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Survey and identification of termites (Insecta, Isoptera) using morphological and molecular methods from eastern, central and western Ethiopia.

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ABSTRACT: The subfamily Macrotermitinae are the largest members among the Family Termitidae which are the fungus growing sub-family and *Odontotermes* are the most abundant genus from the subfamily. The taxonomy of termites is poorly described in Ethiopia. In the present study 168 termite samples were collected from eight locations of Eastern, Western and Central Ethiopia. The collected samples were identified based on morphological and molecular characteristics. Molecular identification was done based on the DNA sequence of a portion of the mitochondrial 16S rRNA gene. A phylogenetic analysis of the collected samples and the outgroup resulted in a consensus tree with four distinct groups. Geographical distribution of the samples also supported the resulting clades. *Odontotermes* were the most widely distributed termites from the collected samples. The genetic distance between the sample showed that *Odontotermes zambesiensis*, Babile 33 is more distantly related with the rest of the samples.

Key words /phrases: Ethiopia, *Macrotermes*, *Microtermes*, *Odontotermes*, Termitidae, 16s rRNA

INTRODUCTION

Termites, order Isoptera, inhabit a wide range of ecosystems and can be found on all continents except Antarctica. They play an important role as one of the only animals that can break down lignocellulose through their symbiotic relationships with flagellates and fungi. Their tendency to eat plant materials, fresh or decayed, has marked them as a nuisance for homeowners and farmers with 10% of described species being labeled as pests (Wood, 1996).

Termite damage is first reported in Ethiopia in the Kiltu Kara District, in Western Oromia Region of Ethiopia (Abdullahi and Haile, 1986; Sanna, 1973). Although damage to crops has been significant, few studies have been conducted to analyze the diversity and distribution of termites endemic to Ethiopia. There is pressure for proper identification and control of termites, the former practice of mound poisoning is discouraged as the chemical compounds used can be quite harmful to non-target organisms and current management by physical destruction of the nests is ineffective at

reducing termite infestations (Fenetahun *et al.*, 2019; Logan *et al.*, 1990; Weise *et al.*, 1973). The type of damage can vary based on the type of termites causing the damage.

In Ethiopia Macrotermitinae termites are greatest threat to agriculture (Cowie *et al.*, 1990). These termites are a subfamily of the Termitidae family, which are sometimes called "higher" termites based on their phylogenetic relationship to other Isoptera families. This subfamily cultivates fungi to facilitate the breakdown of lignocellulose and is comprised of 12 genera with 373 living species and two fossil species (Krishna *et al.*, 2013). Of these genera, *Acanthotermes* Jacobson (1905), *Pseudacanthotermes* Sjöstedt (1924a), *Synacanthotermes* Holmgren (1910), *Allodontotermes* Silvestri (1912), and *Protermes* Holmgren (1910), are unique to the Ethiopian (Afrotropical) region while *Macrotermes* Holmgren (1909), *Odontotermes* Holmgren (1910), *Microtermes* Wasmann (1902), and *Ancistrotermes* Silvestri (1912) are found in both Ethiopian and Oriental regions (Krishna *et al.*, 2013).

The genera *Odontotermes* comprises the majority of the Macrotermitinae subfamily with close to 200

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species (Krishna *et al.*, 2013). *Macrotermes*, *Odontotermes*, and *Pseudocanthotermes* tend to grow large nests that can run 50m long just below the soil's surface, this type of nesting can lead to ring barking and root rot (Wood, 1991). In contrast, *Microtermes* and *Ancistrotermes* create smaller more diffuse nests that are deep underground and tunnel upward to the surface; these termites can tunnel through and up the roots weakening the plants' ability to uptake nutrients. In addition, the type of crop can impact the type of damage caused (Wood, 1991).

Termite infested rangelands can lead to overgrazing of grass that can cause soil erosion and there is an increase in reported diseases in livestock that graze in termite infested rangelands (Cowie and Wood, 1989; Fenetahun *et al.*, 2019; Wood, 1996). The practice of growing exotic trees, such as Eucalyptus, in previously native woodland areas in Ethiopia can also lead to accelerated soil erosion and denudation as these trees are more

susceptible to termite damage (Cowie and Wood, 1989; Fenetahun *et al.*, 2019; Wood, 1991).

The aim of this study is to survey species diversity and distribution of termites in selected agro-ecologies of Ethiopia. Highland, mid-highland, and lowland regions across Western, Central, and Eastern Ethiopia.

MATERIALS AND METHODS

Study areas

The study was conducted in areas rich in termite diversity (Cowie *et al.*, 1990) located in Eastern, Central and Western Ethiopia. Survey was conducted during the 2019/20 cropping season. The survey sites were selected based on representation of the different agro-ecologies viz., highlands (above 2000 m.a.s.l), mid-highlands (1500-2000 m.a.s.l) and lowlands (below 1500 m.a.s.l) as shown in Table 1.

Table 1. Sampling sites in Oromia state, Ethiopia.

Code	Locality name	Latitude	Longitude	Altitude (m)	Habitat
2	Bako	9°05'57"N	37°06'00"E	1634	Grass
4	Bako	9°05'48"N	37°06'03"E	1629	Maize
5	Bako	9°05'30"N	37°06'14"E	1623	Maize
6	Bako	9°06'12"N	37°05'46"E	1704	Grazing land
8	Bako	9°05'47"N	37°06'60"E	2171	Grazing land
10	Ambo	8°58'24"N	37°57'04"E	2386	Grazing land
11	Ambo	8°58'35"N	37°57'55"E	2432	Grazing land
12	Ambo	8°58'35"N	37°58'08"E	2432	Grazing land
13	Holeta	9°03'41"N	38°30'31"E	2340	Forest
14	Holeta	9°03'41"N	38°30'33"E	2329	Forest
15	Holeta	9°03'38"N	38°30'38"E	2397	Forest
16	Holeta	9°03'38"N	38°30'40"E	2472	Forest
17	Bishoftu	8°38'17"N	39°04'52"E	1799	Acacia
24	Metehara	8°54'25"N	39°57'51"E	1262	Arid
27	Metehara	8°54'26"N	39°59'06"E	1105	Arid
28	Asebot	9°23'52"N	41°00'15"E	1626	Arid
30	Haramaya	9°24'53"N	42°01'58"E	1930	Mango
33	Babile	9°13'12"N	42°19'25"E	1328	Groundnut field

From each survey region, two to three districts were selected for sampling based on their accessibility and proximity to roads. From each site, 10 soldiers and 10 worker termites were randomly sampled for identification. Samples were collected by opening termite nests, splitting wood to expose specimens and excavating the soil to a depth of 15-25cm using a shovel.

Microsites surveyed include: surface soil; deep accumulations of leaf litter; decayed and dead wood; and termite nests including subterranean,

epigeal, arboreal and mounds. Field samples were collected from unhealthy looking groundnut crop, other cultivated plants, and natural habitats. Samples were collected in the morning and late evening. Termites were extracted from the sample soil by hand-sorting using a moistened camel's-hair brush and preserved in 97% alcohol.

The collection localities were geo-referenced (latitude, longitude and altitude) using GPS. Voucher specimens were preserved in 85% ethanol and deposited to the Arizona State University

Hasbrouck Insect Collection under accession numbers ASUHC0095057 - ASUHC0095074.

Morphological identification

Soldiers were used for identification to species level using morphological characters. In this study, head color, size, and shape, number of antennae segments, mandible shape, pronotum shape, and gula shape were considered as morphological characters in the identification keys. Species determination was made based on previous descriptions of morphological identification using taxonomic key by Krishna et al (2013) for Macrotermitinae; key described by Bagine (1986) was used to characterize the genus *Odontotermes*; morphological characters described by Rambur (1842) and Grassé (1937) were used for *Macrotermes*; and descriptions used by Chhotani (1997) was used for *Microtermes*. Samples containing only workers were subjected to molecular identification. Coordinates of each collection location were plotted on the map using QGIS 3.10 software.

Molecular characterization

Molecular characterization of the termites was done using 16S rRNA gene sequences. Termite samples were preserved in 97% ethanol for DNA extraction and molecular characterization. DNA was extracted from termite abdomens with the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol. The mitochondrial 16S rRNA gene was amplified by PCR using Econotaq Plus Green PCR Master Mix (Lucigen) and primers LR-N 5'-CGC CTG TTT ATC AAA AAC AT-3' and LR-J 5'-TTA CGC TGT TAT CCC TAA-3' (Kambhampati and Smith, 1995; Simon *et al.*, 1994), under cycle conditions of 3 min at 95 °C followed by 30 cycles of 95 °C for 30s, 46 °C for 30s, and 72 °C for 60s. PCR products were purified using NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel), and sequenced directly on both strands on an Applied Biosystems 3730 capillary sequencer. Termite mt16S sequences generated in this study were submitted to GenBank under accession numbers MW324511-MW324528.

Phylogenetic analysis

New and previously published sequences of Macrotermitinae 16S rRNA were assembled using MEGA 10.1.8 (Kumar *et al.*, 2018) software package and aligned with Muscle using default settings. The sequence alignment had a total of 50 nucleotide sequences in the final dataset. The BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) was used to identify similarities between the sequences obtained in this work and previously published data. *Sphaerotermes sphaerothorax* Sjöstedt (1911a) (Isoptera: Termitidae) (Gen Bank KP026279) and *Sphaerotermes* sp. (Isoptera: Termitidae) (GenBank KY238292.1) were used as the outgroup taxa.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kumar *et al.*, 2018). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA 10.1.8 (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

Morphological identification

In the present study 168 samples were collected where 44 samples had soldiers, 98 samples had workers and 26 samples had both soldiers and workers. Among the collected samples, seven species of termites were identified representing three genera of the family Termitidae viz., *Odontotermes*, *Macrotermes* and *Microtermes* (Figure 1). Among them the genus *Odontotermes* is represented by four species viz., *Odontotermes montanus*, *Odontotermes stercorivorus*, *Odontotermes tanganicus* and *Odontotermes zambesiensis*; the genus *Macrotermes* was represented by two species *Macrotermes jeanneli* and *Macrotermes subhyalinus*; and the genus *Microtermes* was represented by a single species *Microtermes obesi*. Based on morphological characters of the soldier caste, keys were prepared for easy and accurate identification of all the collected termite samples.

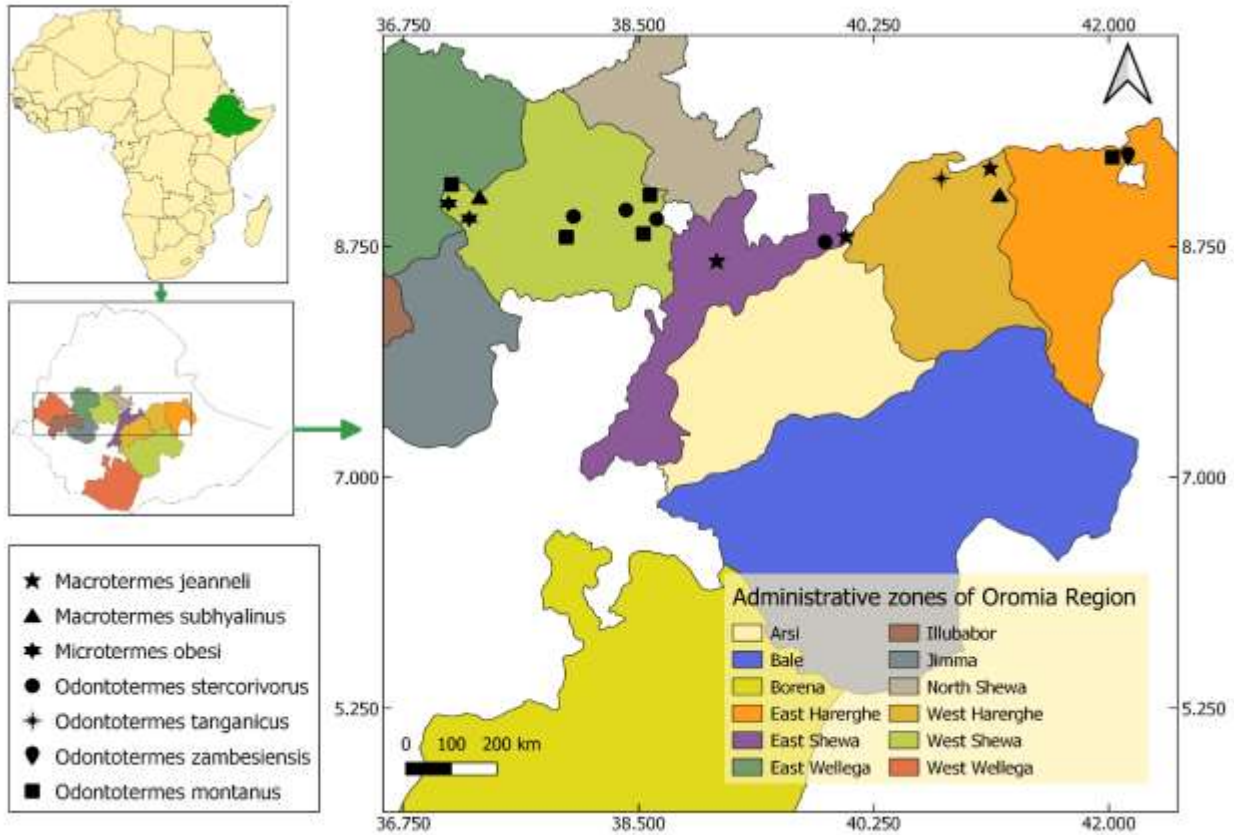


Figure 1 Map of Ethiopia showing geographical distribution of Termitidae: *Microtermes*, *Macrotermes*, and *Odontotermes* included in the study. Dots represents collection sites.

Head yellowish to brownish yellow with brownish tinge; Antennae with 13-14 segments; in specimens with 14 articles, second longer than third; postmentum with scattered bristles and short hairs; pronotum slightly less hairy than head. Head elongate oval, longer than broad, widest a little below middle. Mandibles thin, delicate and weakly incurved apically. Pronotum saddle shaped, longer than half its width; anterior margin distinctly notched in middle; posterior margin very weakly depressed in middle; lateral lobes prominent, broadly rounded (Ahmad, 1965; Chhotani, 1997).

Molecular identifications

A phylogenetic analysis of Termitidae from eight locations, 18 nucleotide sequences and two Rhinotermitidae species (outgroups) having 447 base pair long fragment based on the DNA sequence of a portion of the mitochondrial 16S rRNA gene were performed (

Figure 2). Collections were made from West, Central and East Ethiopia, most of the inferred

relationships had strong quantitative support as indicated by bootstrap analysis. The highly conserved 16S rRNA gene separated the collected samples into three genera *Microtermes*, *Macrotermes*, and *Odontotermes*. *O. stercorivorus* and *O. montanus* share almost identical mitochondrial 16S rRNA gene sequence and they were identified using morphologically

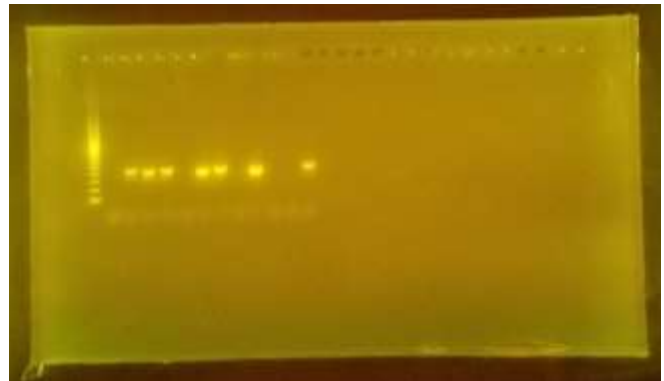


Figure 2 PCR amplified products of 16S rRNA gene of various species of termites

The sequences from *O. stercorivorus* and *O. montanus* were nearly identical, consistent with previous studies, suggesting they should be considered one single species. Molecular characterization of samples including: Bako 8, Ambo 10, Ambo 11, Ambo 12, Holeta 13, Holeta

14, Holeta 15, Holeta 16 and Haramaya 30 provided similar sequences to both *O. montanus* and *O. stercorivorus* but morphological identification of the samples revealed that all belong to the same species *O. montanus* as shown in Table 2.

Table 2 Measurements of soldier caste, n=20.

	Mean \pm SD (mm)	Range (mm)	Minimum (mm)	Maximum (mm)
Total body length	10.32 \pm 0.6	3.96	8.75	12.71
Head length with mandibles	5.42 \pm 0.3	2.009	4.683	6.692
Length of mandible	1.41 \pm 0.14	1.0226	0.9504	1.973
Head width	2.78 \pm 0.09	0.547	2.643	3.19
Head height	4.01 \pm 0.21	1.252	3.467	4.719

Morphological studies showed that *O. montanus* is larger than *O. stercorivorus* (Bagine, 1986). Table 3 shows that comparative measurements of

soldiers of *O. stercorivorus* and *O. montanus* indicating the collected samples are *O. montanus*.

Table 3. Comparative measurements of soldier in mm (Bagine, 1986).

	<i>O. montanus</i> (Bagine, 1986)	<i>O. montanus</i> (Harris, 1960)	<i>O. stercorivorus</i> (Bagine, 1986)
head length	2.62	2.64	2.04
head width	1.96	1.96	1.35
left mandible length	1.58	1.46	1.06
pronotum width	1.36	0.82	1.01
hind tibia length	1.72	1.60	1.05
length of head with mandibles	4.20	4.00	3.10

The minimum divergence of 0.23 was seen between *M. subhyalinus* and *M. jeanneli* species. The maximum divergence of 0.255 was observed between *O. zambesiensis*, Babile 33 and three rift valley isolates *M. jeanneli*, Bishoftu 17, *M. jeanneli*, Metehara 24 and *M. jeanneli*, Asebot 28. Samples from west of the rift valley including Bako, Holleta

and Ambo are different from the central rift valley samples: Bishoftu and Metehara but the Haramaya species, *O. montanus*, Haramaya 30 is genetically identical with the west samples. Outgroup taxa, *R. Arenicola* and *R. virginicus*, have a value of more than one indicating that they are completely different groups (Table 4).

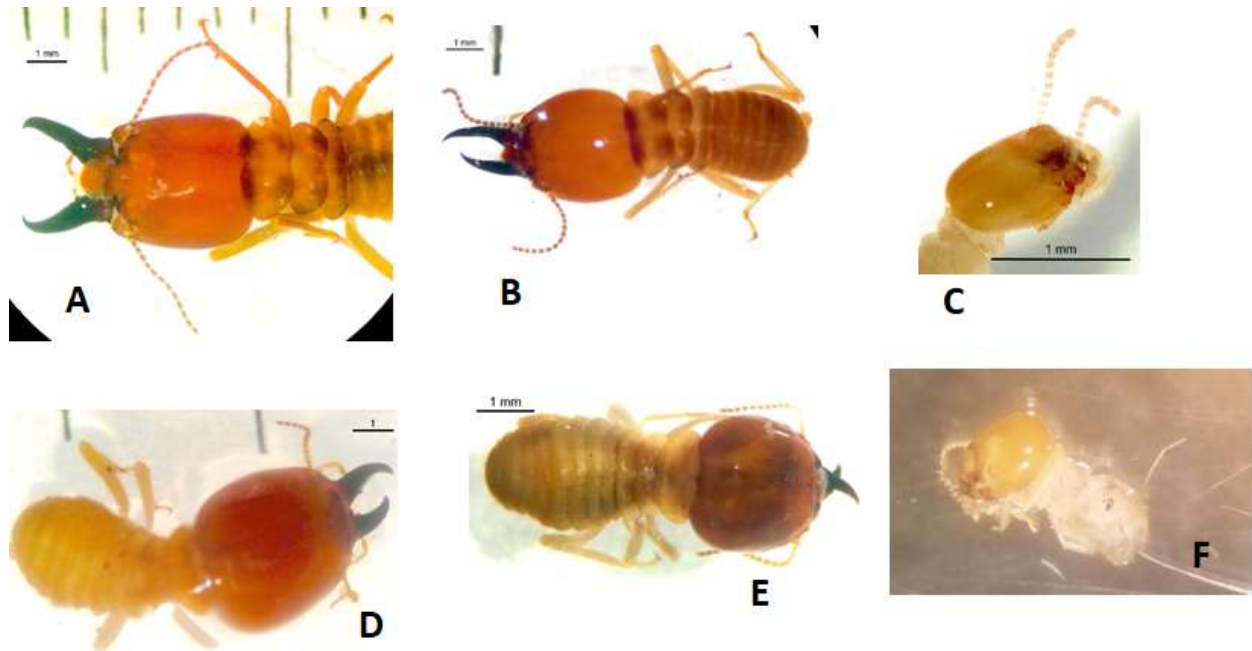


Figure 3. Termitidae species from Ethiopia A. *M. jeanneli*. B. *M. subhyalinus*. C. *M. obesi*. D. *O. montanus*. E. *O. tanganicus*. F. *O. zambesiensis*.

In the present study all the three genera, *Odontotermes*, *Macrotermes* and *Microtermes*, form a monophyletic group and clustered together. However, *O. zambesiensis*, Babile 33 collected from Babile show the greatest diversity among the

Odontotermes species. The tree with the highest log likelihood (-66926.94) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Figure 3).

Table 4. Tajima-Nei pairwise genetic distances.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1																				<i>Macrotermes subhyalinus</i> , Bako 2
2	0.000																			<i>Macrotermes subhyalinus</i> , Bako 5
3	0.023	0.023																		<i>Macrotermes jeanneli</i> , Bishoftu 17
4	0.023	0.023	0.000																	<i>Macrotermes jeanneli</i> , Metehara 24
5	0.023	0.023	0.000	0.000																<i>Macrotermes jeanneli</i> , Asebot 28
6	0.164	0.164	0.168	0.168	0.168															<i>Microtermes obesi</i> , Bako 4
7	0.164	0.164	0.168	0.168	0.168	0.000														<i>Microtermes obesi</i> , Bako 6
8	0.178	0.178	0.193	0.193	0.193	0.144	0.142													<i>Odontotermes montanus</i> , Bako 8
9	0.178	0.178	0.193	0.193	0.193	0.144	0.142	0.000												<i>Odontotermes montanus</i> , Holeta 14
10	0.177	0.177	0.192	0.192	0.192	0.153	0.148	0.006	0.006											<i>Odontotermes montanus</i> , Ambo 10
11	0.181	0.181	0.197	0.197	0.197	0.152	0.152	0.004	0.004	0.008										<i>Odontotermes montanus</i> , Ambo 11
12	0.177	0.177	0.192	0.192	0.192	0.148	0.145	0.003	0.003	0.008	0.000									<i>Odontotermes montanus</i> , Ambo 12
13	0.177	0.177	0.192	0.192	0.192	0.148	0.145	0.003	0.003	0.008	0.000	0.000								<i>Odontotermes montanus</i> , Holeta 13
14	0.177	0.177	0.192	0.192	0.192	0.148	0.145	0.003	0.003	0.008	0.000	0.000	0.000							<i>Odontotermes montanus</i> , Holeta 15
15	0.182	0.182	0.197	0.197	0.197	0.148	0.145	0.000	0.000	0.006	0.004	0.003	0.003	0.003						<i>Odontotermes montanus</i> , Holeta 16
16	0.182	0.182	0.197	0.197	0.197	0.148	0.145	0.000	0.000	0.006	0.004	0.003	0.003	0.003	0.000					<i>Odontotermes montanus</i> , Haramaya 30
17	0.192	0.192	0.208	0.208	0.208	0.168	0.168	0.039	0.039	0.043	0.036	0.035	0.035	0.035	0.039	0.039				<i>Odontotermes tanganicus</i> , Metehara 27
18	0.233	0.233	0.255	0.255	0.255	0.151	0.122	0.126	0.126	0.133	0.158	0.126	0.126	0.126	0.129	0.129	0.150			<i>Odontotermes zambesiensis</i> , Babile 33
19	1.046	1.046	1.083	1.083	1.083	1.083	0.988	1.127	1.127	1.098	1.096	1.119	1.119	1.119	1.132	1.092	1.038	1.024		<i>Reticulitermes arenicola</i> , AY168214.1
20	1.107	1.107	1.147	1.147	1.147	1.144	1.041	1.199	1.199	1.171	1.172	1.190	1.190	1.190	1.204	1.165	1.116	1.083	0.021	<i>Reticulitermes virginicus</i> , AY168224.1

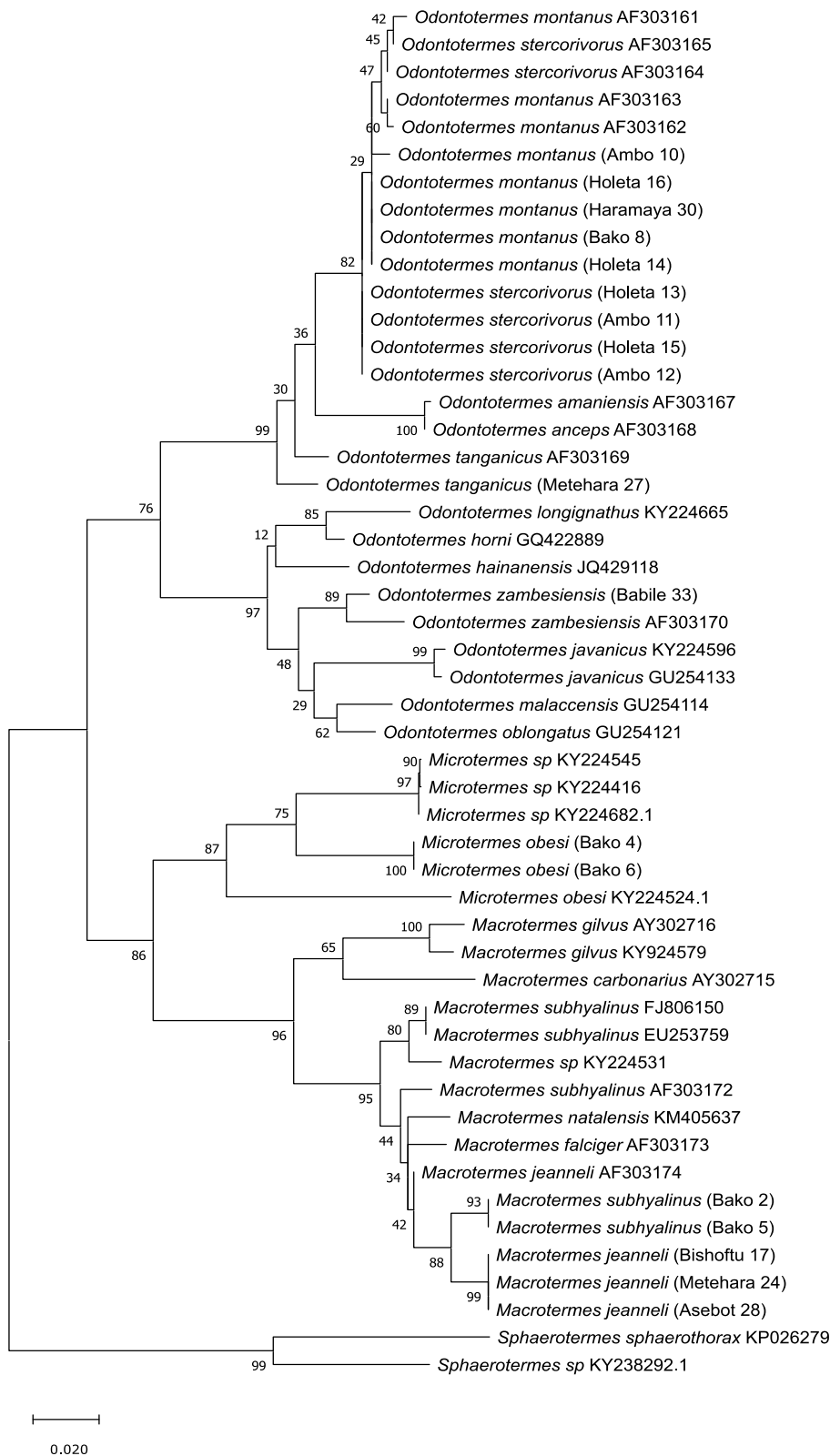


Figure 4. The maximum likelihood tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

The mitochondrial 16S rRNA gene sequences were obtained from each of the 18 Macrotermitinae samples collected in this study. A phylogenetic analysis of new and previously published Macrotermitinae sequences with two Sphaerotermitinae species as out groups was performed. The tree with the highest log likelihood (-66924.38) is shown in Figure 3.

Each of the three genera *Microtermes*, *Macrotermes*, and *Odontotermes* formed a moderately supported clade, with *Odontotermes* as the deepest branch. New *Odontotermes* sequences branched with previously published sequences from the same species, except for *Odontotermes tanganicus*. Instead, *O. tanganicus* sequences were paraphyletic to *Odontotermes amaniensis* Sjöstedt (Sjöstedt, 1924b), *Odontotermes anceps* Sjöstedt (1911b), *Odontotermes stercorivorus* Sjöstedt (Sjöstedt, 1907), and *Odontotermes montanus*.

Our new *Macrotermes* sequences did not branch with previously published conspecifics, but instead formed two supported sister clades within a larger *Macrotermes* clade including *Macrotermes*

natalensis and *Macrotermes falciger* in addition to *M. jeanneli* and *M. subhyalinus*. Similarly, our *Microtermes obesi* sequence did not branch sister to a previously published sequence though it did fall within the same supported clade.

The phylogenetic analysis of Termitidae provided molecular data for the Ethiopian termite species which have been previously reported based on morphological characters only (Wanyonyi *et al.*, 1984; Wood, 1991). Mitochondrial ribosomal RNA sequence has been used for phylogenetic studies of termites (Darlington *et al.*, 2008; Eaton *et al.*, 2016; Kambhampati *et al.*, 1996; Szalanski *et al.*, 2006).

The result now provides evidence to the species diversity of termites in east and west Ethiopia. Three genera from the Termitidae family are identified in this study including *Microtermes*, *Macrotermes*, and *Odontotermes*. Cowie *et al.* (1990) reported that 17 different termite genera from different parts of the country (Figure 4) including those identified in this study.

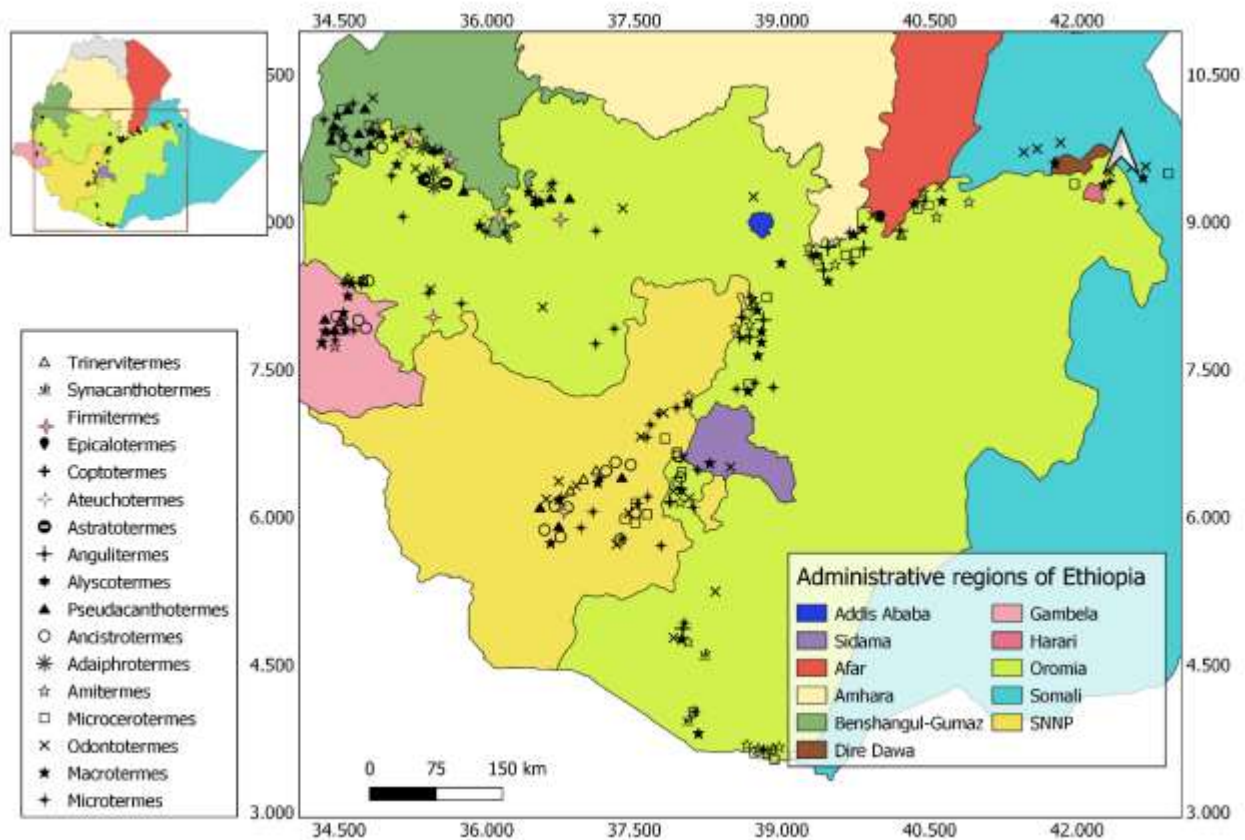


Figure 5. Distribution of termites in Ethiopia (Cowie *et al.*, 1990).

The results demonstrated two things. First, the subfamily Macrotermitinae is the most common termite groups in the survey area. Second, species of *Odontotermes* are widely distributed in Macrotermitinae at the studied locations. In East Africa *Odontotermes* are abundant and diverse groups of termites than any other termite genus with thirty recorded species (Bagine, 1986).

This study also identified new termite species to Ethiopia which includes *O. tanganicus* (Sjöstedt, 1924b), *O. zambesiensis* (Sjöstedt, 1914), *M. obesi* (Holmgren, 1912) and *M. jeanneli* (Grassé, 1937). The *O. tanganicus* and *O. zambesiensis* (Sjöstedt, 1914) are geographically distributed in Kenya (Darlington *et al.*, 1997; Davison *et al.*, 2001; Wanyonyi *et al.*, 1984); Malawi (Munthali *et al.*, 1999); Tanzania (Mathur and Thapa, 1962; Wanyonyi *et al.*, 1984; Weidner, 1960). Whereas *M. obesi* (Holmgren, 1912) is distributed in Bangladesh, India, Pakistan and Sri Lanka (Roonwal and Verma, 1977; Verma and Thakur, 1982); Thailand and Vietnam (Maiti, 1983) and Bhutan, Cambodia, Myanmar (Verma, 1990). *M. jeanneli* on the other hand is distributed in Ethiopian region (Ghidini, 1955; Snyder, 1949), Kenya (Darlington *et al.*, 1997; Davison *et al.*, 2001; Snyder, 1949) and Sudan (Mathur and Thapa, 1962).

O. stercorivorus and *O. montanus* share almost identical mitochondrial 16S rRNA gene sequence, in agreement with previous studies (Davison *et al.*, 2001), though they differ morphologically (Darlington *et al.*, 2008). According to Bagine (1986) *O. montanus* is morphologically larger than *O. stercorivorus* as shown in Table 3. The findings in this report confirms that the collected samples are *O. montanus* where morphometric data of every parameters greater than *O. stercorivorus* as compared to Bagine (1986) as shown in Table 2.

This result ties well with previous studies of Davison *et al.* (2001) that confirms *O. stercorivorus* and *O. montanus* as they belong to a single genetic population but having two distinct morphologies. Genetic similarity between *O. stercorivorus* and *O. montanus* was also observed between Kenyan termite samples (Davison *et al.*, 2001). These samples are morphologically and behaviorally so different but closely related groups and may belong to a single genetic population (Darlington *et al.*, 2008).

CONCLUSIONS

The paper concludes by arguing that termites are important creatures as pests and in their ecological role. Knowing the distribution and diversity of termites will help to understand the kind of species available in the country and will help to identify the pest species easily. Importantly, our results provide evidence that *Odontotermes* are the most abundant termite species in the surveyed areas. In addition, these findings provide additional information about the new species reported in this study for the first time in Ethiopia. Future research on termite survey and identification might extend to untouched parts of the country. We believe that apart from using only morphological identification, future research should focus on molecular identification of termites. In addition, further surveys in additional geographic areas and climatic conditions in the country might prove an important area for future research.

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