academicJournals

Vol. 11(12), pp. 504-515, 28 March, 2017
DOI: 10.5897/AJMR2017.8484
Article Number: 99A19BE63526
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Fungal diversity and community structure in gut, mound and surrounding soil of fungus-cultivating termites

Huxley Mae Makonde[1,2*], Romano Mwirichia[3], Edith Mnyazi Muwawa[4], Hans-Peter Klenk[5] and Hamadi Iddi Boga[6]

[1]Department of Microorganisms, Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.
[2]Department of Pure and Applied Sciences, Technical University of Mombasa, Mombasa, Kenya.
[3]Department of Biological Sciences, Embu University, Embu, Kenya.
[4]Department of Biological Sciences, Pwani University, Kilifi, Kenya.
[5]School of Biology, Newcastle University, Newcastle upon Tyne, UK.
[6]School of Agriculture, Earth and Environmental Sciences, Taita Taveta University, Voi, Kenya.

Received 13.February, 2017; Accepted 9 March, 2017

The fungus-cultivating termites (Macrotermitinae) form part of diverse termite fauna in Africa, but information on their fungal symbionts is inadequate and poorly understood. In this study, the fungal communities and structure between termite gut, mound and surrounding soil were determined using the 454 pyrosequencing-based analysis of the internal transcribed spacer (ITS) gene sequences. Genomic DNA was extracted and purified from the guts of three termites (*Odontotermes* sp., *Macrotermes michaelseni* and *Microtermes* sp.), mound and surrounding soil samples for high-throughput sequencing. A total of 15,256 sequences were obtained and individual samples contained between 4 and 133 operational taxonomic units (OTUs). Termite gut had the least fungal diversity, dominated by members of the *Basidiomycota* (> 98%). More than 98% of the gut sequences were of the genus *Termitomyces*, while < 2% were related to the genera *Chaetomium, Fusarium, Eupenicillium, Cladosporium, Curreya* and *Phaeosphaeria* with between 95 and 98% pair-wise sequence identities. Members of *Ascomycota* (> 94%) were the most abundant in the mound and soil, but significantly differed (P value of 0.04; R value = 0.909) between the mound and soil environments. The results confirm that the genus *Termitomyces* exist in a tight association with their hosts and that *Termitomyces* species are scarcely present in the mound and soil. In addition, by altering soil properties; the fungus-cultivating termites modify the fungal community composition and structure in the mound and surrounding soil environments.

Key words: 454-pyrosequencing, microtermitinae, mutualism, tropical mycology.

INTRODUCTION

The diverse and numerous microorganisms in the soil perform key functions within the environment by participating in the cycling and flux of various nutrients, thereby influencing structure formation and sustenance of

soil properties (Holt and Lepage, 2000; Harry et al., 2001). Termites, a group of social insects consisting of over 2 600 species worldwide (Ahmed et al., 2011), are part of soil organisms that influence soil properties (Holt and Lepage, 2000; Harry et al., 2001; Manuwa, 2009; Muwawa et al., 2014). They are known as "soil engineers" as they have a great influence on the soil characteristics (Holt and Lepage, 2000), hence controlling diversity and activity of other soil organisms (Jones et al., 1997; Lavelle et al., 1997). Their influence on the soil microbial component is as a result of their major construction activities of complex galleries and mounds that result into soil heterogeneity in the tropical regions (De Bruyn and Conacher, 1990; Holt and Lepage, 2000).

The termite mound, thus, forms a specific habitat for soil microbiota since the physical and chemical properties are different from the surrounding soil (De Bruyn and Conacher, 1990; Holt and Lepage, 2000). The type of mound construction depends on the feeding habit of the termite species (Holt and Lepage, 2000). The fungus-growing termites build their mounds using soil and clay cemented by salivary secretions that make the mounds enriched with clay particles but impoverished in carbon (Harry et al., 2001). The nest-walls consist of organo-mineral aggregates, characterized by a low stability hence mineralize easily (Garnier-Sillam et al., 1988). They have a wider range of activity on the surrounding soil of 1 to 3 m in depth and within a range of a 2 to 8 m (Harry et al., 2001), which may influence the soil properties and fertility. The question is whether the fungus-feeding termites can be regarded as metabionts (Waid, 1999).

The Macrotermitinae comprises of the economically important termite species (Ahmed et al., 2011) that have been comprehensively studied (Mathew et al., 2012; Makonde et al., 2013; Otani et al., 2014, 2015; Muwawa et al., 2016). Previous studies have focused on the mutualistic symbiosis between *Termitomyces* sp. (Basidiomycota) and fungus-growing termites (Mohindra and Mukerji, 1982; Zoberi and Grace, 1990; Aanen et al., 2007, 2009; Osiemo et al., 2010; Nobre et al., 2010, 2011), parasitic fungi for termites (Traniello et al., 2002) and saprotrophic fungi such as *Xylaria* species that colonize termite nests (William, 1969; Moriya et al., 2005). Despite the termite activities influencing the microbial diversity and community structure, there is little information on comparative fungal community composition between termite gut, mound and corresponding soil environments. Therefore, in this study, we conducted a 454 pyrosequencing-based analysis of the ITS gene sequences to evaluate the gut fungal diversity associated with three fungus-cultivating termites. In addition, we evaluated on how, by altering soil properties; the fungus-cultivating termites modify the fungal community composition and structure in the mound and surrounding soil environments.

MATERIALS AND METHODS

Study sites and sampling

The samples were collected from Juja in Kiambu County, Kenya (latitude 1° 5′ 54.68″ N, longitude 37° 1′ 1.10″ W). The *Odontotermes* sp. (OTG1) [JQ247986] belonging to mound C, *Macrotermes michaelseni* (MTG4) [JQ247993] and *Microtermes* sp. (MIG7) [JQ247990] both colonizing mound D (~2 km far away from mound C) were sampled by excavating each mound to a depth of approximately 1.0 m and aseptically collecting the termites (n = 200 workers and 50 soldiers). Worker-caste termites were used in the experiments due to their foraging behavior. The identity of the termites was confirmed by sequencing the mitochondria cytochrome oxidase II gene in DNA extracted from the heads of soldiers (Austin et al., 2004) and comparing it to the sequences of previously identified specimens (Inward et al., 2007). In addition, soil samples (~40 g collected at ~5 cm depth) from termite mounds (OTN2 and MTN5) and surrounding soil samples (OTS3 and MTS6, collected at 3 m away from termite mounds C and D, respectively) were included in the analyses.

DNA extraction

DNA extraction was performed as described previously (Makonde et al., 2013). Briefly, the exterior surfaces of the termites were washed with 70% ethanol and then rinsed with sterile distilled water. The guts were aseptically removed with forceps. A total of 165 guts (~1 g) of the *Odontotermes* sp. (OTG1) and *M. michaelseni* (MTG4) and 198 guts (~1g) of *Microtermes* sp. (MIG7) were separately put into sterile micro tubes containing 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). They were then homogenized using a sterile glass rod. The corresponding homogenates were then transferred into sterile tubes and used for total DNA extraction. The soil samples were homogenized separately and debris were removed. Subsequently, soil samples (~4 g) were used for total microbial DNA extraction. Total DNA extraction for all samples was performed using MoBio PowerMax Soil DNA isolation kit (MoBio Laboratories, Inc. CA, USA) according to the manufacturer's protocol. DNA concentration was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA) as recommended by the manufacturer.

Amplification of internal transcribed spacer (ITS) gene region and sequencing

The fungal DNA was PCR amplified using a set of the universal ITS gene primers (the ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS4 [5'-TCCTCCGCTTATTGATATGC-3']) according to White et al.

*Corresponding author. E-mail: makondehuxley@tum.ac.ke, huxcly@yahoo.com. Tel: +254725535161.

(1990). These fungal primer set were modified for 454 pyrosequencing by attaching an adaptor sequence, a key and a unique 12 Nucleotide MID for multiplexing purposes. Each PCR reaction (50 µL) contained forward and reverse primers (10 µM, each), dNTP's (10 mM each), Phusion GC buffer (Finzymes), Phusion high fidelity polymerase (0.5 U μL^{-1}) and 25 ng of template DNA. Amplifications occurred in an Eppendorf Mastercycler Thermal Cycler with the following program conditions: An initial heating at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, after which a final extension step at 72°C for 5 min was performed. The amplification was confirmed using gel electrophoresis of 2 µl of the PCR product on a 1% TAE agarose gel (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, 1.5% (w/v) for 1 h at 100 V. Later three independent PCR products per sample were pooled in equal amounts, separated on a gel and extracted using the peqGOLD gel extraction kit (PeqLab Biotechnologie GmbH, Erlangen, Germany). Quantification of the PCR products was performed by using the Nanodrop (NanoDrop Technologies, USA) method and a Qubit fluorometer mbH, (Invitrogen GmbH Karlsruhe, Germany) as recommended by the manufacturer. Sequencing of the PCR amplicons was done at the Göttingen Genomics Laboratory using Roche GS-FLX 454 pyrosequencer (Roche, Mannheim, Germany) as recommended in the instructions of the manufacturer for amplicon sequencing.

Data analysis

Raw sequences were quality filtered according to Huse et al. (2007) using the QIIME release 1.9.0 software (Caporaso et al., 2010). Briefly, low quality sequences were removed from the analysis if they were less than 200 bp in length, contained ambiguous characters, did not contain the primer sequence or contained an uncorrectable barcode. The remaining sequences were assigned to samples based on the 12-nucleotide barcode. The denoised sequences were evaluated for potential chimeric sequences using UCHIME in the USEARCH package v.4.2.66 (Edgar, 2010). A sequence identity cutoff of 97% was used to pick OTUs from the quality filtered non-chimeric sequences. Representative OTUs were picked using the de novo OUT clustering (Rideout et al., 2014) with standard UCLUST method using the default settings as implemented in QIIME at 97% similarity level. OTU alignment was performed using the python implementation of the NAS algorithm, PyNAST (Caporaso et al., 2010). Taxonomy was assigned to representative sequences from each cluster using BLASTn against the SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013) at dissimilarity levels of 3, 5 and 10%. Rarefied datasets were generated with the multiple_rarefaction function in QIIME in order to remove sample heterogeneity before diversity assessment. Rarefaction curves and diversity indices were calculated and plotted for each sample using QIIME (Caporaso et al., 2010). To determine the amount of dissimilarity (distance) between any pair of bacterial communities, we used the UniFrac metric (Lozupone and Knight, 2005; Lozupone et al., 2007) that incorporates the degree of divergence in the phylogenetic tree of OTUs into Principal coordinates analysis (PCoA). A relatively small UniFrac distance implies that two communities are compositionally similar, harboring lineages sharing a common evolutionary history. In unweighted UniFrac, only the presence or absence of lineages is considered. We used the analysis of similarities (ANOSIM) (Clarke, 1993; Fierer et al., 2010) through 1000 to test for differences in community composition among the groups of samples. Additionally, the relative abundance of the genera was used in hierarchical clustering using the pearson correlation distance metric implemented in MultiExperimentViewer version 4.9.0 (MeV 4.9.0). Fungal communities across the analyzed samples were compared based on the relative abundances of some selected fungal genera, using principal component analysis (PCA) as implemented in R (R Core Team, 2012). All pyrosequencing-derived ITS gene sequences datasets were deposited in the GenBank under accession number SRP019764.

RESULTS

Distribution of Fungal phyla across the samples

The overall reads for the fungal samples were 18,294. After quality filtering and chimera check 15,256, the resulting sequences (≥ 200 bp) were clustered into 287 OTUs (Table 1) at 3% sequence divergence. Taxonomic assignment of the resulting sequences against the SILVA database showed ≥ 2 known phyla, but the major ones (> 90% of the analyzed sequences) were *Ascomycota* and *Basidiomycota* (Figure 1).

Fungal community composition across samples

The abundance of fungal composition at the phylum level differed across the samples (Figure 1; Table 1). Members of the phylum *Basidiomycota* were the most abundant (>98% of the analyzed sequences) in the gut samples [MIG7, MTG4 and OTG1] compared to those of mound [sample OTN2] and soil environments [samples OTS3 andMTS6], which were predominated by members of the phylum *Ascomycota* [>94% of the analyzed sequences] (Figure 1). There were no sequences for sample MTN5 due to some sequencing errors. At the class level, members affiliated with *Agaricomycetes* were the most abundant (>98%) in the gut samples [MIG7, MTG4 and OTG1], but least in the mound (OTN2) and soil (OTS3 and MTS6) samples (Table 2). Members of *Sordariomycetes* and *Eurotiomycetes* were the most abundant in the mound (89%) and soil (54-68%) samples, respectively. Other classes such as *Dothideomycetes* (4.5%), *Eurotiomycetes* (3.6%) and uncultured a*scomycete* (1.1%) were relatively abundant in the mound, while classes such as *Sordariomycetes* (≥12%), *Dothideomycetes* and *Orbiliomycetes* (>5%) were relatively abundant in the soil (Table 2).

At the order level, the relative abundances of the fungal communities in the samples were different. The order Agaricomycetidae was the most abundant group in the termite gut. Notably, the mound was dominated by the order Hypocreomycetidae while the soil was predominated by members of the order Eurotiomycetidae (Figure 2). Other orders such as Dothideomycetidae, Pleosporomycetidae, Chaetothyriomycetidae, Sordariomycetidae, Xylariomycetidae and Orbiliales were detected at varying relative abundances (1 to 22% of the analyzed sequences) in some samples (Figure 2).

At the genus level, the most abundant genus in the gut

Table 1. Number of sequences, observed OTUs, the estimated richness and diversity indices at 3% dissimilarity threshold.

Sample ID	Sample description	Reads before QT	Reads after QT	OTUs	Phyla	Classes	Richness and diversity indices				
							Chao1 index	ACE	Simpson (1/D)	Shannon	Fisher_alpha
OTG1	*Odontotermes* sp. gut homogenate	1,569	1,421	5	3	3	6.5	11	0.002	0.01	0.54
OTN2	Soil from mound C of *Odontotermes* sp.	2,369	2000	53	5	12	59	56.9	0.61	2.4	8.2
OTS3	Soil collected 3 m away from mound C	3,227	2,505	83	4	11	92.3	87.6	0.82	3.7	13.3
MIG7	*Microtermes* sp. gut homogenate	2,614	2373	4	2	2	5	8.1	0.003	0.02	0.42
MTG4	*M. michaelseni* gut homogenate	2,000	1,815	9	2	3	10	10	0.03	0.2	1.2
MTN5	Soil from mound D of *M. michaelseni*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MTS6	Soil collected 3 m away from mound D	6,515	5142	133	5	16	144	145.5	0.79	3.4	23.4
		18, 294	**15, 256**								

QT', Quality trimming; 'OTUs', operational taxonomic units; 'ND', not determined.

samples was *Termitomyces* (>98%), but was detected at low relative abundance (< 0.4%) in the mound and soil samples (Table 3). Notably, fungal species associated with *Eupenicillium limosum*, *Monilinia fructicola* and *Fusarium oxysporum* (with 96 to 98% sequence identities) were detected in the gut of *Odontotermes* sp. (sample OTG1). These fungi, however, constituted about 0.1% of the analyzed sequences. Likewise, in the gut of *Microtermes* sp. (sample MIG7), about 0.1% of the sequences were related to *Trichocoma paradoxa* and *Cladosporium* sp. CF-25 (with 96 to 98% sequence identities). The gut of *M. michaelseni* (sample MTG4), had about 1.3% of the analyzed sequences affiliated with *Chaetomium globosum, Myrothecium* sp. J3, *Monodictys castaneae, Fusarium oxysporum, Penicillium purpurogenum, Cladosporium* sp. CF-25, *Phaeosphaeria avenaria* and *Curreya pityophila* [with 96 to 99% sequence identities] (Table 3).

The genus *Fusarium* [17%] and particularly the genus *Hypocrea* [59%] were the most abundant genera in the mound (Table 3), but the soil samples were predominated by the following genera; *Aspergillus* [45%], *Eupenicillium* [39%] and *Xylaria* [19%]. In the mound (OTN2), most of the fungal species were affiliated with *H. koningii*, *Fusarium* sp. CPCC 1400009 and *C. globosum* (with 97 to 99% sequence identities) while in the surrounding soil, the fungal species were between 97 and 98% affiliated with *E. limosum*, *A. fumigatus*, *Xylaria hypoxylon* and *Hypocrea koningii* (Table 3).

Fungal diversity and richness

Fungal diversity and richness for the analyzed sequences for each sample (Table 1) were evaluated by rarefaction (Figure not shown). At 3% sequence divergence, some rarefaction curves did not reached saturation, indicating that the surveying efforts did not fully cover the extent of taxonomic diversity at this genetic distance, but a substantial fraction of the fungal diversity within individual samples was evaluated. The diversity measures showed that MTS6 had the most genus-level taxa (133; Table 1) and MIG7 the least (4; Table 1), that MTS6 was richest (Chao 1 index), while MIG7 was poorest. There was variation in community composition as indicated by the Simpson (1/D) and Shannon indices (Table 1).

Comparison of the individual samples using unweighted UniFrac PCoA (Figure 3) showed a distinct clustering by environment, but the *p*-value of 0.04 and R value of 0.909 indicated that at an alpha of 0.05; the grouping of samples is relative strong implying that there is dissimilarity between the groups. For instance, the gut samples (MIG7, OTG1 and MTG4) did not cluster together and with those of mound and soil (Figure 3 and 4), indicating dissimilarity in the fungal communities. Likewise, samples OTS3, MTS6 and OTN2 did not cluster together, indicating that each individual sample had almost different fungal communities. Notably, the mound sample (OTN2) did not cluster with its corresponding soil sample (OTS3), implying that the mound fungal community composition was different from that of its surrounding soil (Figures 3 and 4).

The PCA shows that the fungal communities within the termite gut are mainly impacted by the genus *Termitomyces* while those of the mound

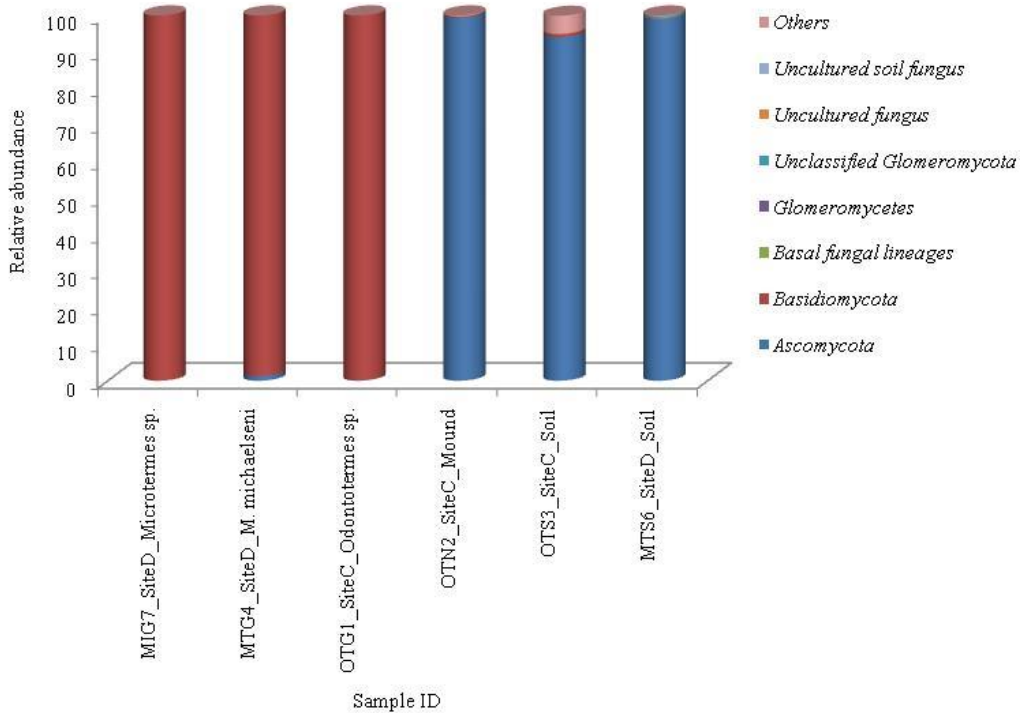

Figure 1. Relative abundances (%) of fungal phyla in the samples. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C. Phylogenetic groups accounting for < 0.4% of the analyzed sequences were included in the artificial group 'others'.

Table 2. Distribution of the fungal sequences into class level after quality trimming.

Class	Termite gut			Mound	Soil	
	MIG7	MTG4	OTG1	OTN2	OTS3	MTS6
Dothideomycetes	0.1	0.9	0	4.5	7	5.2
Eurotiomycetes	0	0	0.1	3.6	67.2	54.9
Lecanoromycetes	0	0	0	0	0.3	0.3
Lichinomycetes	0	0	0	0	0	0.2
Orbiliomycetes	0	0	0	0	5.8	0
Sordariomycetes	0	0.4	0	89	12	36.4
Taphrinomycetes	0	0	0	0.6	0	0.2
Uncultured rhizosphere ascomycete	0	0	0	1.1	0	0.3
Coniosporium	0	0	0	0	0.4	0
Humicola	0	0	0	0	0.8	0
Lecophagus	0	0	0	0	0	0.3
Phoma	0	0	0	0	0.6	0.6
Pseudosigmoidea	0	0	0	0.2	0	0
Agaricomycetes	99.9	98.7	99.9	0.3	0.8	0.2
uncultured Basidiomycota	0	0	0	0.1	0	0
unclassified Mucoromycotina	0	0	0	0	0	0.1
Other	0	0	0	1	5	1.1

MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C. Phylogenetic groups accounting for < 0.1% of the analyzed sequences were included in the artificial group 'others'

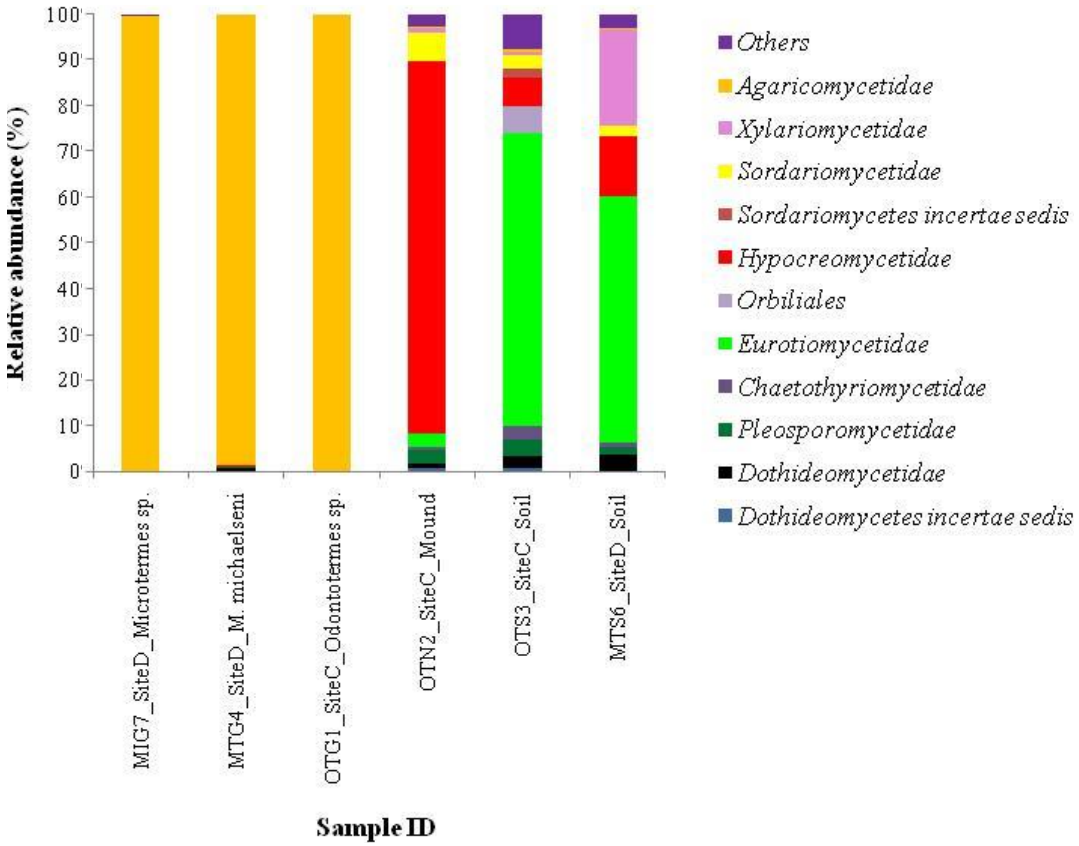

Figure 2. Relative abundances of the orders in the domain Eukaryota. Unknown Phylogenetic groups are included in the artificial group 'others'. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

are impacted by the genera *Fusarium* and *Hypocrea* (Figure 5). The surrounding soil is impacted by the genera *Xylaria, Aspergillus* and *Eupenicillium*. Their relative abundances varied across the samples (Table 3). On one hand, *Termitomyces* species were the most dominant fungal species in the gut of three fungus cultivating termites (*M. michaelseni, Odontotermes* and *Microtermes* species), while members of the genera *Fusarium* and *Hypocrea* were more dominant in the mound. On the other hand, members of the genera *Xylaria, Aspergillus* and *Eupenicillium* were more predominant in savannah soil (Table 3 and Figure 4).

DISCUSSION

Defining the number of fungi on the planet has always been an area of debate (Hawksworth, 2001), but has recently gained prominence in scientific literature. This has provided the foundation for studies aimed at obtaining a better understanding of fungal biodiversity worldwide. Termites and their mounds harbor diverse organisms including fungi. There are two aspects of fungal diversity on termite mounds, namely, the presence of *Termitomyces* versus other fungi such as *Xylaria/ or Pseudoxylaria* species (Moriya et al., 2005; Okane and Nakagiri, 2007; Ju and Hsieh, 2007; Guedegbe et al., 2009; Visser et al., 2009; 2012) and the diversity within *Termitomyces* species.

Our study compared the fungal diversity and community structure in the termite gut, mound and surrounding soil. The results of our study revealed two major fungal phyla; *Ascomycota* and *Basidiomycota* whose members' distribution differed significantly across the samples. The phylum *Basidiomycota* was the most abundant in the termite gut while the phylum *Ascomycota* dominated in the mound and surrounding soil. Furthermore, members of *Ascomycota* differed significantly between the mound and surrounding soil (Table 3 and Figures 2 and 4). The discrepancy of fungal composition between the mound and soil may emanate from the construction activities of the termites. Such activities can chemically modify the

Table 3. Relative abundances (%) of the genera in the domain eukaryota.

Phylum	Genus affiliation	Termite gut			Mound	Soil		%ID
		MIG7	MTG4	OTG1	OTN2	MTS6	OTS3	
Basidiomycota	*Termitomyces* sp. ZA164	99.9	0	0	0	0.1	0	98
Basidiomycota	*Termitomyces* sp. ZA164	0	0	99.9	0.3	0	0.4	98
Basidiomycota	*Termitomyces* sp. ZA164	0	98.6	0	0	0	0	96
Ascomycota	*Xylaria hypoxylon*	0	0	0	0.7	19.6	0.8	98
Ascomycota	*Chaetomium globosum*	0	0.04	0	10.1	1.5	2.6	99
Ascomycota	*Ceratostomella pyrenaica*	0	0	0	0	0	0.5	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0.1	1.6	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0	0.35	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0	0.3	95
Ascomycota	*Fusarium oxysporum*	0	0	0.02	0.7	1.2	0.5	96
Ascomycota	*Fusarium* sp. CPCC 1400009	0	0	0	15.2	0.8	0	97
Ascomycota	*Fusarium* sp. 18014	0	0	0	0.8	0.2	0	96
Ascomycota	*Hypocrea koningii*	0	0	0	59.7	12.5	5.7	98
Ascomycota	*Helicoon fuscosporum*	0	0	0	0	0	6.5	96
Ascomycota	*Aspergillus fumigates*	0	0	0	0.12	42.3	1.4	99
Ascomycota	*Sagenomella humicola*	0	0	0	0.2	0.8	0.5	97
Ascomycota	*Sagenomella humicola*	0	0	0	0	0.5	0	95
Ascomycota	*Sagenomella humicola*	0	0	0	0	2.9	0	95
Ascomycota	*Aspergillus* sp. LQ21	0	0	0	0	1.5	0.5	97
Ascomycota	*Aspergillus clavatus* NRRL 1	0	0	0	0	0.4	0.5	97
Ascomycota	*Eupenicillium limosum*	0	0	0.02	2.4	1.1	39.4	97
Ascomycota	*Phaeosphaeria avenaria f. sp. Avenaria*	0	0.2	0	1.1	0.1	0.2	98
Ascomycota	*Curreya pityophila*	0	0.4	0	1	1.9	4.4	98
Ascomycota	*Curreya pityophila*	0	0	0	0.6	0.2	0.1	95
Ascomycota	*Cladosporium* sp. CF-25	0.01	0.6	0	0.5	1.3	1.6	98
Ascomycota	*Leptoxyphium fumago*	0	0	0	0.8	1.7	0.2	99
Others		0.09	0.08	0.06	5.68	9.3	31.95	

Phylogenetic groups that are (≤ 0.3%) in all samples are included in the artificial group 'others'. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

organic matter in the mound (Holt and Lepage, 2000; Harry et al., 2001), hence creating ecological microniches suitable for more specialized fungi.

The most abundant fungal genus in the termite gut was *Termitomyces*, which was represented by over 98% of the analyzed sequences in each gut sample (Table 3). Notably, the gut *Termitomyces* symbiont differed in the host. For instance, an interesting scenario was noted in mound D, which was inhabited by two different termite species. Each termite species (*M. michaelseni* vs. *Microtermes* sp.) cultivated its own *Termitomyces* strain (Table 3). Since the *Macrotermes* and *Microtermes* termites colonized the lower and upper parts, respectively, the likelihood of horizontal transfer of the fungus should have been high as suggested previously (Makonde et al., 2013). But this was not the case; thus, the affected host-*Termitomyces* relationships are likely to be too specialized to allow host switching. Literature indicates that some termite genera cultivate a restrictive set of fungal symbionts (Aanen et al., 2007; Osiemo et al., 2010). Nonetheless, it remains to be addressed how the termites exclusively select the right *Termitomyces* symbiont for their colony. The mound and soil samples (OTS3, MTS6 and OTN2) did not cluster together, indicating that each individual sample had almost different fungal communities.

Besides, the 454-pyrosequencing approach used in this study revealed other minor fungal species, which were previously often undetected by the traditional Sanger sequencing in the termite gut (Mathew et al., 2012; Makonde et al., 2013). This is because the use of the clone-based approach for microbial analyses in the previously mentioned studies could have been limited by PCR errors and bias in selecting representative clones

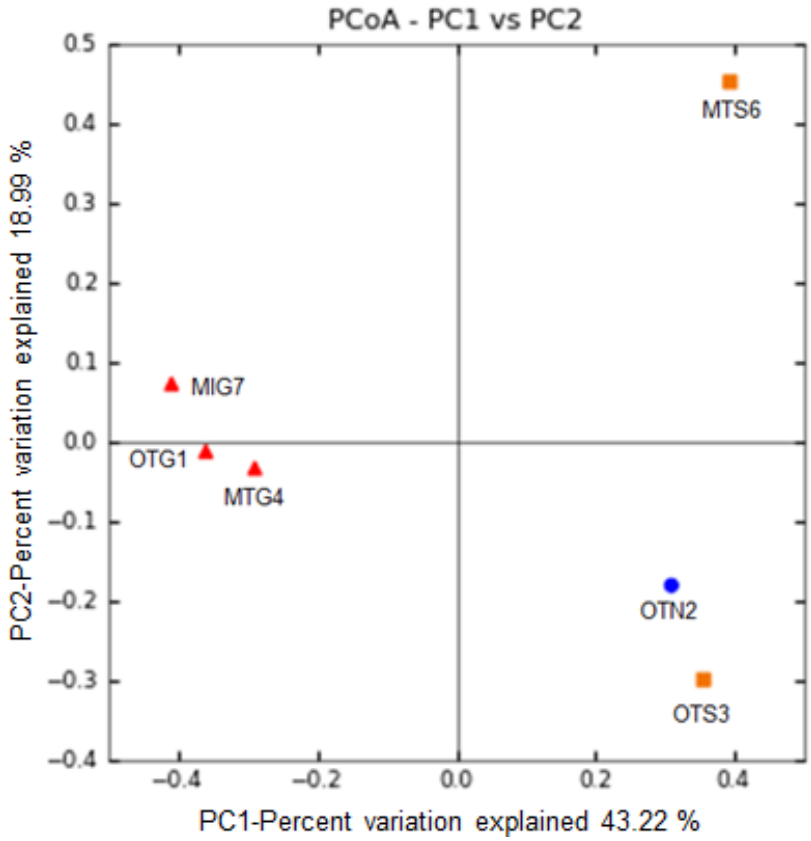

Figure 3. PCoA plots showing the degree of similarity of bacterial communities on termite guts, mounds and soil samples. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

for sequencing. In this current study, fungal species affiliated with *E. limosum, M. fructicola* and *F. oxysporum* were detected in the gut of *Odontotermes* species (Table 3). This, however, constituted about 0.1% of the effective sequences just like in the gut of *Microtermes* species, where 0.1% of the sequences were associated with *T. paradoxa* and *Cladosporium* sp. CF-25 (with 96 to 98% sequence identities). In the gut of *M. michaelseni*, about 1.4% of the sequences were related to *Chaetomium globosum, Myrothecium* sp. J3, *M. castaneae, F. oxysporum, P. purpurogenum, Cladosporium* sp. CF-25, *P. avenaria* and *C. pityophila* (with 96 to 99% sequence identities). Elsewhere, Mathew et al. (2012) isolated yeasts closely related to *Debaryomyces hansenii, Pichia guilliermondii* and *Candida inconspicua* from the comb material and gut of *Odontotermes formosanus* using adapted cultivation techniques. However, these fungi/yeasts were detected insignificantly in quantitative terms, and it is unclear which role they play. In contrast to our findings, some previous studies conducted using clone-based approach (Mathew et al., 2012) could not identify fungal genera in some fungus-cultivating termites' guts other than *Termitomyces*.

Though the results of this study do not support the physiological roles of the symbiotic fungi detected, several researchers have proposed roles associated with symbiotic fungi (*Termitomyces* species) in termites. For example, provision of glycosyl hydrolases (Martin and Martin, 1978), enrichment of nitrogen, which is advantageous as the dead plant material consumed by termites, is poor in nitrogen (Collins, 1983), and lignin degradation, which subsequently allow for cellulose digestion (Hyodo et al., 2000). Nonetheless, the significance of each role differs in value among termite species (Rouland-Lefévre, 2000; Hyodo et al., 2003). Hyodo et al. (2003) suggested that the important role of symbiotic fungi in *Macrotermes* species is to degrade lignin, hence allowing for efficient digestion of cellulose, whereas for *Odontotermes* species, *Hypotermes makhamensis* and *Phidiana militaris*, it is to serve a

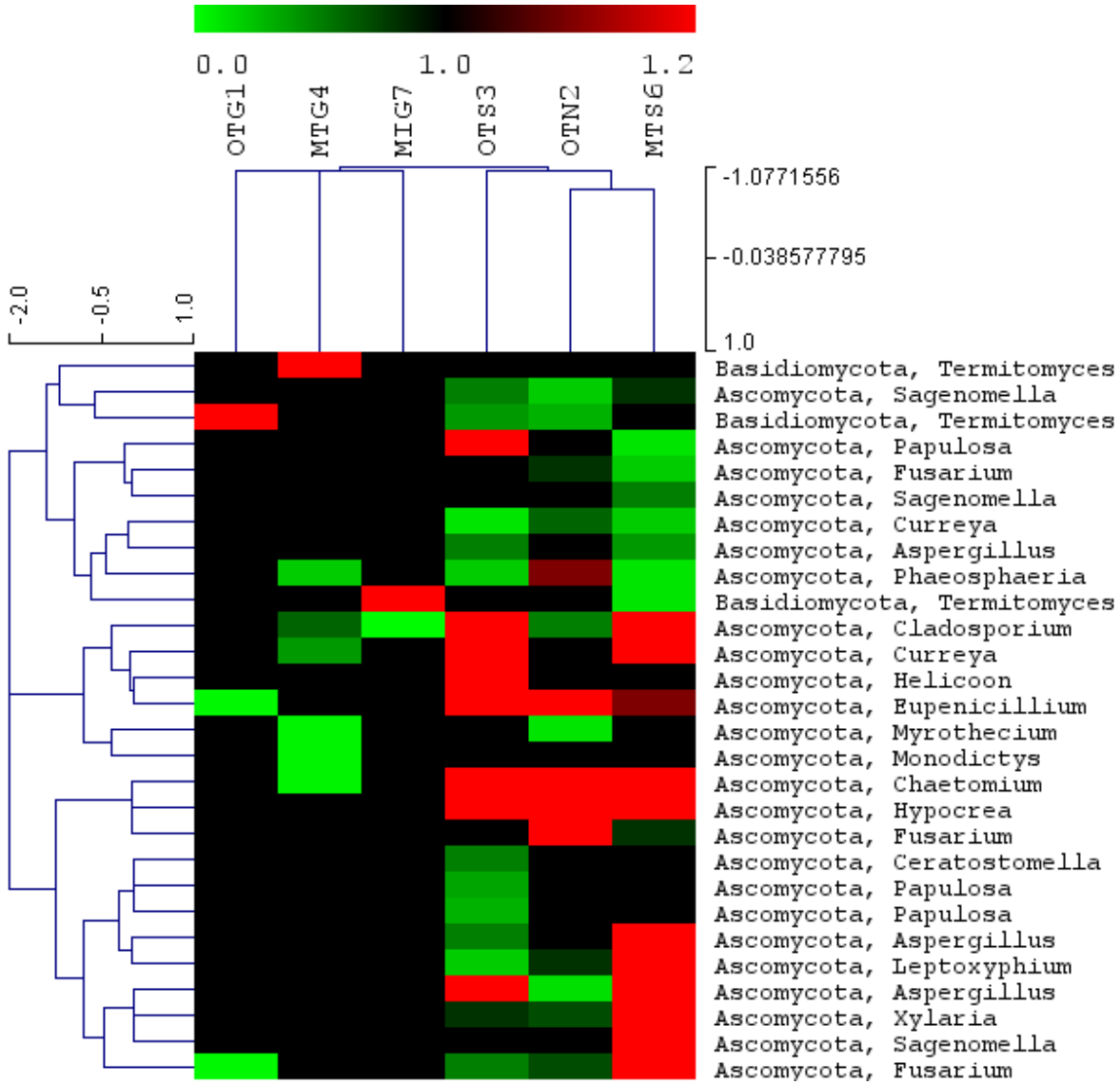

Figure 4. Heatmap shows hierarchical clustering of taxa (relative abundance > 0.1% of the analyzed sequences). The scale bar represents color saturation gradient based on the relative abundances of the fungal genera. The dendrogram at the top shows the weighted Euclidean distance analysis of community similarity. Classification is presented at the genus and phylum levels. OTG1, *Odontotermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; MIG7, *Microtermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C.

nutritional role. However, it is still unclear whether the different roles of such fungi are directly dependent on termite taxonomy or variation in plant biomass used to make fungus comb (Hyodo et al., 2003).

The genus *Fusarium* and particularly the genus *Hypocrea* were the most abundant genera in the mound compared to the soil, which was dominated by the genus *Aspergillus* among others (*Eupenicillium*, *Xylaria* and *Hypocrea*). On one hand, the transformed soil properties in the mound might have favored the proliferation of particular fungi; especially those related to the genera *Fusarium* and *Hypocrea.* On the other hand, it might have limited the growth of other genera such as *Aspergillus, Xylaria* and *Eupenicillium,* which were mostly favored by conditions in the surrounding soil. Thus, the soil harbored a higher diversity of fungi most of which were different from those of the mound and gut. The differences in fungal communities between the mound and surrounding soil may partly be attributed by influence of the fungus-cultivating termites on the soil properties, which

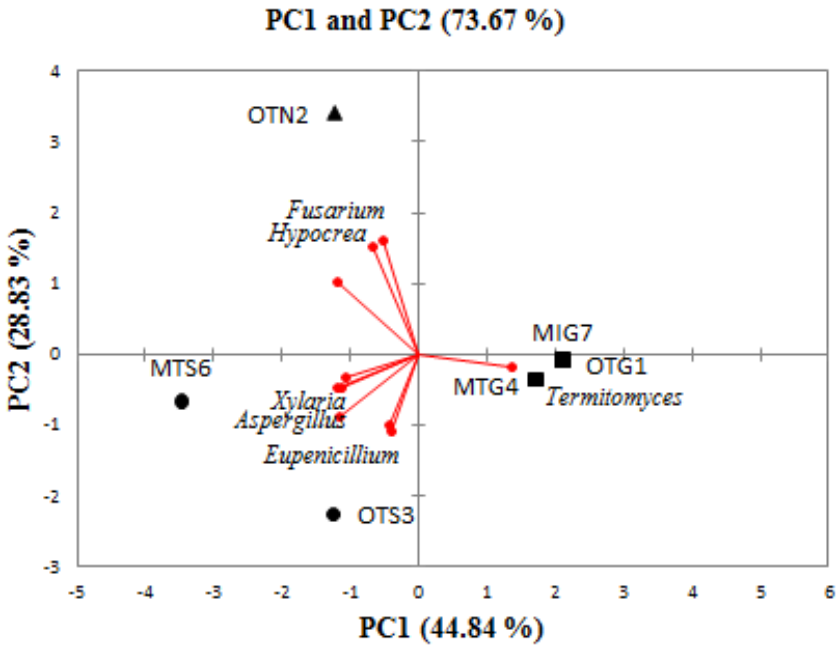

Figure 5. Principal component analysis (PCA) of fungal communities based on the relative abundances of the fungal genera. The vectors indicate the direction and impact of each detected fungal genera on the overall variance. Sample types are marked by the black rectangles, triangles and circles, respectively. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

consequently modify the diversity and composition of fungal communities. Previously, Chen and Cairney (2002) demonstrated that perturbation of Australian forest soils affected the fungal composition while Landeweert et al. (2003) observed difference in basidiomycete community between the organic and mineral horizons. The activities of other biota can modify soil properties and might be the same factor affecting fungal diversity within the same region and vegetation type (McLean and Parkinson, 2000).

The current findings underline the difference on fungal community composition between the gut, mound and surrounding soil. The heterogeneity of the organic matter, occurrence of fungal inhibitors (Chen and Cairney 2002; Lamberty et al., 2001) and the creation of new substrates/or reduced access for fungi in such clay organic complexes could favor some specialized fungal species (Roose-Amsaleg et al., 2004). As a result, fungus-cultivating termites could be regarded, according to Waid (1999), as true metabionts since they create special micro-environments that support specific organisms such as fungi that may adapt, evolve and hence diversify. Such a scenario has been observed on soil-feeding termites; which by modifying the environment drastically affected the soil ascomycete community structure (Roose-Amsaleg et al., 2004).

Soil fungi mediate many biochemical interactions (Bridge and Spooner, 2001) including a variety of associations with plants as pathogens (e.g *Fusarium* spp.), while other genera such as *Aspergillus*, *Penicillium* and *Xylaria* could be saprophytes, necrophilia and even coprophile. Several species of fungi associated with *Reticulitermes flavipes* have been isolated (Zoberi and Grace, 1990), many of which were common saprophytic soil organisms (Barnett and Hunter, 1972). *Mucor mucedo* (L.) Fr. and *Aspergillus niger* Van Tieg. (Steinhaus, 1949) are known to be facultative insect pathogens while *Mucor hiemalis* Weh., was reported as a pathogen of bees. It is worthy of note that the multi-species fungal interactions such as competitive or parasitic interactions (Zoberi and Grace, 1990) among fungi promote termite survival as supported by a number of species associated with living termites.

Conclusion

The findings of this study have demonstrated that members of the genus *Termitomyces* exist in a tight association with their hosts (Rouland-Lefevre, 2000),

hence *Termitomyces* species are scarcely present in the mound and soil. In addition, by altering the habitat, fungus-cultivating termites create microecological niches suitable for some specialized soil fungal species. The use of 454-pyrosequencing has demonstrated the existence of other minor fungal genera in the termite guts other than *Termitomyces,* which is the dominant fungus. This demonstrates that the mutualistic association of the *Termitomyces* with termites is important for their survival. Therefore, further studies should be focused on host-symbiont specificity and physiological roles of the host symbionts for further exploitation particularly in the field of biotechnology.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by a PhD scholarship from the Deutscher Akademischer Austauschdienst (DAAD) and a Grant (NCST/5/003/PG/224) from the Kenya National Commission for Science, Technology and Innovation (NACOSTI). Kenya Wildlife Services (KWS) and National Environmental Management Act (NEMA) provided permits for sample collection in Kenya.

REFERENCES

Aanen DK, de Fine Licht HH, Debets AJM, Kerstes NAG, Hoekstra RF, Boomsma JJ (2009). High symbiont relatedness stabilizes mutualistic cooperation in fungus growing termites. Science. 326:1103-1106.

Aanen DK, Ros VID, De Fine Licht HH, Mitchell J, de Beer ZW, Slippers B, Rouland-LeFevre C, Boomsma JJ (2009). Patterns of interaction specificity of fungus-growing termites and *Termitomyces* symbionts in South Africa. BMC Evol. Biol. 7(115):1-11.

Ahmed BM, Nkunika POY, Sileshi WG, French JRJ, Nyeko P, Jain S (2011). Potential impact of climate change on termite distribution in Africa. Br. J. Environ. Clim. Change. 1:172-189.

Austin JW, Szalanski AL, Cabrera BJ (2004). Phylogenetic analysis of the subterranean termite family *Rhinotermitidae* (Isoptera) by using the mitochondrial cytochrome oxidase II gene. Ann. Entomol. Soc. Am. 97:548-555.

Barnett HL, Hunter BB (1972). Illustrated Genera of lmperfect Fungi. 3rd Ed. Burgess, Min- neapolis. P 241.

Bridge P, Spooner B (2001). Soil fungi: diversity and detection. Plant Soil. 232:147-154.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Meth. 7:335-336.

Chen DM, Cairney JWG (2002). Investigation of the influence of prescribed burning on ITS profiles of ectomycorrhizal and other soil fungi at three Australian sclerophyll forest sites. Mycol. Res. 106:532-540.

Clarke KR (1993). Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117-143.

Collins NM (1983). The utilization of nitrogen sources by termites (Isoptera). *In*: Nitrogen as an Ecological Factor. Eds. Lee JA, McNeill S, Rorison H. 22nd Symposium of British Ecological Society, Oxford. pp. 381-412.

De Bruyn LLA, Conacher AJ (1990). The role of termites and ants in soil modification: A review. Aust. J. Soil. Res. 28:55-93.

Edgar RC (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461.

Fierer N, Lauber CL, Zhou N, McDonald D, Costello EK, Knight R (2010). Forensic identification using skin bacterial communities. Proc. Natl. Acad. Sci. USA. 107(14):6477-6481.

Garnier-Sillam E, Toutain F, Renoux J (1988). Comparison of the action of two termite colonies (soil feeding and fungus growing species) on the structural stability of tropical forest soils. Pedobiology 32:89-97.

Guedegbe HJ, Miambi E, Pando A, Houngnandan P, Rouland-Lefevre C (2009). Molecular diversity and host specificity of termite-associated *Xylaria.* Mycologia. 101: 686-691.

Harry M, Jusseaume N, Gambier B, Garnier-Sillam E (2001). Use of RAPD markers for the study of microbial community similarity from termite mounds and tropical soils. Soil. Biol. Biochem. 33:417-427.

Hawksworth DL (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol. Res. 105:1422-1432.

Holt JA, Lepage M (2000). Termites and soil properties. In Termites: Evolution, Sociality, Symbioses, *Ecology*. Eds. T. Abe T, Bignell DE, Higashi M. Kluwer Academic Publishers Dordrecht. pp. 25-51.

Huse SM, Huber JA, Morrison HG, Sogin ML, Welch DM (2007). Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol. 8(7):R143.

Hyodo F, Inoue T, Azuma JI, Tayasu I, Abe T (2000). Role of the mutualistic fungus in lignin degradation in the fungus-growing termite *Macrotermes gilvus* (*Isoptera; Macrotermitinae*). Soil. Biol. Biochem. 32:653-658.

Hyodo F, Tayasu I, Inoue T, Azuma JI, Kudo T, Abe T (2003). Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (*Macrotermitinae: Isoptera*). Func. Ecol. 17:186-193.

Inward DJG, Vogler AP, Eggleton P (2007). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. Mol. Phylogenet. Evol. 44:953-967.

Jones CG, Lawton JH, Shachak M (1997). Positive and negative effects of organisms as physical ecosystem engineers. Ecology 78:1946-1957.

Ju YM, Hsieh HM (2007). *Xylaria* species associated with nests of *Odontotermes formosanus* in Taiwan. Mycologia. 99:936-957.

Lamberty M, Zachary D, Lanot R, Bordereau C, Robert A, Hoffmann JA, Bulet P (2001). Insect immunity: Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. J. Biol. Chem. 276:4085-4092.

Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003). Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl. Environ. Microbiol. 69:327-333.

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson P, Heal OW, Dhillion S (1997). Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur. J. Soil. Biol. 33:159-193.

Lozupone C, Knight R (2005). UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71:8228-8235.

Lozupone CA, Hamady M, Kelley ST, Knight R (2007). Quantitative and qualitative (beta) diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. 73(5):1576-1585.

Makonde HM, Boga HI, Osiemo Z, Mwirichia R, Stielow JB, Göker M, Klenk HP (2013). Diversity of *Termitomyces* Associated with Fungus-Farming Termites Assessed by Cultural and Culture-Independent Methods. PLoS ONE. 8(2):e56464.

Manuwa SI (2009). Physico-chemical and dynamic properties of termite mound soil Relevant in sustainable food production. Afr. Crop Sci. Soc. 9:356-369.

Martin MM, Martin JS (1978). Cellulose digestion in the midgut of the fungus-growing termites *Macrotermes natalensis*: The role of acquired digestive enzymes. Science 199:1453-1455.

Mathew GM, Ju Y-M, Lai C-Y, Mathew DC, Huang CC (2012). Microbial community analysis in the termite gut and fungus comb of *Odontotermes formosanus*: the implication of *Bacillus* as mutualists. FEMS Microbiol. Ecol. 79:504-517.

McLean MA, Parkinson D (2000). Field evidence of the effects of the epigeic earthworm *Dendrobaena octaedra* on the microfungal community in pine forest floor. Soil Biol. Biochem. 32:351-360.

Mohindra P, Mukerji KG (1982). Fungal ecology of termite mounds. Rev. Ecol. Biol. Sol. 19:351-361.

Moriya S, Inoue T, Ohkuma M, Yaovapa T, Johjima T, Suwanarit P, Sangwani U, Vongkaluang, C, Noparatnaraporn N, Kudo T (2005). Fungal Community Analysis of Fungus gardens in termite nests. Microbes Environ. 20:243-252.

Muwawa EM, Budambula NLM, Osiemo ZL, Boga HI, Makonde HM (2016). Isolation and characterization of some gut microbial symbionts from fungus-cultivating termites (*Macrotermes* and *Odontotermes* spp.). Afr.J. Microbiol. Res. 10(26):994-1004.

Muwawa EM, Makonde HM, Budambula NLM, Osiemo LZ, Boga HI (2014). Chemical properties associated with guts, soil and nest materials of *Odontotermes* and *Macrotermes* species from Kenya. J. Biodiv. Environ. Sci. 4(2):253-263.

Nobre T, Eggleton P, Aanen DK (2010). Vertical transmission as the key to the colonization of Madagascar by fungus growing termites? Proceedings of the Royal Society B: Biol. Sci. 277:359-365.

Nobre T, Fernandes C, Boomsma JJ, Korb J, Aanen DK (2011). Farming termites determine the genetic population structure of *Termitomyces* fungal symbionts. Mol. Ecol. 20:2023-2033.

Okane I, Nakagiri A (2007). Taxonomy of an anamorphic xylariaceous fungus from a termite nest found together with *Xylaria* angulosa. Mycoscience 48:240-249.

Osiemo Z, Marten A, Kaib M, Gitonga L, Boga H, Brandl R (2010). Open relationships in the castles of clay: high diversity and low host specificity of *Termitomyces* fungi associated with fungus-growing termites in Africa. Insect Soc. 57:351-363.

Otani S, Hansen LH, Sørensen SJ, Poulsen M (2015). Bacterial communities in termite fungus combs are comprised of consistent gut deposits and contributions from the environment. Microb. Ecol. 71(1):207-20.

Otani S, Mikaelyan A, Nobre T, Hansen LH, Koné NGA, Sørensen SJ, Aanen DK, Boomsma JJ, Brune A, Poulsen M (2014). Identifying the core microbial community in the gut of fungus-growing termites. Mol. Ecol. 23:4631-4644.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucl. Acids. Res. 41(DI):D590-D596.

R Core Team (2012). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Vienna, Austria. URL http://www.R-project.org/.

Rideout JR, He Y, Navas-Molina JA, Walters WA, Ursell LK, Gibbons SM, Chase J, McDonald D, Gonzalez A, Robbins-Pianka A, Clemente JC, Gilbert JA, Huse SM, Zhou H, Knight R, Caporaso JG (2014). Subsampled open-refer-ence clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. Peer J. 2:e545.

Roose-Amsaleg C, Brygoo Y, Harry M (2004). Ascomycete diversity in soil-feed-ing termite nests and soils from a tropical rainforest. Environ. Microbiol. 6:462-469.

Rouland-Lefévre C (2000). Symbiosis with fungi. In: Termites: evolution, society, symbioses, ecology. Eds. Abe T, Bignell DE, Higashi M. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 289-306.

Steinhaus EA (1949). Principles of Insect Pathology. McGraw-Hill, New York. P 757.

Traniello JFA, Rosengaus RB, Savoie K (2002). The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. Proc. Natl. Acad. Sci. USA. 99:6838-6842.

Visser AA, Nobre T, Currie CR, Aanen DK, Poulsen M (2012). Exploring the potential for actinobacteria as defensive symbionts in fungus-growing termites. Microb. Ecol. 63: 975-985.

Visser AA, Ros VID, De Beer ZW, Debets AJM, Hartog E, Kuyper TW, Laessøe T, Slippers B, Aanen DK (2009). Levels of specificity of *Xylaria* species associated with fungus-growing termites: A phylogenetic approach. Mol. Ecol. 18:553-567.

Waid JS (1999). Does soil biodiversity depend upon metabiotic activity and influences? Appl. Soil Ecol. 13:151-158.

White TJ, Bruns TD, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications. New York: Academic Press. pp. 315-322.

William AS (1969). The association of termite and fungi In: Kummer K, Frances MW (eds) Biology of the termite. Academic Press, New York, USA, pp. 495-524.

Zoberi MH, Grace JK (1990). Fungi associated with the subterranean termite *Reticulitermes flavipes* in Ontario. Mycologia. 82:289-294.