbar, 2000). However, large-bodied slow-reproducing species are more at risk of the effects of human threats than fast-reproducing species (Isaac and Cowlishaw, 2004). The mode of hunting is also important. Trapping with wire snares is still the most common type of hunting practiced in the forests of equatorial Africa. Though trapping generally targets duikers and rodents (Noss, 2000), it also endangers terrestrial primates such as apes and mandrills owing to its indiscriminate nature. Further, the introduction of firearms has contributed to changes in hunting patterns, such that arboreal primates such as red colobus are increasingly hunted with guns (Balinga, 2005). The red colobus monkeys of Central and West Africa are known to be the most threatened than any other taxonomic group of primates in Africa due to the fact that they are vulnerable to hunters (Struhsaker, 2005; Waltert et al., 2002). Overhunting is the main threat to this species, the cause of most of its population decline in Korup National Park (IUCN, 2010; Struhsaker, 2010). Therefore, the reasons as to why PRC is hunted are investigated in this study.

Problem statement

Primate hunting is widespread over many parts of the Guineo-Congolian rainforest zone of Africa and especially in certain West and Central African countries like Sierra Leone, Nigeria, Cameroon and Equatorial Guinea (Isaac and Cowlishaw, 2004). Primates are hunted for both subsistence (local consumption) and commercial purposes (large-scale market). Hunting is considered to be a more serious threat to primates in the Guineo-Congolian rainforest zone of west and central Africa than anywhere else in the world because of its high demand (Abernethy et al., 2013). In KNP, Cameroon, Linder (2008) recorded 648 hunted carcasses of monkeys in Ikenge village inside KNP including 78% of the most frequently recorded species (Putty-nosed monkeys, Mona monkey and Preuss's red colobus). It is almost certain that primates have been greatly reduced or even exterminated from many areas as a result of such hunting pressure. According to Eniang (2002), the population of Preuss's red colobus in Ikpan forest block of the Cross River National Park, Nigeria has been greatly reduced as a result of illegal hunting in that area. More than 10 Preuss's red colobus were hunted within three months during their research in the park, and they noticed an increase in the number of poacher's camps as they moved further into the park. Therefore, this work is aimed at contributing to the sustainable management and conservation of PRC in the southern part of KNP through the analysis of hunting activities on the species. The different methods and tools used in hunting were examined, and the purpose for hunting PRC was determined, considering the perception of the local population with respect to conservation of PRC, and finally, measures to mitigate hunting of PRC were proposed.

MATERIALS AND METHODS

Description of the study area

The study area is located in Korup National Park (KNP), South West Region of Cameroon, between 4°53' to 5°28'N and 8°42' to 9°16' E, with a surface area of 1.260 km^2 mostly undisturbed primary forest (Figure 1). It is adjacent to the border with Nigeria and lies near the center of the Cross-Sanaga-Bioko coastal forests ecoregion (World Wide Fund for Nature, 2001). Korup climate is characterized by two seasons: one dry season from November to mid-March and one wet season from mid-March to October. Temperature varies little throughout the year and the mean annual maximum temperature is 30.2°C. Korup region is known for its taxonomic richness and diversity of primates. These primates also show a high degree of endemism in this region (Linda and Oates, 2011). KNP harbours 14 species of primates, eight are diurnal and six of them are under threats of extinction. Preuss's red colobus is endemic to this part of mainland Africa (Grubb et al., 2003) and it is rated as Critically Endangered (IUCN, 2010).

Hunting is the main threat to large-bodied mammals including PRC in KNP. Commercial logging has never taken place in KNP, even though farm bush surrounds all the villages that are found inside the park, the soil is poor in nutrients, and together with its designated conservation status, have largely protected KNP from widespread cultivation (Ministry of Forestry and Wildlife, 2008).

Data collection

Questionnaires were used to gather information from hunters concerning PRC threats in and out of KNP for a period of one month (January, 2014). All five villages inside the park (Erat, Bera, Esukutan, Ikenge and Bareka Batanga) were surveyed. Another five villages (Mokange, Esoki, Ngenye, Lobe Bat and Ekoneman Ojong) (Figure 1) were randomly selected from the 23 existing villages around the periphery of the park. One hundred and eighty-seven hunters responded to all the questionnaires administered to hunters in these ten villages. The following questions were asked: what are the different tools and methods used in hunting, other occupation of hunters, reasons for hunting PRC, and reasons for not eating PRC. For each village visited, the village chief and council was informed and the purpose of the study was presented prior to the interviews. All the hunters available in each village were gathered, informed about the purpose of the study, including the anonymous and confidential nature of their responses. A questionnaire was then given to each individual to complete, accompanied by photographs of the different species of primates present in and around KNP. An assistant from the team was present to interpret questions in their local dialect when necessary. Each village was visited at least twice for the whole period of data collection to make sure that all the hunters in the selected villages were properly/satisfactorily administered questionnaires.

Analysis

Data obtained from questionnaires administered to hunters (tools,

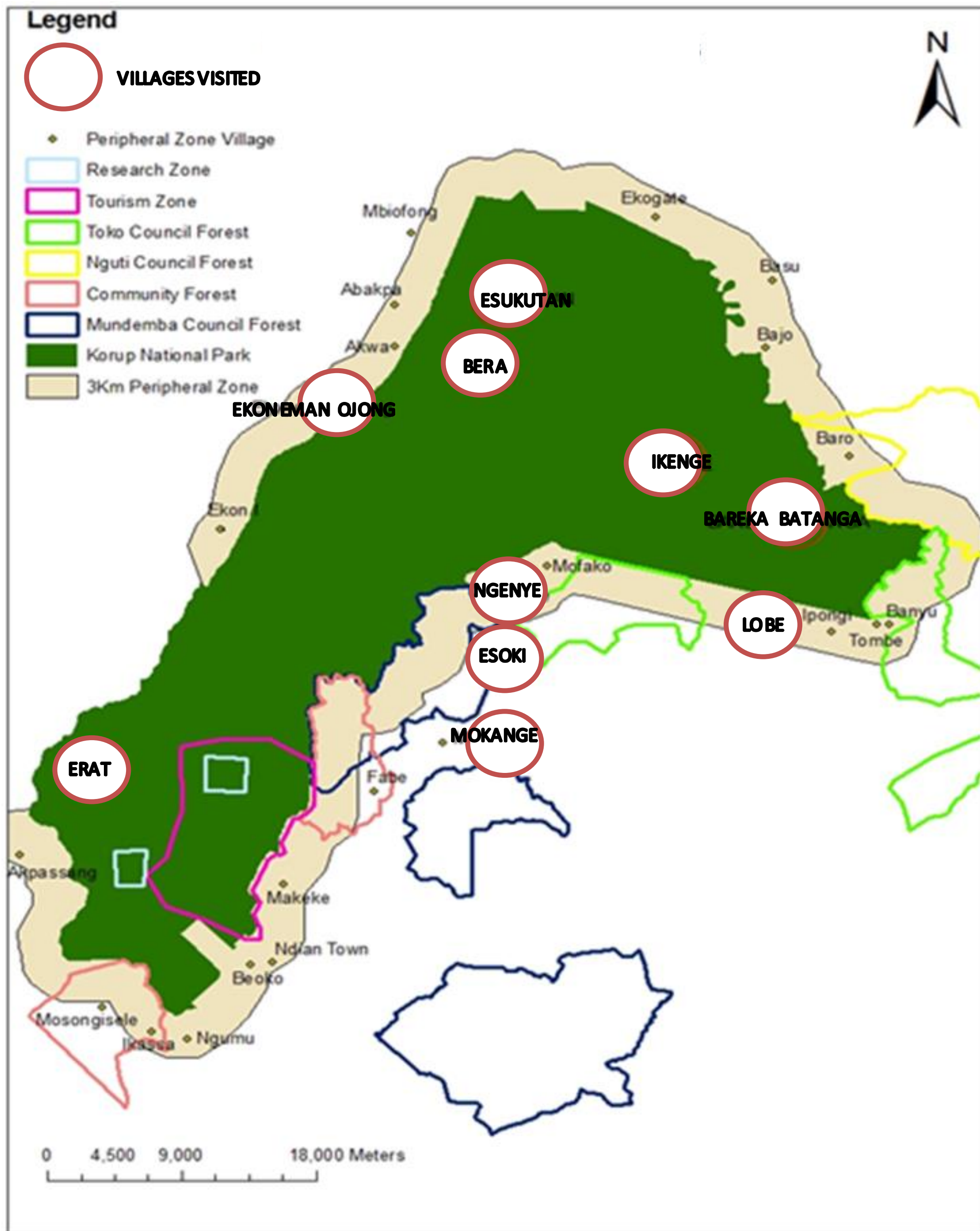

Figure 1. Map of Korup region showing villages of the study site where questionnaires were administered to the hunters (Koruo management 2007-2013).

methods, other occupations, hunting seasons, reasons for hunting and why hunters do not eat PRC) were analysed using Excel software. Analysis consisted of inserting all variables into an Excel worksheet. Each variable represented a title and all related titles were grouped in a new Excel table and calculations of relative proportions were done.

RESULTS

Tools and hunting methods

Several tools and methods are used in carrying out

Table 1. Reasons why some hunters do hunt PRC.

Reason for hunting Preuss's red colobus	Percentage of hunters
Hunt for food	24.72
Hunt to generate income	17.23
Hunt for food and income	9.76
Use as pet	10.3
For cultural rites	7.07
For medicinal purposes	2.26
For passion/ recreation	1.19
No response	27.39
Total	100

Table 2. Reasons why some hunters do not eat PRC.

Reason for not eating Preuss's red colobus	Percentage of hunters
Bad odour	45.40
Meat is tough	22.70
Sensitize by KRCS not to kill or eat primates	9.00
Parent don't like it because it is tasteless	4.50
Don't like it	4.50
Resemble human beings	4.50
Did not respond	9.00
Total	100.00

hunting in Korup National park such as shotguns used in hunting and wire snares use in trapping.

According to the 187 hunters that were administered questionnaires, 53.5% mentioned that they hunt solely with shotgun and 46.5% of them use both shotgun and wire snares in hunting.

Occupation of respondents

The people of Korup region practice mainly hunting, farming and fishing as sources of livelihood. The occupation of the respondents from the questionnaires shows that 46% of them practice hunting and farming, 32% practice solely hunting, 18% practice both hunting and farming and 4% practice hunting, farming and fishing. In addition to the three main occupations (hunting, farming and fishing), some of the respondents do carry out menial jobs such as small trades, porting, guiding, craftwork, making modern furniture, tailoring and teaching.

Reasons for hunting

Out of the 187 hunters administered questionnaires, 73.26% gave reasons as to why they hunt PRC. Table 1 shows the various reasons given by hunters as to why they hunt PRC. A majority (24.72%) of the hunters do hunt for food, followed by 17.23% of hunters who hunt to generate income. However, very few hunters (1.19 and 2.26%) hunt for passion/recreation or for medicinal purposes, respectively.

Reasons why hunters do not eat Preuss's red colobus

Even though majority (88.2%) of the hunters do eat PRC, however, few (11.6%) of them do not. As shown in Table 2, majority (45.4%) of the hunters in KNP do not eat Preuss's red colobus because of its bad and irritating odour, and 22.7% of hunters said the flesh of Preuss's red colobus is too hard, and takes longer time to get ready as compared to other primates. Moreover, 4.5% of the hunters do not eat it because it resembles humans.

Perception of local population of the Korup region on KNP

Some of the hunters (10.70%) said the local communities

in KNP depend more on forest activities for their survival and that they are proud of the KNP. It is their source of development and a natural gift from God. The animals (especially drill, PRC and Chimpanzees) in the park provide income that is used to educate children, build houses, pay for medication and even marry women with part. Much of the bush meat is sold in local markets, towns in Cameroon and in Nigeria. Most of the species taken to Nigeria are endangered (Drill, PRC and chimpanzees). Some locals feel happy shooting or killing animals in the forest but only one hunter said during the questionnaire session that he feels good killing PRC. In as much as this forest is lucrative to the people, it is also dangerous to them. Some men and women complained that these wild animals destroy their crops. A greater number of hunters (80%) do not see the benefit of conservation; the reason being that KNP is their source of living.

DISCUSSION

Hunting is an all year activity in Korup region. However, the intensity of this activity declines from December to March because it is the peak-farming season (Okon and Ekobo, 2007). During this season, hunters concentrate more on farming and only those who have no other occupation are involved fully in hunting. According to Lindsey et al. (2011), bush meat hunting is affected by the patterns in agricultural activity, which dictates household food availability and amount of time people have available for hunting. However, hunters in KNP carry out hunting year round even during peak farming season although there is a drop in the intensity during such period. Fishing activity in this area is done mostly for home consumption by men and women and less for sale. However, fishing in this area does not really have a significant effect on hunting because it is carried out only on a small scale for home consumption. Sometimes the rivers are polluted with Gammalin 20 that renders the rivers devoid of fish, thereby discouraging fishing activity in the area. Farming brings in additional income to hunters. Some of the produce like plantains, bananas, cassava, peppers, etc. are consumed at home and sold locally, while others like cocoa are entirely for export. Farming in this area can be a means to reduce hunting pressure due to the fact that these hunters sometimes concentrated on their farms during the peak period of farming. Therefore, if farming activity is encouraged by giving the hunters farm equipment, and large farmland out of the park and other necessities they need to improve their yields, then it will play an important role in conservation (Walter et al., 2002). Fishing could also help in reducing hunting pressure if strong roles and regulations are put in place to stop the use of Gammalin or any other poisonous substance used in fishing. Control should be done in the size of fishes caught and the sizes of nets used to catch these fishes (Dunn and Okon, 2003). Gun hunting is the only reliable method that can be used to hunt PRC and it is one of the common tools used in hunting in both day-time and night in KNP. According to Ntumwel (2012), gun hunting is common in KNP because cartridges are cheap and many hunters can afford them. A large part of hunters (74.47%) admitted that they do hunt PRC for food and income. They have ready market in villages around the park and in Nigeria that brings in income that they use to supply basic needs for their family. Also, this species serves as a source of protein to the local people. This is supported by Linder (2008) who attests that PRC accounts for a greater proportion (25%) of the total primate sales in Mundemba town. Hunting provides 30 to 80% of income and almost 100% of protein to the rural households in Central and West Africa (Fa et al., 2009). This shows that they will continue to hunt this species to sustain their family if they continue to rely on hunting without any other alternative source of income and protein. Therefore, they do not see anything wrong in killing this species as long as it will give them money and protein.

Some hunters accepted that they keep PRC as pets for the fact that they want their children to play with them and when matured they can be sold. They went further and explained that even if young PRC are kept as a pet, it is only for a short period of time. This is because it is difficult to feed them and most often, they die. None of them admitted to have ever sold a live young PRC. Study of Ntumwel (2012) and this study portray a low percentage (21.9 and 1.6%, respectively) of hunters using parts of PRC for medicinal purpose, and other purposes in KNP, which is to the advantage of PRC. The lesser the demand of parts of red colobus for medicinal purposes, the lesser the hunting of the species.

Bad odor, hardness of PRC meat and sensitization can serve as a means to discourage PRC hunting, even though very few hunters are aware that PRC is a Critically Endangered Species and are forbidden to hunt. Therefore, hunters need to be sensitized and create awareness of the danger of killing this species so as to reduce the rate of hunting. Very few people in KNP consider PRC as taboo and these few people believed that any pregnant woman who eats PRC will give birth to a child having the orange colour of PRC. PRC as a taboo is gradually dying out in Korup region as is confirmed by Ntumwel (2012) where less than 20% of the people believed that PRC is a taboo to the community. This species has no significant cultural value to the people in Korup. This is important for conservation because there will be less demand for PRC.

Conclusion

In Korup National Park, guns and traps are the tools used

in hunting, and gun is the major tool used in hunting PRC because of their arboreal nature. This implies that gun hunting in KNP has an impact on the survival of PRC.

The majority of the hunters do hunt PRC because it serves as a source of protein and provides income to sustain their families. With this, PRC are at risk of declining in population size or being exterminated in KNP if action is not taken.

Factors such as farming, fishing, bad odour, meat hardness of PRC and sensitization can serve as a means of reducing the rate of hunting in KNP, because these factors offer alternative means of sustenance, and/or make hunting of this species less attractive.

RECOMMENDATIONS

In order to properly conserve primate species in KNP, it would be important for the park management to identify primate biodiversity hot spots. The southern part of KNP would therefore be the most appropriate areas to focus primate conservation efforts.

People who are involved in bush meat trade should be identified. The trading of PRC meat should be a serious offense that will result in a higher fine. This fine should be made known and announced to all villages in Korup region. The public should also be informed that hunting or selling of PRC meat is considered as a high offense. For example, PRC poachers should receive higher fines or be denied bail.

Provision of an alternative activity for hunters such as using them during research work in the park will go a long way to discourage them from this increasingly large threat from hunting. This will help increase their skills as guides and alleviate their poverty stricken-situation.

PRC is one of the species of primates that is rare to see in protected areas in Cameroon. The conservation of this species will attract ecotourism and research work in KNP.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Impacts of waste on macroinvertebrate assemblages of Msimbazi River, Tanzania

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The status of waste and its impact on macroinvertebrate assemblages along Msimbazi River was assessed between May 2014 and August 2014. The aim was to identify the type of waste, their potential sources, and the assemblage of macroinvertebrate taxa that have been affected by waste disposal in the river. The study involved field study and laboratory analysis. Results indicated that organic and inorganic waste are the main types of waste dumped into the river; industries and communities living adjacent and along the river are the main waste producers. Macroinvertebrates belonging to eight orders and 27 families were recorded. Macroinvertebrates of the Gastropoda and Diptera orders were the most dominant in occurrence of all macroinvertebrate taxa, contributing 27 and 21%, respectively of the total macroinvertebrates. Based on macroinvertebrates sensitivity, 21 taxa (77.77%) were identified as being highly tolerant to pollution, six taxa (22.23%) as moderately tolerant and none of the most sensitive taxa. Macroinvertebrate diversity indices yielded a slightly lower Shannon-Weiner diversity index (H'=0.42) and the Shannon Evenness Index (0.2050) amongst sites. These findings are indicative of disturbed systems whose severity seems to be driven by the on-going waste disposal within and along the river continuum.

Key words: Benthic macrofauna, diversity indices, organic waste, inorganic waste, pollution, waste management.

INTRODUCTION

Large scale pollution of rivers has resulted in strict enforcement of waste disposal legislation in most industrialized countries, where wastewater is to be disposed of only after the quality meets certain criteria. This is in contrast to most developing countries where sewage goes untreated (Fakayode 2005; Bernhardt and Palmer, 2007; Kassenga and Mbuligwe, 2009; Mrutu et al., 2013). Rivers have a natural, though limited, capacity

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to restorewater quality to pre-pollution levels, through dilution, die-off, sedimentation and biological processes (Anyona et al., 2014). However, with the growth of cities, the amount of waste disposed into rivers often grows beyond their self-purifying ability (Fakayode, 2005; Kassenga and Mbuligwe, 2009). In these instances, untreated wastewater disposal poses serious risks to aquatic ecosystem health (Anyona et al., 2014; Elias et al., 2014).

Excessive loading of both industrial and domestic waste into rivers can alter the physical, chemical and biological characteristics of the aquatic system beyond their natural self-purification capacity. Higher levels of turbidity, nutrients, suspended and dissolved solids as well as coliform bacteria in rivers are all indicative of compromised systems attributed to increased pollutant load, resulting largely from anthropogenic activities (Adams and Papa, 2000). Such changes in water quality can alter the community structure of benthic macroinvertebrates and other aquatic biota therein (Boyle and Fraleigh, 2003).

Freshwater macroinvertebrate species are therefore at higher risk of extinction due to habitat degradation following overwhelming human activities (that is, invasive industrialization, agriculture, and urban development) near rivers (Jamil, 2001; L´evˆeque and Balian, 2005; Likens, 2010; Elias et al., 2014). It is unlikely that there is a substantial number of freshwater bodies remaining that have not been irreversibly altered from their original state as a result of anthropogenic activities (Elias et al., 2014). In Tanzania, for example, most of the industries are located in Dar es Salaam city and discharge their waste into Mzinga, Msimbazi, Yombo, and Kizinga rivers, which eventually flows into Indian Ocean (Ak'habuhaya and Lodenius, 1989; Kassenga and Mbuligwe, 2009; Mrutu et al., 2013; Elias et al., 2014). This, in turn, affects the occurrence, composition, and the distribution of freshwater macroinvertebrate species, depending on their levels of tolerance and adaptability (Suleiman and Abdullahi, 2011; Elias et al., 2014).

Tanzania is one of the richest countries in the world in terms of biodiversity and endemism (Lovett, 1998). In spite of its rich biodiversity, some areas, especially urban communities, are currently being threatened by pollution resulting from improper waste management. The Msimbazi River in Dar es Salaam is one such area that has been heavily polluted by domestic and industrial waste, thus negatively impacting the biodiversity and possibly resulting in the disappearance of certain plant and animal species (Mashauri and Mayo, 1989). Despite the ecological importance of Msimbazi River, few studies (Ak'habuhaya and Lodenius, 1989; Kassenga and Mbuligwe, 2009; Mwegoha et al., 2012; Mrutu et al., 2013) have been conducted on the ecological effects and current ecosystem health resulting from the continuous discharge of solid and liquid waste into the river.

The aim of this study was to identify the type of waste and provide information about the ecosystem health of the Msimbazi River using macroinvertebrates as ecological indicators. An overview of selected physico-chemical conditions present in the river is also provided. This paper presents the findings of investigation on the effects of waste on macroinvertebrate taxon diversity and composition along the Msimbazi River, Tanzania.

MATERIALS AND METHODS

Study site

The study was conducted in the Msimbazi River, Tanzania. The river originates in Kisarawe highlands in the Coast-Pwani region of Tanzania, to the south-west of Dar es Salaam city. It flows between latitudes 6°48' 51.3"S and 39°16' 43.5" E in a North-Easterly direction, and enters the Indian Ocean within the northern part of Dar es Salaam City (Figure 1).

The river has a wide flood plain (as wide as 1100 m) in some areas and covers a total area of approximately 260 km^2 (Hobbelen, 2001). The Msimbazi valley provides diverse ecological, socio-economic and agricultural functions to residents of Dar es Salaam, a city with an estimated population of approximately 4 million people. The Msimbazi valley is an important ecological area, largely due to its extensive wetlands (Rwenyagira, 1988). Six sampling sites were selected along the river. Site SS1 was located in the upper Msimbazi River before industrial waste and sewage discharge points, and therefore serves as a reference point. Sites SS2, SS3 and SS4 were at the points of industrial waste discharge (midstream) while sites SS5 and SS6 were along the sub-catchment (downstream) where wastes had already mixed thoroughly. A summary description of the sampling sites is provided in Table 1.

Macroinvertebrate assessment

Field surveys to collect macroinvertebrate data on the Msimbazi River took place from May 2014 to August 2014 at six established sampling sites, where 20 sampling stations at each site were sampled on a monthly basis. Six sites each were chosen along with the river continuum; three (SS2 – SS4) within the industrial area, to capture the contributions from the industries to water quality degradation, two sites (SS5 and SS6) were located along the sub-catchment where wastes had already mixed thoroughly, one site (SS1) was on the upstream reach for comparison purposes. A total of 120 samples were thus taken on a monthly basis, totaling 480 samples during the course of the study.

Macroinvertebrates were sampled in a 100 m stream reach where benthic and free floating invertebrates were collected using a standard hand net (0.3 m deep with 500 µm mesh size) attached to a metal frame (area 0.625 m^2) and handle (1.48 m long). The net was used to collect macroinvertebrates from the water column, mud, on stones and macrophytes. Each hand netting round took one minute. To avoid bias resulting from spatial variations or patchiness, three random samples were collected from each of the microhabitats by establishing a transect at each sampling reach, with five equally spaced points from which a sampling point was selected using random numbers. This procedure was replicated three times for each microhabitat, making nine samples per reach and samples were pooled to make one composite sample per habitat per station. Samples were emptied into a white tray where

Table 1. Description of the sampling stations along the Msimbazi River.

Sampling station	Site description
SS1	The most upstream site before industrial and sewage discharge points with habitat diversity (riffles, pools and runs) (reference site). There was overhead canopy along the stream banks.
SS2 - SS4	These points located in an industrial area; they were located in the waste discharge points (effluent streams) with limited habitat diversity (few pools). The river banks were devoid of forest cover.
SS5 - SS6	These sites were eroded and devoid of vegetation; they are situated along the sub catchment where waste had already mixed thoroughly. The stream bed was made of cobbles and boulders with some woody debris. The area was dominated with agricultural and domestic activities.

large objects were removed by forceps and the samples were filtered into plankton net.

Mesohabitats, which are distinct areas within a stream that provide habitat heterogeneity and increase invertebrate diversity, were identified as either stream banks where roots were present, vegetation or submerged objects; substrate areas of glides, riffles and pools; or accumulated organic material in glides and riffles. Riffles and pools were sampled separately to account for habitat variations. The sampled materials were emptied into specimen containers and preserved in 70% alcohol for laboratory analysis at the University of Dar es Salaam. Macroinvertebrate samples were sorted and identified to family level using general invertebrate textbooks and identification manuals (Gerber and Gabriel, 2002; Day et al., 2003).

Water quality assessment

Water quality parameters such as dissolved oxygen and conductivity were measured *in situ* using a YSI 556 MPS Handheld Multi Parameter Instrument (YSI Incorporation, Yellow Spring, USA) and pH using a hand held pH/mV meter (Model: SX 711). Additional water samples for total suspended solids analysis were collected in 1000 ml bottles and refrigerated at low temperatures (< 4°C) for 24 h prior to laboratory analysis. Total suspended solids (TSS) were analyzed from water samples following methods outlined in APHA (1998) by filtering 50 ml of the sample through a pre-weighed standard glass-fiber filter into a dry and pre-weighed evaporating dish.

The residue retained on the filter paper was dried to a constant weight while the filtrate in the evaporating dish was evaporated in an oven at 103 to 105°C for 1 h. These were cooled in a desiccator and weighed. The cycle of weighing was repeated until a constant weight was obtained, after which TSS was calculated and presented in milligram per litre. All the analyses were conducted at the Department of Aquatic Sciences and Fisheries Laboratory at the University of Dar es Salaam.

Identification of type of waste and their potential sources

Data on the type of waste was obtained from both primary and secondary sources. Primary data was obtained using a structured questionnaire that served as the main source of information. Questionnaires were given to households to obtain information on the type of waste and their potential sources (Table 2). In addition, formal interviews were conducted with residents living along and adjacent to the river. The household samples for the study were drawn using systematic sampling whereby a house was selected randomly then after in every two households a sample for the study was selected. The households were picked in relation to where water samples were taken at the distance of 500 m from both sides of the sub-catchment. Various stakeholders including Dar Es Salaam Water Supply Company (DAWASCO) were also interviewed. A total of two hundred and thirty (n=230) respondents were interviewed using questionnaires. The sampling units were obtained from four wards, located in two municipalities where the river crosses. The wards were Tabata in Ilala Municipal, and Kigogo, Mzimuni, and Magomeni in Kinondoni Municipality. Variables collected in the field included types of waste and their sources and residents' awareness of proper sewage disposal mechanisms and interest in environmental conservation.

Data analysis

Descriptive statistics presented as means and their standard deviations were used to summarize the data characteristics. Students't-test was used to establish differences among variables between sites. The Shannon–Wiener diversity index (H'), as described by Magurran (1988), was used to assess diversity as follows:

$$H' = \Sigma \left[(n/N) \times \ln (n/N)\right]$$

Where n = number of individuals of a taxon and N = total number of individuals in the sample. An associated evenness $J' = H'/H'max$ (Pielou, 1975) was also calculated, where $H'max$ is the maximum possible value of H'. The collected data from questionnaires were verified, coded, and later analyzed using Statistical Package for Social Science (SPSS).

RESULTS

Macroinvertebrate taxa diversity and abundance

Macroinvertebrates belonging to eight orders and 27 families were encountered in the samples collected from six sampling sites. Macroinvertebrates of the Gastropoda and Diptera orders were the most dominant of all

Table 2. Questionnaires provided to households along the Msimbazi River.

Section I. General information of respondents		
Respondent information		
Name of district Name of ward Name of street	**Response**	**Code**
Age	18-25	1
	25-33	2
	33-40	3
	40-47	4
	47-54	5
	Above 54	6
Gender	Male	1
	Female	2
Education	Non	1
	Primary	2
	Secondary	3
	College/University	4
Section II. Questions		
Questions	Answer	Code
For how long you have been living in this area?	Less than a year	1
	Three years	2
	Five years	3
	More than five years	4
What do you do to earn your living?	Unemployed	1
	Employed	2
	Self-employed	3
	Small businesses	4
	Small medium enterprises	5
	Urban agriculture	6
	House wife/husband	7
If you are self-employed what livelihood activities do you practice?	Urban Agriculture	1
	Livestock keeping	2
Do you use water from the river?	Never	1
	Sometimes	2
	Always	3
	Hardly	4
If yes; What do you use for?	Drinking	1
	Cooking	2
	Cleanness (washing and mopping)	3

Table 2. Contd.

	Irrigation (gardens)	4
	Construction	5
	Recreational (swimming)	6
	Others	7
For the time you have lived here have you observed any changes in the cleanness/ quality of water in the river?	Yes	1
	No	2
If yes; what do you think is the cause of those changes?	Industrial waste discharge	1
	Domestic wastes	2
	Population increase/ change	3
	Sewerages	4
If the answer is industrial pollutants; does it affect macroinvertebrates community structure?	Yes	1
	No	2
If the answer is Yes: How does it affect macroinvertebrates?	Abundance	1
	Diversity	2
	Others	3
Which macroinvertebrate species do you think are more affected?	Annelida	1
	Ephemeroptera	2
	Hemiptera	3
	Diptera	4
	Coleoptera	5
	Tricoptera	6
	Gastropoda	7
	Odonata	8
	Others	9
What can be done to control industrial/domestic waste discharge into water systems?		
What can be done to reduce the effects of industrial/domestic waste discharge?		

macroinvertebrate taxa, contributing 27 and 21%, respectively of the total macroinvertebrates abundance (Table 3). However, Chironomidae accounted for 79% of the dipteran abundance along the river continuum.

In addition, Oligochaetes and Hirudinae (considered being pollution tolerant taxa) in the phylum Annelida were collected at all sampled sites. There were significant differences in abundance between sites ($p < 0.001$). When the 27 macroinvertebrate taxa were further divided into three arbitrary groups based on their sensitivity or pollution tolerance using Gerber and Gabriel (2002), 21 taxa (77.77%) were identified as being highly tolerant to pollution (those taxa known to occur in severely polluted or disturbed streams) and six taxa (22.22%) as moderately tolerant (those taxa that are found in streams with intermediate degrees of pollution or disturbance) (Table 4). Macroinvertebrate diversity indices yielded a slightly lower (0.42) Shannon-Weiner diversity index (H') between sites. Likewise, the Shannon Evenness Index was also low (0.2050) (Table 5) and still fell far below the ideal metric value of 1 which is indicative of similar proportions of all taxa in the system.

Physico-chemical water quality parameters

Table 6 presents the mean and standard deviations of various physico-chemical parameters between the sampled sites in the Msimbazi River. Dissolved oxygen (DO) levels ranged between 0.01 and 9.20 mg/L. There

Table 3. Macroinvertebrates from all sampling sites in the Msimbazi River (The numbers refers to occurrence of the taxonomic group).

Taxonomic group	Family/class	SS1	SS2	SS3	SS4	SS5	SS6	Total	% contribution (Family/class)	% contribution (Taxa)
Annelida	Hirudinae	20	17	14	10	5	2	68	7.3	13
	Oligochaeta	12	14	10	8	4	5	53	5.7	
Ephemeroptera	Caenidae	0	0	0	0	2	2	4	0.4	3.5
	Baetidae	8	20	0	0	0	0	28	3.0	
Hemiptera	Veliidae	0	0	0	0	1	3	4	0.4	7.1
	Pleidae	0	0	0	0	1	1	2	0.2	
	Gerridae	1	0	0	0	0	2	3	0.3	
	Corixidae	3	2	0	0	0	1	6	0.6	
	Belostomatidae	10	0	0	8	9	1	28	3.0	
	Naucoridae	0	0	3	0	0	12	15	1.6	
	Nepidae	0	0	0	5	0	2	7	0.8	
	Notonectidae	0	0	0	0	1	0	1	0.1	
Coleoptera	Dytiscidae	0	4	7	0	0	7	18	1.9	6.5
	Elmidae	9	0	0	0	8	9	26	2.8	
	Gyrinidae	0	0	0	0	12	5	17	1.8	
Trichoptera	Hydropsychidae	0	0	15	12	5	8	40	4.3	5.6
	Ecnomidae	0	0	8	0	0	4	12	1.3	
Odonata	Corduliidae	0	0	0	0	2	0	2	0.2	15.9
	Libellulidae	0	5	3	0	0	7	15	1.6	
	Coenagrionidae	30	15	28	8	17	0	98	11.0	
	Aeshnidae	0	3	4	4	6	12	29	3.1	
Gastropoda	Planorbinae	28	20	18	19	17	0	102	11.0	27
	Physidae	22	17	10	12	8	20	89	9.6	
	Lymnaeidae	8	19	13	9	0	11	60	6.4	
Diptera	Chironomidae	40	30	38	28	22	0	158	17	21.4
	Ceratopogonidae	0	0	0	3	1	25	29	3.1	

Table 3. Contd.

	Simuliidae	3	9	0	0	0	0	12	1.3	
Total occurrence		194	155	171	126	141	139	926	100	100

*SS- Sampling station

Were significant differences in DO levels between sites (Student's t-test, P<0.05). Electrical conductivity values ranged between 2350 and 11943 µS/cm. Conductivity levels varied significantly between sites (Student's t-test, P<0.05). pH ranged from 8.02 to 11.50, with no significant difference in pH levels among sites (Student's t-test, P>0.05). There were no significant differences in TSS levels between sites (Student's t-test, P = 0.289).

TSS concentrations in the range of 25 to 100 mg/L is considered to represent moderate water quality, with an average concentration of 25 mg/L suggested as an indicator of unimpaired stream water quality (Nevers and Whitman, 2004). The concentrations of TSS recorded at the sampling sites are considered higher when assessed against the Tanzania Municipality and Industrial Effluents standards, and Tanzania Water Quality Standards (Table 6).

Waste disposal regimes in place near the Msimbazi River

Most respondents (67%) disposed their domestic waste directly into the river, while only 33% claimed that they have other forms of waste disposal mechanisms which are not connected to the river. It was identified from the field survey that, several factors influence waste disposal into the river; approximately 20% of respondents noted that there were no disposal grounds available for their use, while approximately 15% of respondents observed that there are disposal sites, however these were inaccessible to them.

Source of waste to Msimbazi River

Based on the household survey, it was noted that most of the waste disposed into the river is from domestic sources, although waste from sanitation facilities and industries were also recorded.

DISCUSSION

The Msimbazi catchment is characterized by a variety of land uses, including agriculture for green vegetables and many manufacturing and processing companies such as breweries, food processing plants, textile industries, soap and detergent producers, footwear and rubber product industries, paper manufacturers, sand mining for construction, waste stabilization ponds and so on (M. Shimba and I. Thomas, Personal Observations). Observations made at sampling points showed that all sites were impacted, though to varying degrees. Anthropogenic activities contribute to water quality deterioration, impacting macroinvertebrate assemblages (Pallela, 2000). It was established that the most common solid waste disposal method among residents along Msimbazi River was open dumping, owing to a general lack of clearly demarcated waste disposal sites. Dumping of garbage along the river banks and in storm drains may have facilitated their transportation and subsequent deposition in the Msimbazi River, resulting in water quality degradation.

According to Inanc et al. (1998), Martin et al. (1998) and Mwenda (2014), surface water contamination within urbanized areas is attributed to solid and liquid waste resulting from anthropogenic activities and deposited either along the banks, in storm drains or directly into the river channel. Kassenga and Mbuligwe (2004) and Mwenda (2014), also identified uncontrolled disposal of solid waste and excreta from informal settlements along the river continuum, inappropriately sited disposal pits, blockages and/or breakages of sewer lines at major junctions, increased human activities and urban surface runoff as among the sources of pollution into the Msimbazi River. This is in agreement with the present study where highest proportions of solid waste were recorded at the upper part of Kigogo ward along Msimbazi River that was characterized by elevated anthropogenic activities. Emere and Narisu (2007) further reported that, sections of the river flowing through urbanized areas and informal settlements are highly prone to pollution owing to their proximity to numerous pollutant sources such as waste water discharge points, solid waste disposal sites, raw sewage spills, and urban runoffs.

The type of solid waste encountered along the banks of the Msimbazi River was typical of

Table 4. Taxa of some common Benthic Macroinvertebrates recorded while sampling during the study, categorized in general groupings of pollution sensitivity (Adapted from Gerber and Gabriel, 2002).

Taxonomic order	Family/class	Highly tolerant to pollution	Moderately tolerant to pollution	Very low tolerance to pollution
Annelida	Hirudinae	√		
	Oligochaeta	√		
Ephemeroptera	Baetidae	√		
	Caenidae		√	
Odonata	Aeshnidae		√	
	Coenagrionidae	√		
	Libellulidae	√		
	Corduliidae		√	
Coleoptera	Dytiscidae	√		
	Gyrinidae	√		
	Elmidae		√	
Hemiptera	Notonectidae	√		
	Pleidae	√		
	Nepidae	√		
	Belostomatidae	√		
	Gerridae	√		
	Veliidae	√		
	Corixidae		√	
	Naucoridae	√		
Diptera	Chironomidae	√		
	Simuliidae	√		
	Ceratopogonidae	√		
Gastropoda	Physidae	√		
	Lymnaeidae			
	Planorbidae	√		
Tricoptera	Hydropsychidae	√		
	Ecnomidae		√	
Total		21	6	0
Taxa % of the total		77.7	22.22	0

the ongoing activities in the immediate vicinity. Common sanitation facilities used in these areas are pit-latrines, which are constructed close to the river in such a way that all the contents are directly disposed of into the river. Due to the fact that the water table is high, the pits are not sunken too deep and large parts are above the ground.

The results of the present study conform to some previous studies on pollution of Msimbazi River and their major sources. For example, Pallela (2000) and Mwenda (2014) cited untreated domestic wastes, pit-latrine effluent, and industrial effluent as the major source of pollution in the Msimbazi River. Several industries were identified as potential point sources of sewage including the breweries, food processing and textile industries (I. Thomas, Personal Observation). Although Lakhani Textile Industries has its sewage directed to Vingunguti oxidation ponds, there has been allegations that it is the

Table 5. Diversity of macroinvertebrates along Msimbazi River.

Parameter	Status
Number of occurrence	926
Taxa richness (d)	27
Shannon-Weiner Diversity Index (H')	0.42
Shannon Evenness Index (E)	0.2050

main polluter of the river in recent times. This is due to the fact that the industries lack wastewater treatment facilities and perhaps the government is not much concerned with the issue of pollution control in this river ecosystem. The situation is worse, since the effluent from Vingunguti oxidation ponds is also discharged to the river on its way into the Indian Ocean. The Vingunguti oxidation ponds serve all industries along Nyerere road housing estate and most likely influences sites SS5 and SS6.

Changes in the water quality of streams can reflect increased nutrient loads associated with sewage discharge and probably, to a lesser degree and agricultural runoff (Gaufin and Tarzwell, 1956; Gaufin, 1958; Olive and Dambach, 1973). In this study, the observed decreases in dissolved oxygen levels from SS2 to SS6 indicated a potential increased organic load in the stream.

Macroinvertebrates comprising of 27 taxa were encountered along with the river continuum. The 27 taxa recorded were however relatively low compared to those that have been reported for other rivers in other parts of Tanzania, for example 48 taxa in Karanga River (Elias et al., 2014) and 96 taxa in Pangani River (Kaaya et al., 2015). The low taxa diversity and the dominance by few taxa (particularly pollution tolerant taxa) could have been due to poor water quality resulting from changes in physico-chemical parameters as well as nutrient levels contributed by increased solid waste and sewage disposal into the river channel. The dominance of pollution tolerant taxa i.e. (Chironomidae, Planorbidae and Coenagrionidae) over the sensitive and non-tolerant taxa could be a pointer to poor water quality along the river continuum.

According to Allan (1995), Cortes et al. (2002) and Hamada et al. (2002), distribution and assemblages of benthic macroinvertebrates may be attributed to small scale variability in a variety of water quality parameters. However, Sanseverino and Nessimian (1998), Nessimian et al. (2003), and Chatzinikolaou et al. (2006) established that pollution and excessive nutrient enrichment from anthropogenic sources, in particular sewage and solid waste, can affect benthic macroinvertebrates' trophic relationships as well as their habitats and thus change their community structure and composition. Adakole and Annune (2003) reported that changes in physico-chemical parameters can disrupt life and reproductive cycles of aquatic fauna, impact on food chain and migration patterns or worse still impose physiological stress on even the tolerant macroinvertebrates. According to Mhatre and Panthurst (1997), Bonada et al. (2006), Choudhary (2012) and Sharma et al. (2012), the continuous exposure of benthic macroinvertebrates communities to pollution changes their species composition in response to the magnitude, duration and frequency of the pollutants.

The physico-chemical conditions, taxa diversity and the number of taxa encountered during this study might be linked to degradation of Msimbazi River largely as a result of the discharge of untreated waste into various sections of the river. Chironomidae presence at sites SS2 to SS5 may be due to its ability to survive in a wide range of pH spectrum and anoxic conditions which were prevalent there (Choudhary, 2012; Sharma et al., 2012). The sewage disposal at this point could have contributed to deterioration of its waters especially because, the area was not protected and wastewater could therefore contaminate the water. Studies also show that Chironomidae are common inhabitants of sewage polluted waters that are often rich in nutrients and poor in oxygen (Chakraborty and Das, 2006). Even though Chironomidae comprised a large portion of the macroinvertebrate taxa at all sites except at site SS6, it is likely that the species present were different, particularly between sites SS1 to SS4. Winner et al. (1980) recorded similar differences. If the same Chironomidae member occurred at all sites, their abundance in one site should have occurred in synchrony with the others.

Effects of the decline in water quality downstream of the Msimbazi River were also evident in other macroinvertebrate taxa (Hirudinae, Oligochaeta, Planorbinae and Physidae), which exhibited reduced taxa numbers. Differences in macroinveretebrate taxa between sites SS1 to SS4, SS5 and SS6 were probably related to water chemistry, since there was a gradual change in physico-chemical water characteristics to further down the catchment where sites are located. Studies have shown that in streams similar to the Msimbazi River, constant or decreasing numbers of macroinvertebrate taxa are observed along the gradient from upstream to downstream (Elias et al., 2014; Kaaya et al., 2015). The loss of taxa owing to man-related impacts on the stream environment was confirmed in the present study.

Taxa diversity has often been related to the stability of the environment (Kaaya et al., 2015). In this study, the macroinvertebrate communities were subjected to environmental stress (e.g. changes in water quality), which was less predictable and more severe at sites SS4, SS5 and SS6 than the other sites. The dominance of a few macroinvertebrate taxa resulted in variation in

Table 6. Variation in the selected water quality parameters (Mean±SD) along Msimbazi River.

Parameter (Unit)	SS1	SS2	SS3	SS4	SS5	SS6
pH	8.50±0.70	8.60±0.50	10.40±0.14	11.50±0.12	9.10±0.01	8.02±0.11
DO (mg/L)	9.20±0.70	0.90±0.22	1.70±0.11	0.10±0.17	0.01±0.08	3.00±0.18
TSS (mg/L)	328.7±2.0	434.5±3.9	738.0±2.10	1727.50±5.3	2240.0±4.5	942.30±3.5
Conductivity (μS/cm)	2350±20.1	6870±2.12	3080±2.5	11943±5.8	3500±7.55	2509±4.3

+SS-Sampling station, ●Permissible TSS (mg/L) by Tanzania Water Quality Standards [TZS 789:2008]: Released in May 2009 (Upper limit values) = 100, ●Permissible TSS (mg/L) by Tanzania Limits for Municipal and Industrial Effluents stipulated in Environmental Management (Water Quality Standards) Regulations, 2007 = 100.

macroinvertebrate abundance at sites SS4 to SS6 Site SS2 recorded a higher abundance of Baetidae as compared to Site SS1. It is impossible to determine whether this increase resulted from successful reproduction at the site or from drift originating upstream. The latter explanation seems most probable, as macroinvertebrates from Baetidae were absent in sites SS3 to SS6. The other observed differences among the macroinvertebrate communities may have also been related to changes in water quality.

Continued accumulation of dissolved and suspended solids in aquatic systems reduces water transparency, effectively impacting on primary productivity, thus affecting benthic macroinvertebrates (Emere and Narisu, 2007). It can also clog gills of aquatic fauna (Edokpayi and Nkwoji, 2007) and destroy critical habitats by settling at the bottom of the water body, forming a blanket over critical spawning and breeding ground, thus interfere with the life cycle of aquatic organisms including macroinvertebrates. Increases in total suspended solids from SS1 to SS5 appeared to have detrimental effect on the macroinvertebrates. The overall low diversity of macroinvertebrates (H' = 0.42) reflected that, taxa composition was very different between sites; a fact that was observed by the dominance and abundance of pollution tolerant taxa (Chironomidae, Planorbinae and Coenagrionidae) over other taxa. In the present study, macroinvertebrates were not uniformly distributed along the river continuum, neither did they exhibit consistent increase or decrease downstream, but instead, varied between sites along the river continuum. This was partly caused by changes in water quality along the river continuum.

Conclusion

The Msimbazi River is partly impacted by waste disposal from nearby and fringing industries. A change in physico-chemical water quality parameters, driven mainly by these practices along the river continuum influence macroinvertebrates. Our results suggest that some of the observed taxa are highly tolerant to pollution while others are moderately tolerant to pollution. These indicator taxa can be used in water-quality evaluation and in monitoring the recovery of streams in the study area.

Msimbazi River with habitat diversity has experienced significant impact as a result of the influx of sewage and chemicals and organic pollution disposed off in it, of which it contributed to the change in macroinvertebrates assemblages. Maintenance of ecological integrity by controlling anthropogenic activities, protection of the river channel and its basin and increased public education and awareness with regard to environmental integrity is therefore recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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