Molecular phylogenetic study of fungal symbionts of fungus-growing termites and DNA barcoding of termites for biodiversity assessment in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my husband Christopher and baby Esther, parents, brother John and other siblings for your prayers, support and encouragement during the study.

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ABBREVIATIONS

A	Adenine
AIC	Akaike Information Criterion
BLAST	Basic Local Alignment Search Tool
BioEdit	Biological Editor
bp	base pairs
C	Cytocine
°C	centigrade Celsius
COI	Cytochrome c oxidase subunit I gene
COII	Cytochrome c oxidase subunit II gene
CNI	Close-Neighbor-Interchange
DNA	DeoxyriboNucleic Acid
dNTP	deoxyribonucleoside triposphate (dATP, dCTP, dGTP, dTTP)
EB	Elution Buffer
G	Guanine
GTR+G	General Time Reversible plus Gamma
h	hour
HKY+G	Hasegawa-Kishino plus Gamma
hLRT	hierarchical Likelihood Ratio Tests
ITS1	Internal Transcribed Spacer1
ITS2	Internal Transcribed Spacer2
K2P	Kimura-2-Parameter
ln L	log Likelihood
m	minutes

MCMCMC	Metropolis Coupled Markov Chain Monte Carlo
ML	Maximum Likelihood
MP	Maximum Parsimony
mtDNA	mitochondrial DNA.
MEGA	Molecular Evolutionary Genetics Analysis
MOTU	Molecular Operational Taxonomic Units
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
NUMTs	nuclear mitochondrial DNA sequences
PCR	Polymerease Chain Reaction
rDNA	ribosomal DNA
sec	seconds
SH	Shimodaria and Hasegawa
T	Thymine
TBR	Tree-Bisection-Reconnection
TL	Tissue Lysis
TrN+G	Tamura-Nei plus Gamma
µl	micro-litre

ABSTRACT

Termites are among the most important decomposers in tropical ecosystems. Their taxonomy to the species level mostly relies on characters of soldiers. The workers are morphologically uniform, and thus exhibit only few traditional taxonomic characters for species identification. However, they are the most frequently sampled caste during field sampling. Therefore, the diversity of termites is poorly understood. Recently, the use of DNA-sequences (barcoding) has become more important for inventory and biodiversity assessment of hyperdiverse taxa and those which are difficult to identify. An approach towards establishing a DNA barcode library for termite species identification and biodiversity assessment using sequences of the mitochondrial COII gene is presented in this study. At least 16 termite species were observed in Kakamega Forest by morphological identification whereas at least 22 species (MOTUs) were found by molecular species delimitation. This study highlights the advantage that molecular based species delimitation reveals some morphological cryptic species. Furthermore, termite workers can be assigned to their respective phylogenetic clusters. Recent studies on fungus-growing termites based on genetic markers have largely increased knowledge about the evolutionary history of the symbiosis between fungus-growing termites and their fungi. Studies focusing on interaction specificity have been conducted elsewhere in Africa but none so far has been done on Eastern Africa despite the high generic richness of host termites in this area. The phylogenetic relationship among *Termitomyces* sampled in Kenya showed strong sequence divergence among host termite genera, showing high interaction specificity. However, within the clade associated with the host genus *Macrotermes*, fungi lineages occurred in several species indicating low host specificity. Furthermore, same lineages occurred across steep environmental gradients. Therefore, the association of the fungal lineages with

several host species is not the result of an allopatric distribution of fungi among climatic regions.

CHAPTER ONE

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Termites (Phylum, Arthropoda; Class, Insecta and Order, Isoptera) are the dominant organisms in tropical and subtropical ecosystems (Wood and Sands 1978). Their complex social lifestyles have made them incredibly successful throughout the tropics, although some can be found in the temperate zones and very few in cooler climates. They are recognized to be the most important decomposer animals in lowland tropical ecosystems. They can make up to 95% of the soil insect biomass (Eggleton *et al*., 1996), where they decompose dead wood, helping it to be reclaimed quickly by the soil. They play a key role in controlling carbon and nitrogen fluxes both in semi arid and humid ecosystems such as savannas and tropical rain forests. In the tropical forests, they have a positive effect on soil fertility. Their potential impact on agriculture is receiving increasing attention (Black and Okwakol, 1997). They are phylogenetically divided into lower (primitive) and higher (advanced) termites. Higher termites comprise three quarters of all described termite species. They differ from lower termites (all families except Termitidae) by having bacteria rather than protozoa in their guts. About 95% of higher termites occur in a single family, the Termitidae which not only constitute the majority of species, but also exhibit the widest range of ecological and behavioral diversity, including many traits fundamental to the evolution of the Isoptera, such as soil-feeding and fungus-growing (Abe *et al.*, 2000).

The fungus growing termites (Macrotermitinae) are the largest and important groups within family Termitidae. They are the only groups which have developed an ectosymbiotic relationship with fungi *Termitomyces*. They play major role in decomposition of dead plant material by living in an obligate mutualistic symbiosis with the *Termitomyces* fungi. The close association between Macrotermitinae and *Termitomyces* seems to be the result of a long coevolutionary process, which has a single African origin with a lack of secondary domestications of other fungi or reversal of mutualistic fungi to a free-living state (Aanen, *et al*., 2002). One posed question is "At what level is the hypothesis of host termite and symbiont coevolution acceptable?" There are still some uncertainties about the level of the host specificity in the fungi.

Global surveys have shown that termites in general have their highest species densities in tropical rain forests, where much of the termite group's radiation has occurred (Abe *et al.*, 2000*).* The ecological importance of termites, together with their uniquely derived social structures and cooperative behavior, made it appropriate for an investigation on their true diversity in tropical forests to be carried out. Subsequently, Kakamega forest, a forest which is a hotspot of biodiversity in Kenya, and which is threatened by the increasing human population was chosen for this study. There is increasing loss of biodiversity caused by human activities which may lead to extinction. Termites constitute one of the hyper diverse invertebrate groups, which are difficult to classify, and so their species diversity is poorly understood. Most biodiversity studies have remained vertebrate centered and yet the invertebrate animals comprise over 90% of animal species and over 80% of all of life on earth,

accounting for their importance in ecosystem functioning (LaPolla and Sinclair, 2007).

Termites comprise of a caste system based on their functional roles. The reproductive caste consists of the queen and the king. The soldier soldier caste consists of non fertile males and females with special morphological adaptations for defense of the colony. The worker caste comprises of immature (nymphs) male and female offspring of the king and queen, with potential to molt into replacement soldiers or reproductives if needed. These are the largest caste in the colony. The taxonomy as well as the classification of termites to the species level mostly relies on characters of soldiers. However, workers are the most frequently sampled caste during ecological surveys. Recently, the use of DNA-sequences (barcoding) has become more important for inventory and biodiversity assessment of hyperdiverse taxa as well as taxa which are difficult to determine. While DNA barcoding can never replace other types of data such as morphology and behavior in the delineation of species, it promises to provide a quick, informative tool that will greatly facilitate species identification and adds another tool available to taxonomists (Smith *et al.*, 2006). Furthermore, it is promising to speed up the processing of biodiversity studies, which is essential if diverse groups of invertebrates are to be used in conservation planning (Groombridge, 1992).

1.2 Literature review

1.2.1 Ecological importance of termites

Termites have a great impact on the environment and humans as a result of their feeding. They affect plant communities, soil fertility and human activities and nutrition (Lee and Wood, 1971). Ecologically, termites are important in nutrient recycling, as direct mediators of decomposition, humification, soil formation and conditioning, fragmentation of organic detritus and formation of clay-mineral complexes (Lee and Wood, 1971; Martius *et al.*, 1994; Tayasu *et al.*, 1994). Majority of termite species have humivorous mode of nutrition. They play a key role in controlling carbon and nitrogen fluxes both in semi arid and humid ecosystems such as savannas and tropical rain forests (Wood and Johnson, 1986; Collins, 1989). Humivore mounds may contain phosphorus concentration over four times that of adjacent soil (Anderson and Wood, 1984). Water and fine clay particles are brought up from the soil depths and subterranean galleries may trap water otherwise lost to superficial layers (Lee and Wood, 1971). By fixing nitrogen, termites bring atmospheric element into the terrestrial domain (Collins, 1981). Their potential impact on agriculture especially in enhancing soil fertility is receiving increasing attention (Black and Okwakol, 1997).

Termites serve as food for countless predators, for example they serve as animal feed in aquaculture or poultry production. They also serve as diet supplement for humans in the tropical areas, especially flying alates and large bodied species. In particular seasons, *Termitomyces* mushrooms appear which are unique in nature blooming only

from termite nests (Plate 1.1). They are commercially important due to their high price especially the edible ones, contributing to the local economy. Since they have long pseudorhiza which connects to the surface of the fungus comb, they have been considered to be symbiotic fungi found on fungus comb as mycelia and fungus nodules. They also help in degradation of plant cellulose debris to useful end product thus eliminating problems associated with accumulation of debris (Lee and Wood, 1971). Their pest status comes from the damage they wreak on wooden structures, plantation trees and crops.

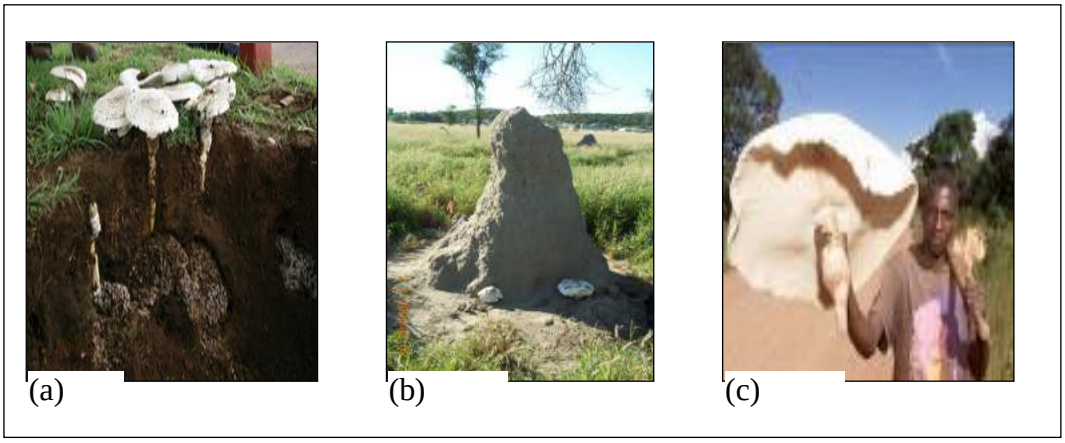

Plate 1.1 Termitomyces mushrooms blossoming from a fungus comb inside a termite mound (a, b). The sizes varry depending on the species, some can be so large (c).

Termites build gigantic mounds inside of which they farm a fungus that is their primary food source. The fungus must be kept at exactly 87 ºF, while the temperatures outside range from 35 ºF at night to 104 ºF during the day. The termites achieve this remarkable feat by constantly opening and closing a series of heating and cooling vents throughout the mound over the course of the day. With a system of carefully adjusted convection currents, air is sucked in at the lower part of the mound, down into enclosures with muddy walls, and up through a channel to the peak of the termite mound. The industrious termites constantly dig new vents and plug up old ones in

order to regulate the temperature. The Eastgate Centre (Shopping Mall) in Harare, Zimbabwe, largely made of concrete, has a ventilation system which operates in a similar way. This is a good example of an architectural marvel which has been constructed using biomimicry principles in Africa. The outside air that is drawn in is either warmed or cooled by the building mass depending on which is hotter, the building concrete or the air. It is then vented into the building's floors and offices before exiting via chimneys at the top. The complex also consists of two buildings side by side that are separated by an open space that is covered by glass and open to the local breezes. The building, designed by architect Mick Pearce, has no conventional air-conditioning or heating, yet stays regulated year round with dramatically less energy consumption using design methods inspired by indigenous Zimbabwean masonry and the self-cooling mounds of African termites!

1.2.2 Recent advances in termite phylogeny and taxonomy.

Recent molecular phylogenetic analyses of termites to establish their relationship with other insects has revealed that termites (Class Insecta, order Isoptera) could be treated as a family (Termitidae) of order Blattodea comprising of cockroaches (Inward *et al*., 2007). Previously, termites (Isoptera) were known to be phylogenetically related to cockroaches and mantids (Abe *et al*., 2000). Taxonomically termites comprise of 7 families, 21 subfamilies, around 280 genera and over 2800 described species (Table 1.1). They are phylogenetically divided into lower (primitive) and higher (advanced) termites. Higher termites are the most evolved and divergent group comprising of only Termitidae. Lower termites comprise of Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae and Serritermitidae.

A number of termite phylogenetic studies have already been attempted based on both morphological characters (Ahmad, 1950; Donovan *et al*., 2000; Krishna, 1968; Miller, 1986; Noirot, 1995a and b) and also molecular methods (Kambhampati and Eggleton, 2000; Austin *et al*., 2002; Aanen *et al*., 2002). All these are aimed at solving long-term taxonomic problems concerning termites. There are significant disagreements and contradictions among the proposed taxonomy and phylogeny of termite genera. Even family- and subfamily-level classifications sometimes discord, preventing them from being reliable and convincing, due to poor taxon sampling. Most of them are sampled either from one biogeographic region (Ohkuma *et al*., 2004), or bias towards the less species-rich non-termitid families (e.g. Kambhampati *et al*., 1996). Other problems include a lack of any rigorous cladistic analysis of the available data, difficulty of dealing with the soldierless termites (Termitidae: Apicotermitinae) which generally lack good diagnostic characters but which comprise large numbers of species, many of them undescribed (Eggleton *et al*., 2001). Morphological characters, especially those of the soldier caste, are strongly influenced by defense or feeding strategies, so homoplasious convergence in form is frequent (Donovan *et al*., 2000). Molecular sequence data on the other hand, provide a wealth of characters that should be free of such non-heritable variation, and can be produced in an unambiguous manner. It is now universally accepted that DNA sequences are a rich source of taxonomic characters for estimating phylogenies (Kambhampati and Eggleton, 2000). The sequences are not expected to vary among castes and developmental stages, hence solving the main problem of absence of soldier castes in some specimens and groups.

1.2.3 Distribution and ecology of termites

The termites inhabit approximately 75% of the Earth's land surface and are distributed between latitudes 45°N and 45°S (Lee and Wood, 1971; Wood, 1988). Of the approximately 275 genera in the order, only thirteen have cosmopolitan distributions. The vast majority of genera are restricted to one or two continents.

The lower termites eat primarily woody materials, with exception of grass-feeding Hodotermitidae, and harbor both protozoans and bacteria as intestinal digestive symbionts (Krishna, 1968). The higher termites show considerable variation in their feeding behaviour, consuming wood, grass, litter, which is not limited to xylophagy (Table 1.1). Some feed exclusively on soil, presumably deriving nutrition from the humic compounds therein, and others 'cultivate' and consume cellulolytic fungi (Ohkuma *et al*., 1999).

Table 1.1 Classification of termite with their distribution, nest sites and major digestive symbionts

		Genera	Species	Distribution	Nest site	Food	Symbionts
Order Isoptera							
Family	Mastotermitidae	1	1	Australia	DW, LW, S, TN	DW, LW	B, P
Family	Hodotermitidae	3	19	Africa, Eurasia, Orient	E, S	G	B, P
Family	Termopsidae						
	Subfamily Porotermitinae			Australia, Chile, Africa	DW, LW	DW, LW	B, P
	Subfamily Stolotermitinae			Africa, Australia	DW	DW	B, P
	Subfamily Termopsinae	5	20	Eurasia, North America	DW, LW	DW, LW	B, P
Family	Rhinotermitidae						
	Subfamily Coptotermitinae			Worldwide	DW, E, LW, S	DW, LW	B, P
	Subfamily Heterotermitinae			Worldwide	S	DW, LW	B, P
	Subfamily Prorhinotermitinae			Worldwide	DW	DW	B, P
				(islands and shores)			
	Subfamily Psammotermitinae			Africa, Eurasia, Orient,			
				South America (arid areas)	S	DW	B, P
	Subfamily Stylotermitinae			Orient	DW, LW	DW, LW	B, P
	Subfamily Termitogetoninae			Orient	DW	DW	B, P
	Subfamily Rhinotermitinae	14	343	Worldwide	DW, LW, S, TN	DW, LW,	B, P
Family	Serritermitidae	1	1			S, TN	
Family	Termitidae	236	1958	Worldwide			
	Subfamily Macrotermitinae	14	349	Africa, Orient	E, S, TN	DW, FC,	B, FC
						G, L	
	Subfamily Sphaerotermitinae						
	Subfamily Foraminitermitinae						
	Subfamily Apicotermitinae	43	202	Worldwide	A, S, TN	H	B
	Subfamily Syntermitinae						
	Subfamily Termitinae	43	288		A, E, S, TN	DW, G, H,	B
					L, TN		
	Subfamily Nasutitermitinae	91	663	Worldwide	A, E, DW,	A, E, DW,	B
					LW, S, TN	LW, S, TN	
	Subfamily Amitermitinae	17	295				
	Subfamily Cubitermitinae	28	161				
Family	Kalotermitidae	22	419	Worldwide	DW, LW	DW, LW	B, P

Abbreviations: A, arboreal; B, bacteria; DW, dead wood; E, epigeal mounds; FC, fungus comb; G, grass; H, humus; L, litter; LW, live wood; P; protozoans; S, subterranean; TN, termite nests (adopted from Inward et al., 2007).

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1.2.4 The fungus-growing termites

Macrotermitinae, a subfamily within Termitidae, is a large and ecologically important group in Africa and southern Asia (Davison *et al*., 2001). It is the only subfamily which cultivates fungi hence called fungus-growing termites. The family Termitidae, which is considered to be the most evolved group of termites, is the largest with approximately 85% of all the known genera and nearly 70% of the species. Macrotermitinae is divided into 11 taxonomically well supported genera with approximately 330 species. The genera include Macrotermes, Odontotermes, Microtermes, Pseudacanthotermes, Acanthotermes, Megaprotermes, Parahypotermes, Protermes, Shaerotermes, Synacathotermes, Allodotermes, Ancistrotermes, Euscaiotermes and Hypotermes. More than half of the described species are assigned to the genus Odontotermes (Holmgren, 1912).

The distribution of Macrotermitinae indicates a fairly late evolutionary origin (compared to other termite subfamilies) in the late Oligocene (Emerson, 1955). The subfamily appears to have originated in Africa and later spread to the Middle East and southern Asia. It has not yet reached Ausralasia or the New World (Darlington, 1994). Most of the diversity occurs in Africa, where 10 of the 11 genera are found (Davison *et al*., 2001). Five genera occur in Asia (one of these exclusively) and two genera in Madagascar (Kambhampati and Eggleton, 2000). They have great impact in most Africa and Asian ecosystems, with Macrotermitinae reprocessing more than 90% of dry wood in Kenya, where it exists in abundance (Buxton, 1981).

1.2.5 Termite-fungus interactions in Macrotermitinae

Macrotermitinae is the only subfamily within family Termitidae, which has developed an ectosymbiotic relationship with fungi of genus *Termitomyces* (Darlington, 1994). Their relationship is highly efficient and sophisticated, exemplifying a stable symbiosis with an even higher degree of interdependence and co-adaptation, mainly to decompose dead wood especially in Asian and African tropics (Davison *et al*., 2001). The fungus-growing termites and the fungi appear to be obligately dependent on each other (Batra and Batra, 1966*;* Johnson, 1981*)*. The termites feed on dead plant tissues, which are rich in cellulose and lignin, but poor in nitrogen. They are able to obtain nutrient-rich food through this relationship (Darlington, 1994). The fungi degrade lignin, plant-derived material, into substances that can easily be used by the termites, increasing the Nitrogen:Carbon ratio in the termites' diet. The termite in turn provides optimal microclimate for fungal cultivation by their elaborate thermoregulation in the nests. They also selectively inhibit other fungi and microbial infections by their secretions (Darlington, 1994*)*.

The fungus grows on a special convoluted structure in the nest, the fungus comb (1.1a). Fungus combs are housed in specially constructed termite mounds (plate 1.1b), maintained by the termites through continuous addition of pre-digested plant substrate. They are largely organic constructs with very distinct architecture that varies in different taxa of termites. Fecal pellets produced by termite workers are added to the top rim of the comb. Fungal mycelium develops rapidly within it. After a period of about two weeks the fungus begins to produce vegetative fruiting bodies in form of white small nodules (plate 1.2a). The nodules and older comb material

containing fungal hyphae are consumed by the termites (Darlington, 1994; Bignell and Eggleton, 2000).

As compared to host termite, approximately 40 species of the *Termitomyces* symbionts have been described (Kirk, 2001). However, DNA-based phylogenetic studies (Aanen *et al.*, 2002; Froslev *et al.*, 2003) suggest that many more species could be existing. Although additional strains have been recognized by molecular data, the low number of fungal species suggests that many of these fungi are shared by different termite species in the symbiotic relationship.


(a)


(b)

Plate 1.2 The fungus comb (a) where the fungus grows, the nodules are attached on it. Fungus combs are housed inside the termite mound (b).

1.2.6 Evolution of fungus growing termites

DNA sequence data have been used to estimate phylogenies of fungus-growing termites and their associated *Termitomyces* species (Rouland-Lefèvre, 2000; Aanen *et al.*, 2002). The latter authour provided strong evidence for a single origin of

symbiosis because both termites and their fungal symbionts were well-supported monophyletis groups. Therefore the close association between Macrotermitinae and *Termitomyces* seems to be the result of a long coevolutionary process which has a single African origin with a lack of secondary domestications of other fungi or reversal of mutualistic fungi to a free-living state (Aanen *et al.*, 2002). The high degree of specificity of termite-fungus associations mainly at genera level clearly demonstrates that coevolution has occurred. Some distinct traits of these termites and their fungi are likely to be a direct consequence of this long coevolution. For example the elaborate thermoregulation in nests leading to constantly high temperatures and high relative humidity, hence providing the optimal microclimate for cultivation of the fungi (Lüscher 1961; Korb and Linsenmair 1999, 2000a, 2000b). Furthermore, the strong enzymatic capacities of *Termitomyces* allow the termites to feed on plant structural components hard to degrade such as cellulose or lignin (Rouland-Lefèvre *et al.*, 1991). The fungal cellulases and xylanases work synergistically and complementarily with the endogenous gut enzymes. This has been suggested to be the result of tight coevolution (Martin and Martin, 1978, 1979; Rouland-Lefèvre *et al.*, 1988a)

Rouland-Lefèvre *et al.*, (2000) suggested that the roles of acquired enzymes in termite nutrition may be variable and that digestion and decomposition cascades may operate within the same genus of fungus-growing termites. Within the enzyme-producing *Termitomyces* in general, two different substrate specificities can be distinguished: (1) generalist symbionts that produce a large diversity of enzymes irrespective of the substrate and are associated with several host species; (2)

specialist symbionts that only produce a single enzyme on their 'natural' comb substrate and are thus associated with a single termite host.

Complex phylogenetic relationships (patterns) have been reported by several workers between host and their fungal symbionts (Aanen *et al*., 2002; Katoh *et al.,* 2002; Rouland-Lefèvre *et al.,* 2002; Taprab *et al.,* 2002). Most of the findings are limited in sampling and lack of robust statistics to reconstruct these phylogenies in resolving the relationship. There are still some uncertainties about the level of the host specificity in fungi (host genera and host species). A reliable phylogeny using current and improved phylogenetic analyses is required in order to understand this symbiotic relationship and determine whether coevolution forces have shaped the relationship in a host species specific way or genus specific.

1.2.7 Termite DNA barcoding for taxonomy and biodiversity assessment

One idea that has grown in popularity to overcome both the taxonomic impediment and the large scale processing of biodiversity samples has been the advent of DNA barcoding (Herbert *et al.,* 2003). This involves molecular identification and cataloguing of species based on a defined part of genome (Floyd *et al*., 2002; Hebert *et al*., 2003a). Barcoding has been considered a rapid and effective tool towards biodiversity assessment of various organisms by enhancing morphological taxonomic efforts (Tautz *et al*., 2003; Seberg *et al*., 2003). Recently, one study has been conducted for barcoding ants from Madagascar. It shows that barcodes are effective and rapid tool for biodiversity assessment of the ants (Smith *et al*., 2005).

DNA barcoding as first proposed by Hebert *et al.* (2003a), was founded in September 2004 and implemented in the "Barcoding Life" consortium (Hebert *et al*., 2004a). It intends to create a global biodiversity barcode database of animal life on Earth in order to facilitate automated species identifications. It was suggested that sequencing of a small (648 bp) fragment at the 5' end of the Cytochrome Oxidase I (COI) gene from the mitochondrial genome would be sufficient in most Metazoa to identify them to the species level (Herbert *et al.*, 2003). This will form a library of sequences linked to vouchered specimens and make a sequence an identifier for species that will be known as a "DNA barcode" (Hebert *et al*. 2003a). In a barcoded world, an unidentified specimen will be determined based on its COI sequence, whichwill be matched to an identified DNA barcode from a publicly available database. DNA barcoding and DNA taxonomy utilize sequences from GenBank (Barrett and Hebert, 2005; Hebert *et al*., 2003b). Although the GenBank is known to include misidentified sequences (Ruedas *et al*., 2000; Harris, 2003; Hebert *et al*., 2003b; Vilgalys, 2003; Seberg, 2004) currently it is the only database that can provide data that are rich in congeneric sequences and have a similar submission profiles as future barcode databases. For termites, use of Cytochrome Oxidase II (COII) gene sequences for barcoding is appropriate because a lot of such sequences have been published in GenBank.

1.2.8 Defining a molecular threshold for termite species delimitation

Genetic distances of sequence data showing sequence divergences within and among taxonomic groups can be delineated into Molecular Operational Taxonomic Units (MOTUs) to give species identification tags based on sequence divergence thresholds in barcoding system. Sequence divergence threshold represents just one possible

means to determine MOTUs (Sites and Marshall, 2003; DeSalle and Amato, 2004). There has been considerable interest recently in the use of haplotype phylogenies from DNA sequence data to infer species boundaries (Avise and Ball, 1990; Baum and Donoghue, 1995; Graybeal, 1995). The major problem with defining this threshold value is when there is a wide range of overlap between intra- and interspecific divergence values. The choice of threshold value for distinguishing intra- from interspecific distances has been largely arbitrary in evaluating species limits (Ferguson, 2002; Will and Rubinoff, 2004; DeSalle *et al*., 2005). It is necessary to define threshold values that ideally provide a sharp distinction between intraspecific and interspecific divergence values. If an unknown sequence differs from the closest reference sequence by a divergence above the threshold, the individual from whom the sequences were obtained belongs to a candidate species, which means that its taxonomic status merits further investigation. For mammals, a threshold at 11% of the cytochrome *b* gene, whereas a cox1 threshold of only 2.7% for birds was proposed (Hebert *et al*., 2004b). These authors proposed a standard sequence threshold which should be 10 times the mean intraspecific variation observed. By giving species molecular identification tags (MOTUs) known as DNA Barcodes, they could be used as type specimen for future reference to identify other specimens by analyzing their DNA rather than analysing DNA from morphologically identified specimens.

Another possibility is using tree-based species identification techniques, which rely on clustering as an indication for identification. Clustering requirement assumes that species should be monophyletic (Wheeler and Meier, 2000). Most studies use tree-based identification tools based on NJ trees (Floyd, 2002; Hebert *et al*., 2003a; Tautz *et al*., 2003; Barrett and Hebert, 2005). Queries are considered successfully identified

when they cluster with conspecific barcodes. Both sequence divergence and NJ trees can be used in combination with morphological data to give barcodes in various organisms. Hebert and Gregory (2005) have emphasized that DNA barcoding seeks merely to aid in delimiting species i.e to highlight genetically distinct groups exhibiting levels of sequence divergence suggestive of species status and that by themselves, they are never sufficient to describe new species. At some stage, clearly divergent DNA barcodes, in combination with morphological and physiological information, will be used as the basis for providing new Linnaean names (Smith *et al*., 2005).

1.3 Statement of the problem

The identification of termites and their associated fungal symbionts using classical taxonomic approaches is sometimes almost impossible because of extensive morphological similarity among termite species (especially the soildiers) or absence of basidiocarps in the fungi. Therefore, the diversity of termites and termitomyces is poorly understood and especially in tropical forests which are threatened by increasing human destruction. The recent establishment of molecular markers for their determination and phylogenetic reconstruction, has opened new horizons for studying the evolutionary history of the symbiosis between fungus-growing termites and their cultivated fungi. Phylogenetic studies have so far been conducted in South, West and Central Africa but none has focused specifically on Eastern Africa despite the high richness of host termites in this area. This underlines the need for recent research in this region.

1.4 Justification of the study

Recently there has been an increasing interest in the relationship between fungus-growing termites and their *Termitomyces* fungal symbionts. This revival is caused mainly by the establishment of molecular markers, which has opened new horizons for the study of these unique mutualistic ectosymbiosis. With classical taxonomical approaches, certain determination of the interacting partners is sometimes almost impossible because of extensive morphological similarity among termite species or lack of basidiocarps of the fungi. But recent studies based on genetic markers have largely increased our knowledge about the evolutionary history of the symbiosis between fungus-growing termites and their fungi (Rouland-Lefèvre *et al.*, 2002; Aanen *et al.*, 2002).

Although comparable studies on *Termitomyces* have been conducted in West and Central Africa, South Africa and Southeast Asia, no one so far has focussed specifically on Eastern Africa, Kenya, despite the highest generic richness of host termites in this region. At the moment the only known taxonomic study on *Termitomyces* with focus on Eastern Africa was carried out by Otieno (1964) who described four new species of the genus found in Kenya and referred to one more species from Ethiopia. But many more species especially in Eastern Africa are expected. A review by Wood and Thomas (1989) listed four *Termitomyces* species for East Africa associated with at least two termite species but disregarded the fungus species described by Otieno (1964). This still fragmentary knowledge underscores the need to conduct further research in this region.

Termites are among the most important decomposer in tropical ecosystems, where they can make up to 95% of the insect biomass in soil. Specialized taxonomic knowledge is rarely available for their routine identifications. Species level identification and analyses are required for one to do accurate biodiversity assessment. The level of overall biodiversity is thought to be highest in tropical forest ecosystems (Marshell, 1992). However, these ecosystems are among the most threatened by changes in land use (Harcourt, 1992). Kakamega forest is the eastern extension of the Congo forest block reaching western Kenya. This isolated forest is a hotspot of biodiversity in Kenya and threatened by the increasing human population. The species composition of termites in this forest is not yet known. This may be largely because of the difficulty of species-level identifications. The taxonomy as well as the determination of termites to the species level mostly relies on characters of soldiers. However, workers are the most frequently sampled caste during ecological surveys. Therefore, the diversity of termites in general is poorly understood and especially in tropical forests. Recently, the use of DNA-sequences (barcoding) has become more important for inventory and biodiversity assessment of hyperdiverse taxa as well as taxa which are difficult to determine. Hence DNA sequences can be used to barcode termites starting with available sequences in GenBank which are published for phylogenetic studies, together with new sequences to help in contributing to solving termite taxonomy impediment and biodiversity assessment.

1.5 Hypotheses

1. The fungal symbionts are strongly associated with their termite hosts at genus level.

2. *Termitomyces* strains are widely distributed across Kenya and not restricted to certain biome.

3. Molecular based termite species delimitation will reveal morphologically cryptic species using a COII molecular divergence threshold.

4. There is higher termite species diversity in Kakamega forest using DNA barcodes as compared to morphological identification.

1.6 Objectives

1.6.1 General objective

To investigate the phylogenetic relationship of the fungal symbionts of the fungus-growing termites and assess diversity of termites using DNA barcodes.

1.6.2 Specific objectives

1. To establish the taxonomic level at which fungal symbionts are associated with their termite hosts

2. To determine the diversity of fungal symbionts cultivated by the fungus-growing termites in Kenya

3. To define a molecular divergence threshold for termite species delimitation.

4. To assess termite species diversity in Kakamega forest, Kenya using DNA barcodes.

CHAPTER TWO

2.0 GENERAL MATERIALS AND METHODS

2.1 Sampling of *Termitomyces* and termites

Termitomyces symbionts of fungus growing termites were collected directly from nests of their respective termite host across different geographical regions in Kenya. Termite nests (Plate 2.1a) were dug and opened up to the nest chamber to expose the fungus combs containing nodules (Plate 2.1b). Fungus nodules from fungus combs or with fungus combs, were carefully picked up using sterile forceps, initially rinsed with 0.6% sodium hypochlorite solution and then washed with sterile water and either stored in absolute alcohol or air dried to await DNA extraction. For each strain of fungi, soldiers and workers of the associated termites were collected and classified using morphological features. Termites in Kakamega forest were sampled across a gradient from primary forests to farmland to assess the species diversity in the forest.

(a) (b)

Plate 2.1 The termite mound of (a) *Macrotermes jeanneli* (Marigat) and (b) *Macrotermes michaelseni* (Kajiado), Kenya. The fungus garden is housed inside the mounds.

2.2 Termite identification

The specimens sampled were identified immediately upon collection using morphological features up to genera level by inventory specialists in the forest. They were then preserved in absolute ethanol. The termites were then morphologically identified to species level by J.P.E.C. Darlington of Cambrige University, UK. The specimen which could not be identified, due to the absence of soldiers were grouped using special features, mostly in regard to their size.

2.3 DNA isolation from *Termitomyces*

DNA was isolated by crushing about two fungal nodules (~15mg) in sterile 1.5 ml micro centrifuge tube using sterilized micro pestles using DNeasy® Tissue Kit (Qiagen). 20 µl Proteinase K was used to digest the tissue in 1.5-ml micro centrifuge tube containing 180µl of ATL Buffer, which was then incubated at 55ºC overnight.

2.4 DNA isolation from Termites

Each termite specimen was washed with distilled water in a petri dish to remove ethanol from the previously ethanol-preserved specimens. Whole specimen or head and thorax or all six legs, were used for DNA extraction. In some cases the presence of certain mineral or organic matter in the gut was found to inhibit PCR amplification, particularly in soil-feeding termites, so the abdomen or gut was subsequently removed from specimens before extraction. Soldiers or alates were preferentially used for DNA extraction because their large muscles gave more DNA. The specimen was homogenized in 180 µl of ATL Buffer, to release DNA followed by incubation with Proteinase K at 55 ºC for at least 3 hrs. DNA solution was appropriately eluted by

using EB buffer using DNeasy® Tissue kit. The eluted DNA was measured using Photometer and stored at –23°C for use as template for PCR.

2.5 DNA amplification of the ITS region of *Termitomyces*

The PCR reaction was performed in 20µl of PCR mix composed of 4.0µl, 10× PCR-buffer, 3.0µl of dNTP mix (2mM of each dNTP), 1µl of each primer (10mM), 0.2µl of *Taq* polymerase ($5U\mu l^{-1}$), 8.8µl of PCR water and 2.0µl of DNA ($10ng\mu l^{-1}$) template. Temperature profile for DNA amplification included, initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 1.3 min, an annealing step at 48°C for 1.5 min, an extension step at 72°C for 2 min and a final elongation of 72°C for 10 min then 4°C soak. The following primers by White *et al.*, (1990) were used:

forward, 5'CTTGGTCATTTAGAGGAAGTAA3'(ITS1-F),
'TCCGTAGGTGAACCTGCGG3' (ITS1).

reverse, 5'CAGGAGACTTGTACACGGTCCAG3'(ITS4-B),
5'TCCTCCGCTTATTGATATGC3' (ITS4).

For every experiment, a negative control was set up containing PCR water instead of the template DNA.The PCR cycling parameters were, initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 1.3 min, an annealing step at 48°C for 1.5 min, an extension step at 72°C for 2 min and a final elongation of 72°C for 10 min then 4°C soak. Amplifications were performed in a thermocycler (Eppendorf Mastercycler, Hamburg, Germany).

2.6 DNA amplification of COII gene region of Termite mtDNA

PCR was performed in 20 µl of PCR mix composed of 4.0 µl, 10× PCR-buffer, 3.0 µl of dNTP mix (2mM of each dNTP), 1 µl of each primer 0.4 µM of each (10mM) primer (A-Leu-modified: forward: 5´ CAG ATA AGT GCA TTG GAT TT, B-Lys: reverse 5´ GTT TAA GAG ACC AGT ACT TG), 0.2 µl of *Taq* polymerase ($5U\mu l^{-1}$), 6.8 µl of PCR water and 4.0 µl of DNA ($10ng\mu l^{-1}$) template. Temperature profile for DNA amplification include, initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 sec, an annealing step at 45°C for 1 min, an extension step at 65°C for 3 min and a final elongation of 72°C for 10 min then 4°C soak.

2.7 Gel electrophoresis and DNA purification

The successful amplifications were checked by electrophoresis in 1% agarose gels immersed in 1xTBE buffer at a constant voltage of 100V for about 45 min. The size of amplified DNA fragments were determined by size ladder (e.g. λ DNA, Hind III). After electrophoresis, the gels were stained using ethidium bromide (0.5 µl/ml) to bind the DNA molecule for 15 min. They were then subsequently washed under distilled water bath for 5 min. Band pattern images were recognized under ultraviolet radiation lamp and photographs taken for documentation. Samples containing clean single bands were purified for sequencing. Purification was done using QIAquick® PCR Purification Kit (Qiagen). Amount of DNA was measured using Photometer, after which again electrophoresed on 1% agarose gel. The purified PCR products were sequenced in both directions by one of two commercial companies, GENterprise GmbH, Mainz, Germany or Scientific Research and Development GmbH, Bad Homburg, Germany.

2.8 Data analyses

2.8.1 Termite sequences

Primer ends were removed and sequences pruned to a maximum size of 681 bp equivalent triplet codons for this protein coding gene. Sequences with gaps which could not be divided by three were detected as possible pseudogenes and deleted from the data set. The sequences were translated into amino-acid sequences using invertebrate mitochondrial genetic code in MEGA 4.0 (Kumar *et al*., 2001). Those with numerous stop codons were omitted from the data set too. Identical haplotypes were also omitted from the data set but their numbers indicated in brackets in the tree reconstructed. GenBank sequences of the COII gene from NCBI website (up to March 2008) were used. Only sequences meeting a priori defined criteria of length, position, similarity and taxonomy were analysed.

2.8.2 *Termitomyces* sequences

This is a protein non-coding region, and so codon structure could not be used to aid in the alignment process. A region of about 30 nucleotides was excluded from the analysis because it could not be aligned unambiguously, together with regions with both primers fragments. Alignment of this variable region is ambiguous with insertions or deletions (indels positions). These gaps which were produced due to alignment were treated as having phylogenetic information, therefore the simple indel coding approach introduced by Simmons and Ochoterena (2000) and realized in the SeqState software package (Müller, 2005) was used.

2.8.3 Sequence alignments

Pairwise alignments of the termite and *Termitomyces* sequences obtained were performed with Clustal W (Larkin *et al*. 2007), with default parameters in BioEdit 7.0.9.0 (Hall, 1999) and then adjusted deletion positions by eye for gaps created due to the alignment. BLAST searches were done at NCBI website http://www.ncbi.nlm.nih.gov/blast/ to test for similarity of the query sequences with the databases in GenBank.

2.9 Phylogenetic Analyses

2.9.1 Neighbour Joining

Phylogenetic tree reconstructions were performed by Neighbour Joining (NJ) as implemented in MEGA 4.0. (Kumar *et al.* 2001). Kimura-2-Parameter (K2P) model (Kimura, 1980) of nucleotide substitution was used to calculate uncorrected pairwise genetic distances and phylogeny. Support for internal branches within the NJ tree was assessed using bootstrap resampling with 1000 replicates.

2.9.2 Maximum Likelihood analyses

Maximum likelihood analyses were performed using PAUP* 4.0β version 10. (Swofford, 2001). MODELTEST 3.7 (Posada and Crandall, 1998) was used to select the best-fit model and model parameters of nucleotide substitution for the data. MODELTEST evaluates the 56 different substitution models using hierarchical Likelihood Ratio Tests (hLRT) and the Akaike Information Criterion (AIC). The two tests favoured two different models, Hasegawa-Kishino-Yano model of nucleotide substitution with gamma-distributed rate variation (HKY+G) by hLRT and Tamura-

Nei and gamma-distributed rate variation (TrN+G) by AIC. The latter was used for subsequent analyses because AIC offers important advantages, simultaneously compares multiple nested or non-nested models, assessed model selection uncertainty, and allows for the estimation of phylogenies and model parameters using all available models (Posada and Buckley, 2004). Heuristic searches employed Tree–Bisection-Reconnection (TBR) branch-swapping with stepwise addition algorithm by 10 random taxon addition sequences. The level of stastistical support for different clades and robustness of the internal branches was assessed by using nonparametric bootstrapping obtained from 1000 bootstrap replicates

2.9.3 Maximum Parsimony analyses

Maximum parsimony analyses were performed in Mega 4.0, using the Close-Neighbor-Interchange algorithm with 10 random taxon addition replicates. Gaps/missing data were eliminated only in pairwise sequence comparisons (pairwise deletion). Bootstrap support for the most parsimonious topology was evaluated using 1000 replicates. Parsimony tree construction is based on searching all alternative trees for minimum total length. It performs best when tree branch lengths do not vary substantially (Holder and Lewis, 2003).

2.9.4 Bayesian analyses

Bayesian posterior probabilities of phylogenetic reconstruction employs Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) to approximate the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations (data). MRMODELTEST version 2.2 (Nylander, 2005) was used to select the best-fit model of nucleotide substitution for the observed data.

MRMODELTEST also evaluates different substitution models using hLRT and AIC, but over 24 models. The General Time Reversible model of nucleotide substitution with gamma-distributed rate variation (GTR+G) was favored for phylogenetic reconstruction by the two tests.

Bayesian analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) for estimation of the Bayesian topology for the data. Bayesian approach only requires a substitution model without fixed base frequencies and rate matrix of substitution model.

The data was partitioned into two; the first part was the normal matrix, the second part was the gap-coded partition. Markov chains were run for two million generations with four chains (three heated and one cold) starting from a random tree. Samples of the Markov chain were taken every 100 generations giving a total sample of 20,001 trees. The first 5000 trees (25%) were discarded as burnin. The four independent analyses converged on similar log-likelihood scores and reached "stationarity" (lack of improvement in likelihood values in cold chains). Stationarity was judged by visually inspecting the plots of the likelihood scores. The discard cut-off point was determined by the rule that the average standard deviation of split frequencies approach zero or less than 0.000001. A 50% majority-rule consensus tree was constructed from the remaining 15,001 saved trees (total samples minus the burnin) by running two differentially heated chains simultaneously to ensure a good sample from the posterior probability distribution. The parameters and the trees were then summarized by "sump" and "sumt" commands in MrBayes Version 3.1.2.

2.9.5 Shimodaria and Hasegawa (SH) test

To identify the best tree out of all the tree constructed, the log likelihood of all topologies was conducted using SH test (Shimodaira and Hasegawa, 1999) in PAUP.

CHAPTER THREE

3.0 SPECIFITY OF SYMBIOTIC RELATIONSHIP IN FUNGUS-GROWING TERIMTES-ISOPTERA:MACROTERMITINAE

3.1 Introduction

Recently, there has been an increasing interest in the phylogenetic relationship between fungus-growing termites (Macrotermitinae) and their fungal symbionts of the genus *Termitomyces* (Basidiomycota, Tricholomataceae). This has been mainly by the establishment of molecular markers, which opens new horizons for the study of these unique mutualistic ectosymbiosis. Use of classical taxonomical approaches, certain determination of the interacting partners is sometimes almost impossible because of extensive morphological similarity among termite species or lack of basidiocarps of the fungi (Frøslev *et al.*, 2003). However, recent studies based on genetic markers have largely increased knowledge about the evolutionary history of the symbiosis between fungus-growing termites and their fungi (Rouland-Lefèvre *et al.*, 2002; Aanen *et al*., 2002).

The ecological interactions within the obligate mutualistic symbiosis between fungus-growing termites and *Termitomyces* fungi have been the content of numerous studies and reviews (Leuthold *et al.*, 1989; Woods and Thomas, 1989; Rouland *et al.*, 1991; Darlington, 1994; Rouland-Lefèvre, 2000; Hyodo *et al.*, 2003; Aanen and Boomsma, 2005). The termites cultivate their fungal symbionts on special structures (fungus-combs) within the nest. Here, the termites provide substrate, optimal microclimate and

a competition free space for fungal growth. The fungi aid in digesting complex plant-derived macromolecules in particular lignin. Fungal nodules (vegetative fruiting bodies) as well as old comb material serve as food for the termites, decreasing the Carbon/Nitrogen ratio of their diet. The association between Macrotermitinae and *Termitomyces* seems to be the result of a long co-evolutionary process, which has a single origin in African rain forest with no reversals of mutualistic fungi to a free-living state (Aanen *et al.*, 2002; Frøslev *et al.*, 2003; Aanen and Eggleton, 2005). Fungus-growing termites are found throughout the Palaeotropics (Eggleton, 2000), but are ecologically dominant in savannas (Wood and Sands, 1978; Buxton, 1981; Collins, 1981) where symbiosis has achieved its major adaptive radiation (Aanen and Boomsma, 2006).

There exist at least 332 species of fungus-growing termites in 14 genera (Kambhampati and Eggleton 2000). But only approx. 46 species of *Termitomyces* have been described so far (www.indexfungorum.org; Kirk *et al.*, 2001). Taxonomy of the genus has been difficult (Heim, 1977). Thus it is widely accepted that there will be many more species, some morphologically cryptic or without the existence of taxonomical important basidiocarps. However, it is obvious that there are less fungal symbionts than termite host species which contradicts the strict system of one host and one fungal species proposed by Heim (1977). Instead certain symbionts are shared by different host species and even genera (Wood and Thomas, 1989). Already in the second half of the last century a review by Sands (1969) indicated some degree of specificity between termite genera and species of fungus. Recently, a few other studies, which examined the relationships between termites and their *Termitomyces* symbionts found complex patterns in particular at lower taxonomic levels (Aanen *et*

al., 2002, 2007; Rouland-Lefèvre *et al.*, 2002; Taprab *et al.*, 2002). Host switching of the fungi is frequent and single host species can cultivate different *Termitomyces* lineages albeit seldom in the same nest (Katoh *et al.*, 2002). In the most comprehensive study so far carried out, Aanen *et al* (2002) proposed considerable congruence of this symbiosis at the genus level of the hosts but much more complex relationships below this level.

An integral aspect of the specificity of the relationship between fungi and termites is the acquisition of the fungal symbiont during colony foundation. Two modes of symbiont transmission are reported (Korb and Aanen, 2003). In *Macrotermes bellicosus* and at least five species of *Microtermes*, the fungus is carried from the mother colony to the new founded colony by the reproductive male or female respectively (Johnson, 1981; Johnson *et al.*, 1981). This uniparental vertical symbiont transmission could lead to a strong specificity between host and fungi. However, most of the fungus-growing termites are reported to acquire basidiospores of the fungi after colony formation from the environment by the first foraging workers (Sands, 1960; Johnson *et al.*, 1981; Sieber, 1983). This horizontal transmission of symbionts should lead to new combinations of termites and fungal lineages in each generation unless the termites are able to discriminate their mutualistic partner actively or passively.

The aim of this study was to investigate the interaction specificity and diversity of *Termitomyces* and their termite hosts in East Africa. Although comparable studies have been conducted in West and Central Africa (Aanen *et al.*, 2002; Rouland-Lefèvre *et al.*, 2002), South Africa (Aanen *et al.*, 2007) and Southeast Asia (Aanen *et al.*, 2002; Taprab *et al.*, 2002), no such study so far has focused specifically on Eastern

Africa despite the high generic richness of host termites in this area. The only taxonomic studies on *Termitomyces* with focus on Eastern Africa were carried out by Otieno (1964, 1969) who described six new species of the genus from Kenya and made reference to one more species from Ethiopia. But the authour projected the existence of many more species especially in Eastern Africa. A review by Wood and Thomas (1989) listed four *Termitomyces* species for East Africa associated with at least two termite species but disregarded the fungus species described by Otieno. So far, about 50 species of fungus-growing termites and 13 species of *Termitomyces* were reported from East Africa (Table 3.1). This fragmentary knowledge underscores the need for further research.

Table 3.1 Previous reports of *Termitomyces* fungi from East Africa.

species	**synonyms**	**distribution** (East Africa)	**reported by** *
T. aurantiacus	*T. cylindricus*	Tanzania, Uganda	5, 6
T. clypeatus		Kenya, Tanzania, Uganda	2, 3
T. eurhizus	*T. cartilagineus*	Kenya, Tanzania, Uganda	3, 5, 6
T. globulus		Kenya	3
T. letestui	*T. biyii*	Kenya, Tanzania, Uganda	1, 5, 6
T. mammiformis		Tanzania (Zanzibar)	3
T. microcarpus	*T. narobiensis, badius, orientalis*	Kenya, Tanzania, Uganda	1 - 6
T. rabuorii		Kenya	1
T. robustus	*T. fuliginosus*	Kenya, Uganda	2, 3
T. schimperi	*T. magoyensis*	Ethiopia, Kenya, Tanzania	1 - 4
T. singidensis		Tanzania	5
T. striatus		Kenya, Uganda	3
T. tyleranus	*T. tyleriana*	Kenya	1

* 1: Otieno, 1964; 2: Otieno, 1968; 3: Pegler, 1977; 4: Wood & Thomas, 1989; 5: Härkönen *et al.*, 1995; 6: Katende *et al.*, 1999

The current research aimed to answer the following questions:

(1) How many lineages of *Termitomyces* occur are associated with the various fungus-growing termites of Kenya?

(2) At which taxonomic level of the host are the lineages of the fungal symbiont associated with their hosts?

(3) Are lineages of the fungi widely distributed across Kenya or do they have restricted distributions e.g. to certain biomes.

To address these questions *Termitomyces* were sampled from 40 colonies across different termite genera, species, and populations, from geographically diverse regions in Kenya. One main focus of the sampling procedure centred on the species-rich genus *Macrotermes*, which is believed to have been under-represented in previous studies. The ITS region of the fungi was sequenced afterwhich phylogeny reconstructed. A randomization approach was then used to test for the distribution of host genera and host species across the phylogenetic trees of the fungi.

3.2 Materials and Methods

3.2.1 Sampling of *Termitomyces*

Termitomyces symbionts of fungus growing termites were collected directly from nests of their respective termite host across different geographical regions in Kenya (Figure 3.1). Termite nests were dug and opened up to the nest chamber to expose the fungus combs containing nodules. Fungus nodules from fungus combs or with fungus combs, were carefully picked up using sterile forceps, initially rinsed with 0.6% sodium hypochlorite solution and then washed with sterile water and either stored in

absolute alcohol or air dried to await DNA extraction. For each strain of fungi, soldiers and workers of the associated termites were collected and classified using morphological features.

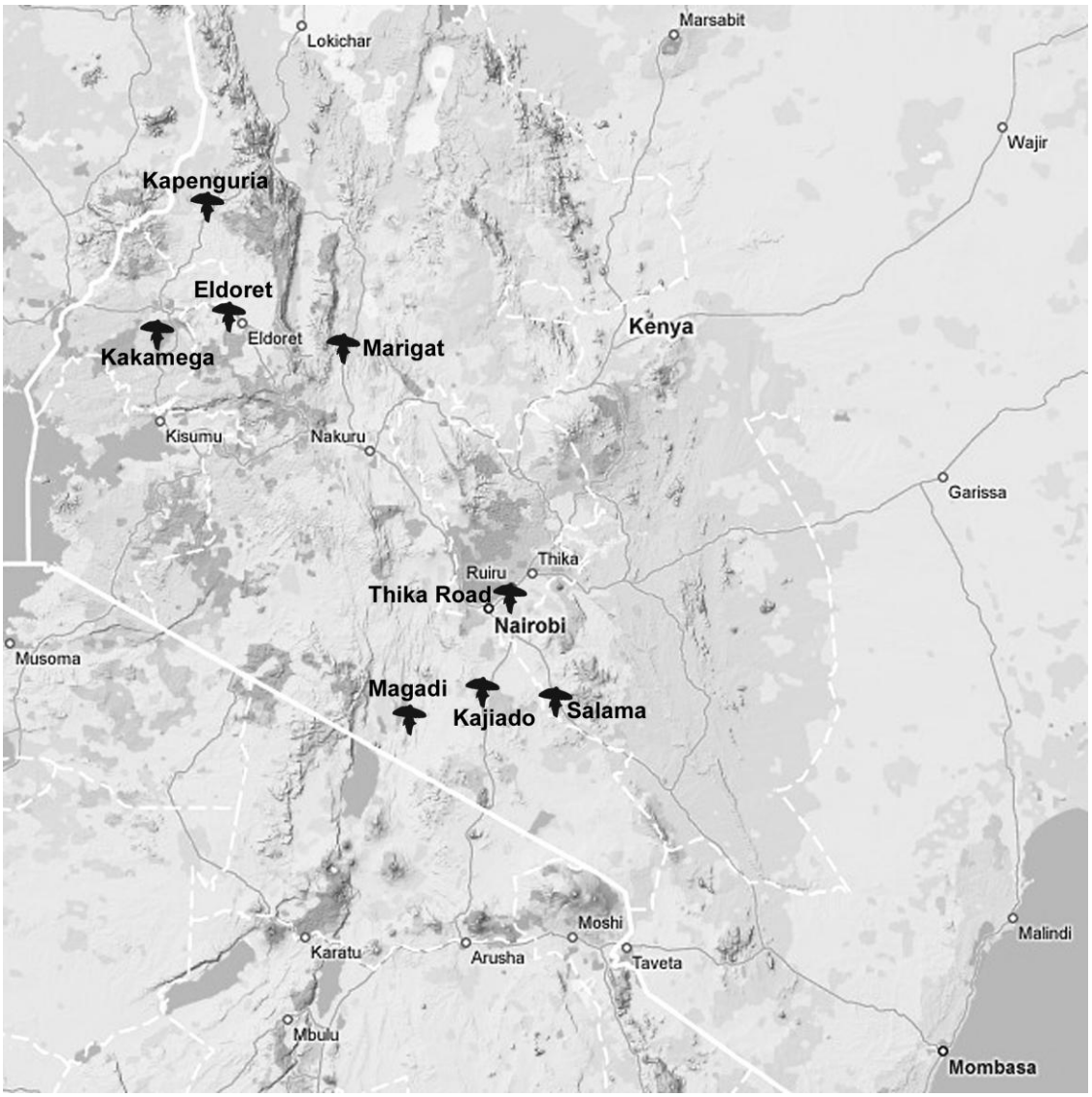

Figure 3.1 Map of Kenya with sample sites of *Termitomyces* symbionts. Location of sampling regions is indicated by symbol of black fungi and the corresponding names. (map source: http://maps.google.de)

3.2.2 Extraction and Sequencing of DNA

DNA was isolated as described in DNeasy® Tissue Kit (Qiagen). Two fungal nodules (~15mg) were homogenized in sterile 1.5 ml micro centrifuge tube using sterilized micro pestles. Hilden, Germany). The following primers were used to amplify the ITS region of the fungal symbionts;

forward, 5'CTTGGTCATTTAGAGGAAGTAA3'(ITS1-F),

'TCCGTAGGTGAACCTGCGG3' (ITS1).

reverse, 5'CAGGAGACTTGTACACGGTCCAG3'(ITS4-B),

5'TCCTCCGCTTATTGATATGC3' (ITS4).

(White *et al.,* 1990).

The sequenced length for the entire ITS region (including 5.8S) ranged from 638 to 780bp. The average base composition was A=0.236; C=0.206; G=0.268; T=0.290. This is very similar to the value generally reported for fungi (Gardes and Bruns, 1996). The length of ITS1 ranged from 298 to 312bp and ITS2 from 269 to 297bp (The 5.8S sequence was 176bp long and identical for all the taxa.

The PCR cycling parameters were as follows: initial denaturation (94°C, 5 min), 40 cycles of denaturation (94°C, 1.3 min), annealing (48°C, 1.5 min), and extension (72°C, 2 min), final elongation (72°C, 10 min). All amplifications were performed in a Mastercycler (Eppendorf, Hamburg, Germany). The successful amplifications were checked by gel electrophoresis. Samples which yielded multiple bands or not strongly stained bands of the expected size of the ITS region were rejected. Amplified PCR products were purified using QIAquick® PCR Purification Kit (Qiagen). The purified PCR products were sequenced in both directions by one of two commercial companies

(GENterprise GmbH, Mainz, Germany or Scientific Research and Development GmbH, Bad Homburg, Germany).

3.2.3 Sequence alignment

Nucleotide sequences obtained from the ITS region were checked separately manually to exclude primer regions and revaluate ambiguities. This is a non-coding region, and so codon structure could not be used to aid in the alignment process. Alignment of this variable region is sometimes ambiguous with numerous indel positions. Thus an ambiguous region of about 30 nucleotides was excluded from the analysis. Sequences were further aligned by the CLUSTAL W package (Thompson *et al.,* 1994) using default parameter settings in Bio Edit 7.0.5.3 (Hall, 1999). BLAST search at NCBI website (http://www.ncbi.nlm.nih.gov/) was done to check for closely related sequences in GenBank. In contrast to most of the other genetic studies on the ITS region of *Termitomyces,* gaps which were produced due to alignment were considered to contain phylogenetic information. Therefore simple indel coding approach was used by as introduced by Simmons and Ochoterena (2000) and realized in the SeqState software package (Müller, 2005). In addition to the amplified sequences, some ITS sequences of *Termitomyces* with African origin published in GenBank were used to construct a phylogeny (Table 3.2). As outgroup species *Tricholoma portentosum* (AF241517) from a sister genus within *Tricholomatacea* family was used, which is close enough phylogenetically to the genus *Termitomyces*.

Table 3.2 GeneBank ITS sequences included in the phylogeny

Termite host	GenBank Accession	Reference
Macrotermes muelleri Gabon 635 (MG1)	AF321368	Rouland-Lefevre *et al*., 2002
Macrotermes nobilis Gabon 630 (NG1)	AF321373	Rouland-Lefevre *et al*., 2002
Macrotermes subhyalinus Zanzibar Island 620	AF321370	Rouland-Lefevre *et al*., 2002
Macrotermes subhyalinus Tanzania 620 (ST2)	AF321369	Rouland-Lefevre *et al*., 2002
Macrotermes subhyalinus Se´ne´gal 620 (SS1)	AF321362	Rouland-Lefevre *et al*., 2002
Macrotermes bellicosus Se´ne´gal 618 (BS1)	AF321371	Rouland-Lefevre *et al*., 2002
Odontotermes nilensis Senegal 618 (OS1)	AF321364	Rouland-Lefevre *et al*., 2002
Odontotermes sylvicolus Se´ne´gal 637 (OS2)	AF321375	Rouland-Lefevre *et al*., 2002
Protermes minutus Gabon 630 (PG1)	AF321372	Rouland-Lefevre *et al*., 2002
Pseudacanthotermes spiniger Congo 613 (PS1)	AF321366	Rouland-Lefevre *et al*., 2002
Pseudacanthotermes militaris Congo 612 (PM1)	AF321367	Rouland-Lefevre *et al*., 2002
Ancistrotermes guineensis Senegal 618 (AnS1)	AF321365	Rouland-Lefevre *et al*., 2002
Microtermes sp. Gabon 618 (MiG1)	AF321376	Rouland-Lefevre *et al*., 2002
Microtermes subhyalinus Senegal 628 (MiS1)	AF321363	Rouland-Lefevre *et al*., 2002
Macrotermes natalensis South Africa	DQ436946	De Fine Licht *et al*., 2007
Microterme s sp.1 South Africa	EF636926	De Fine Licht *et al*., 2007
Odontotermes travaalensis South Africa	EF636916	De Fine Licht *et al*., 2007
Odontotermes obadius South Africa	EF636917	De Fine Licht *et al*., 2007
Odontotermes latericuris South Africa	EF636914	De Fine Licht *et al*., 2007
Tricholoma portentosum	AF241517	Suh & Kim, 2000

3.3 Phylogenetic Analyses

3.3.1 Neighbor Joining

Phylogenetic tree reconstruction was performed by Neighbor Joining (NJ) as implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 4.0. (Tamura *et al.*, 2007). The Kimura-2-Parameter (K2P) model of nucleotide substitution was used to calculate pairwise genetic distances and phylogeny. Support for internal branches within the NJ tree was assessed using bootstrap resampling with 1000 replicates.

3.3.2 Maximum Likelihood analyses

Maximum likelihood analyses were performed using PAUP* 4.0b10 (Swofford, 2001). Modeltest 3.7 (Posada and Crandall, 1998) was used to select the best-fit model and model parameters of nucleotide substitution for the data. Evaluated by Akaike Information Criterion (AIC) Modeltest favored the Tamura-Nei model of nucleotide substitution (Tamura and Nei, 1993) with gamma-distributed rate variation (TrN+G). AIC offers important advantages, simultaneously compares multiple nested or non-nested models, assess model selection uncertainty, and allows for the estimation of phylogenies and model parameters using all available models (Posada and Buckley, 2004). Heuristic searches employed Tree–Bisection-Reconnection (TBR) branch-swapping with stepwise addition algorithm by 10 random taxon addition sequences. The level of statistical support for different clades and robustness of the internal branches was assessed by using nonparametric bootstrapping obtained from 1000 replicates

3.3.3 Maximum Parsimony analyses

Maximum parsimony analyses were performed in Mega 4.0, using the Close-Neighbor-Interchange algorithm with 10 random taxon addition replicates. Gaps/missing data were eliminated only in pairwise sequence comparisons (pairwise deletion). Bootstrap support for the most parsimonious topology was evaluated using 1000 replicates.

3.3.4 Bayesian analyses

Bayesian analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) for estimation of the Bayesian topology for the data. Bayesian approach only requires a substitution model without fixed base frequencies and rate matrix of substitution model. MrModeltest version 2.2 (Nylander, 2005) was used to select the best-fit model of nucleotide substitution for the observed data. The General Time Reversible model of nucleotide substitution with gamma-distributed rate variation (GTR+G) was favored for phylogenetic reconstruction.

The data was partitioned into two; the second part was the gap-coded partition. Markov chains were run for two million generations with four chains (three heated and one cold) starting from a random tree. Samples of the Markov chain were taken every 100 generations giving a total sample of 20,001 trees. The first 5000 trees (25%) were discarded as burn in. The four independent analyses converged on similar log-likelihood scores and reached "stationarity" (lack of improvement in likelihood values in cold chains). Stationarity was judged by visually inspecting the plots of the likelihood scores. The discard cut-off point was determined by the rule that the average standard deviation of split frequencies approach zero or less than 0.000001. A 50% majority-rule consensus tree was constructed from the remaining 15,001 saved trees by running two differentially heated chains simultaneously to ensure a good sample from the posterior probability distribution. The parameters and the trees were then summarized by "sump" and "sumt" commands in MrBayes.

3.3.5 Shimodaira-Hasegawa (SH) test

To identify the best tree out of all the trees constructed, we computed the log-likelihood of all topologies and compared them using SH test (Shimodaira and Hasegawa, 1999) in PAUP.

3.3.6 Test of host specificity

The ancestral character history of the *Termitomyces* lineages was traced and characters (host genera and host species) were randomized across the phylogenetic tree of the fungi, using 'reshuffle' option in the software Mesquite version 2.5 June 2008 (Maddison and Maddison, 2008). This randomization procedure was done to obtain the distribution of the minimum tree lengths for the randomized characters (among host genera and host species). The distribution of the frequencies of the resulting number of steps was used to test whether the minimum tree length generated from real data (observed number of steps) was significantly smaller than the trees generated from the randomized characters (expected number of steps).

3.4 Results

3.4.1 Sequence attributes and divergence

The average base composition of the sequenced ITS region was A=0.22, C=0.23, G=0.23, T=0.32. Across the final alignment with 802 positions, 322 positions were variable and 226 of these were parsimony informative. 241 different gaps were coded by SIC, thereof 63 as single base gaps. 156 of these gaps were parsimony informative. Strains H3 and H7 were most frequently observed (Table 3.3).

The proportion of nucleotide differences between pairs of all sequences of *Termitomyces* for ITS1-5.8S-ITS2 rDNA ranged from 0 to 15.4%. Sequence variations (genetic distances among recognized *Termitomyces* symbionts) were generally high ranging from 4.0% to 15.4% (Table 3.4) showing deep sequence divergence. In contrast, the variation within all clades was shallow and ranged from 0 to 2.3% (Table 3.4). The length of the sequenced ITS region (including 5.8S) ranged from 607 to 632bp. The length of the ITS1 fragment ranged from 224 to 248bp, and the ITS2 from 200 to 224bp followed by a 22bp fragment of the 25S rRNA gene at the end. The 5.8S rRNA gene was 158bp long and identical for all Kenyan samples.

Table 3.3 Sampled *Termitomyces* symbionts with there termite host species, the sampling locality and the detected ITS haplotypes from 39 termite colonies in Kenya.

host species	sample site	sampled host colonies	ITS haplotype (H1- H22)
Macrotermes bellicosus	Kapenguria	1	2
Macrotermes herus	Kakamega	2	11
	Marigat	5	2,10
Macrotermes jeanneli	Kapenguria	1	6
	Marigat	1	4
Macrotermes michaelseni	Kajiado	12	3,5,7,8,9,12,13,14
	Salama	2	1,3
	Thika Road, Nairobi	3	3
Macrotermes subhyalinus	Kajiado	1	7
	Magadi	2	3
Microtermes sp.	Kakamega	1	23
Odontotermes sp.	Kajiado	2	21,22
	Kakamega	1	20
Pseudacanthotermes spiniger	Eldoret	1	15
	Kakamega	2	18,19
	Marigat	2	16,17

Table 3.4 Percentage pairwise sequence divergence (K2P distances) of ITS region of *Termitomyces*.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Macro I-a													
2. Macro I-b	8.3												
3. Macro I-c	5.9	5.6											
4. Micro II-a	9.1	9.8	10.9										
5. Micro II-b	11.7	12.4	11.9	8.0									
6. Macro I-d	4.0	10.5	5.0	9.8	11.8								
7. Macro I-e	10.1	8.7	11.2	9.2	12.9	12.0							
8. Odonto IV-a	12.5	12.1	13.0	9.4	10.7	12.7	13.1						
9. Odonto IV-b	13.5	13.0	15.0	8.7	10.9	13.4	13.2	8.3					
10. Odonto IV-c	10.9	11.2	12.2	8.2	10.5	11.2	11.6	7.2	4.9				
11. *O.latericius*	15.0	14.1	14.8	11.3	12.7	15.2	15.4	10.9	9.7	8.0			
12. *O.sylvicolus*	11.5	11.3	12.8	8.3	9.7	11.9	12.6	7.1	7.1	5.6	6.3		
13. Pseuda	10.7	11.0	11.8	7.7	8.5	11.3	11.2	8.1	9.3	7.5	10.3	6.9	
14. Outgroup	20.3	21.5	21.6	21.1	22.8	21.7	22.3	23.0	22.5	20.8	26.1	22.9	17.2

3.4.2 Phylogenetic Analyses

All phylogenetic approaches revealed almost similar tree topologies. The only differences were details of positioning fungal sequences of different host species within host genera.Therefore, only the consensus tree of the Bayesian analysis was shown. When the Shimodaira-Hasegawa (SH) test was performed, the ML tree produced the highest likelihood (ln L = -3887.58) as compared to the other tree topologies. However, the topologies were not significantly different from each other (Table 3.5). ML tree was calculated using the TrN+G model; gamma distribution shape = 2.2931; rate matrix: [A-C] = 1.0000; [A-G] = 1.8403; [A-T] = 1.0000; [C-G] = 1.0000; [C-T] = 2.4670 and [G-T] = 1.0000; Proportion of invariable sites = 0. Base frequencies were as follows A=0.23655; C=0. 0.2307; G=0.2215; T=0.2689.

The *Termitomyces* fungi clustered into four well supported monophyletic groups. Each clade comprised of fungal symbionts affiliated to one specific termite host genera i.e. *Macrotermes* (I), *Odontotermes* (IV), *Pseudacanthotermes* (III) and *Microtermes* (II), except for fungal sequences of *Odontotermes nilensis* from Senegal and *Ancistrotermes guineensis* from Gabon which clustered together within the *Microtermes* clade. Within the monophyletic group of fungi associated with *Macrotermes,* five well supported lineages were found which probably belonged to different *Termitomyces* species. The genetic distances showed deep divergence among the recognized lineages of *Macrotermes* symbionts regardless of their geographical distribution. Deep sequence divergence was also realized within the monophyletic group of fungi associated with the genus *Odontotermes*, comprising of at least three lineages. In total, at least ten well supported lineages were recognized within the

analyzed *Termitomyces*, out of which eight were sampled from Kenya. The bootstrap values and posterior probabilities are also shown in Figure 3.2.

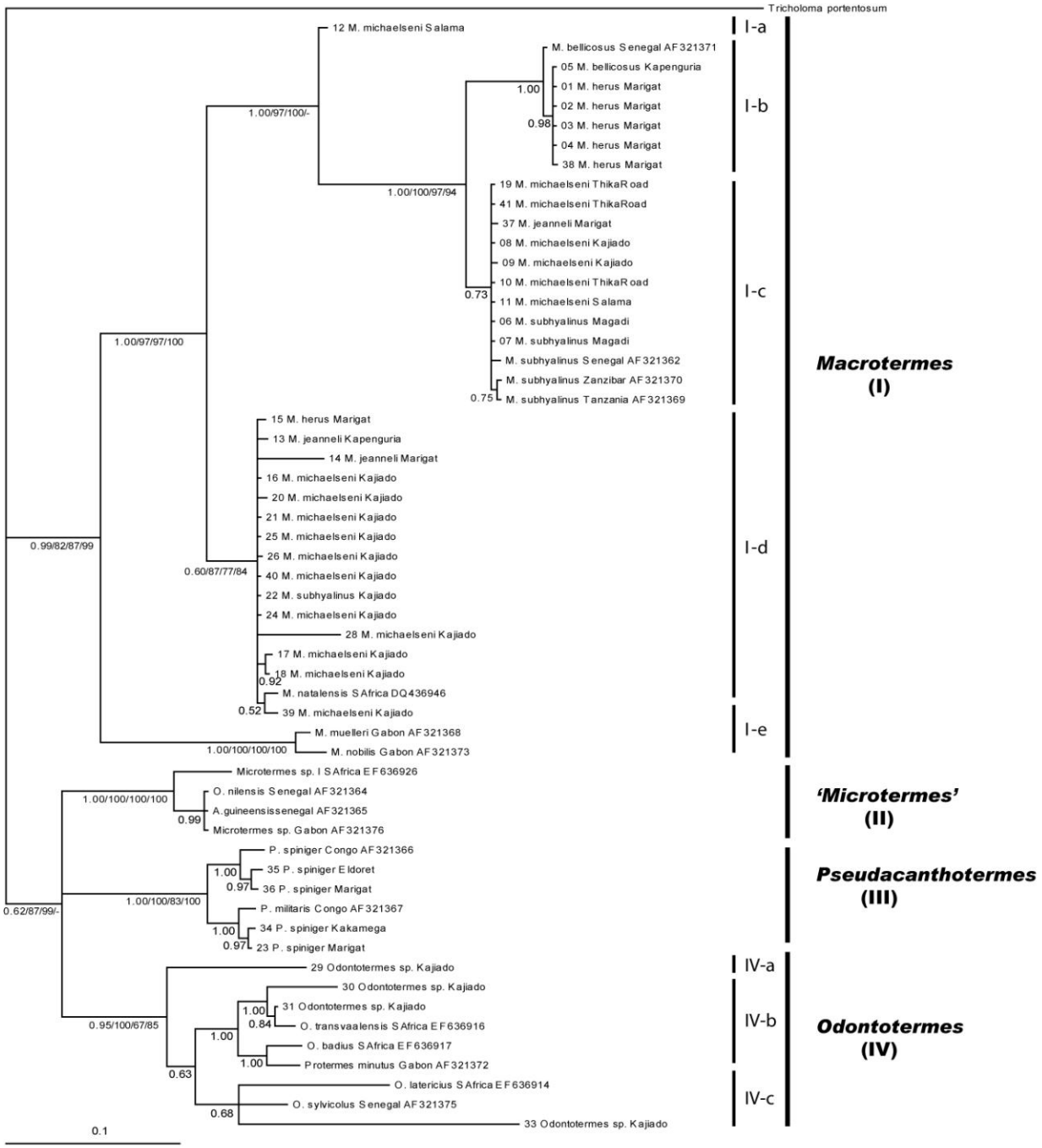

Figure 3.2 Bayesian tree of Termitomyces phylogeny in relation to their host termites based on ITS1-5.8S-ITS2 rDNA gene sequences. Posterior probabilities or bootstrap values are indicated below branch nodes for Bayesian/ML/MP/NJ respectively, with 1000 replicates. Tricholoma portentosum was used as outgroup to root the tree.

3.4.3 Test of host specificity

The distribution of the resulting number of steps for minimum tree length for the randomized characters among host genera across the phylogeny is shown in table 3.6. A randomization test showed that the observed number of steps for the reference trees was significantly smaller ($P<0.05$) than that of the trees generated from the randomized characters (expected number of steps) among genera. The resulting number of steps for minimum tree length was 6 for all the trees compared to 17 steps from randomization (Table 3.6). The distribution of the host species (within genus *Macrotermes*) was not completely randomly distributed across the fungal trees. The resulting number of steps for minimum tree length was 9 for all the trees compared to 11 steps from randomization (Table 3.7).

Table 3.5 Comparison of different topologies evaluated by SH test with confidence P values at the 5% level.

Test of topologies			
Phylogenetic tree	**lnL**	**Diff -ln L**	**P**
Bayesian	-3919.84	32.31	0.091
ML	-3887.54	(best)	
MP	-3914.43	26.89	0.131

Table 3.6 Resulting number of steps for minimum tree length from randomized trees with distribution of their frequencies. The different topologies for symbionts of Macrotermitinae are also given.

	No. of steps of from randomization with ther frequency distribution				
Topologies	**No. of steps of reference tree**	**17**	**18**	**19**	**20**
Bayesian	**6**	1	9	27	63
ML	**6**	1	12	24	63
MP	**6**	2	4	29	65
NJ	**6**	5	14	38	43
Master tree	**6**	1	6	19	74

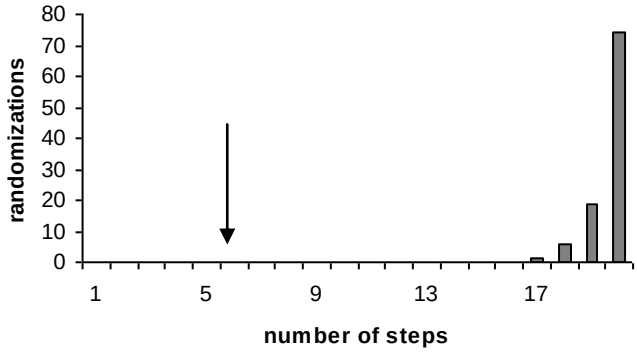

Figure 3.3 Frequency distribution of randomized characters (host genera) on phylogeny (Arrow points to the observed number of steps for minimum tree length for the master tree).

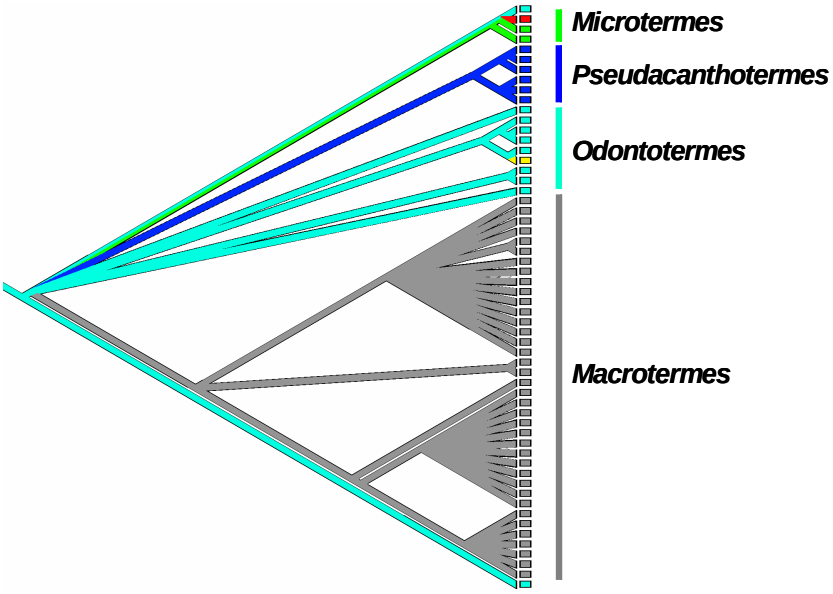

Figure 3.4 Phylogeny of Termitomyces in relation to their host termites showing specificity at genera level. Different colors show different host termite genera.

Table 3.7 Resulting number of steps for minimum tree length from randomized trees with distribution of their frequencies. The different topologies for symbionts of Macrotermes species are also given.

	No. of steps of from randomization with their frequency distribution			
Topologies	**No. of steps of reference tree**	**11**	**12**	**13**
Bayesian	**9**	2	10	65
ML	**9**	1	12	61
MP	**9**	2	9	59
NJ	**9**	1	14	41
Master tree	**9**	1	13	86

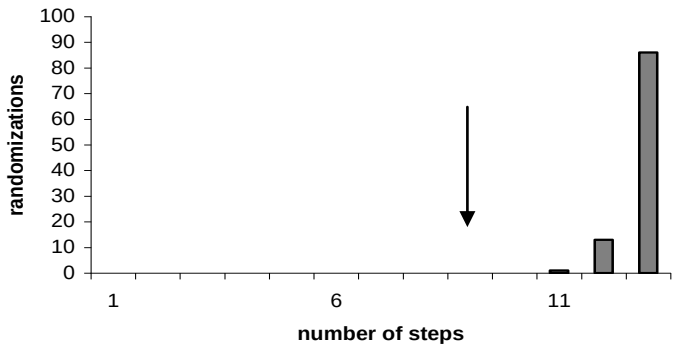

Figure 3.5 Frequency distribution of randomized characters (host species) on phylogeny.

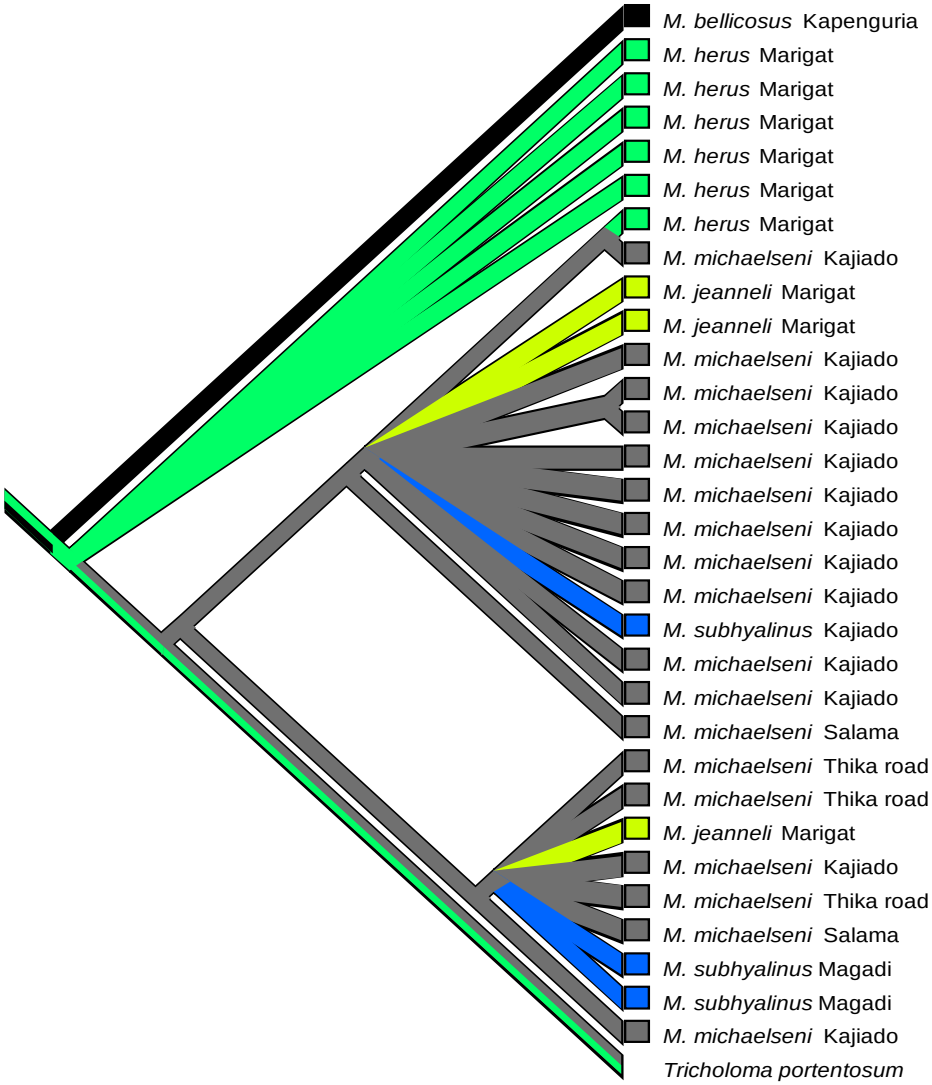

Figure 3.6 Phylogeny of Termitomyces in relation to their host termite species within the genus Macrotermes. Different colors show different host termite species.

3.5 Discussion

Phylogenetic relationship of *Termitomyces* symbionts of the fungus-growing termites was investigated in this study in order to establish the level at which the symbionts are associated with their termite hosts. Due to taxonomic dificulties of these fungi, comprehensive understanding of symbiosis between fungal symbionts and their host termites has been problematic. From this study, genetic characterization of *Termitomyces* by direct sequencing of the ITS region from fungal nodules collected directly from the nest was possible without necessarily having to cultivate them.

Eight *Termitomyces* lineages were found in Kenya which probably represent different species. Four of them were associated with *Macrotermes* species, three with *Odontotermes* species and one with *Pseudacanthotermes spiniger*. Species affiliation of the sequenced fungi was not determined because of using fungal nodules from the fungus-comb. Considering the limited number of sampled species and colonies compared to the high species richness and abundance of fungus-growing termites in Eastern Africa, many more *Termitomyces* species are expected. The knowledge about the diversity of *Termitomyces* in Eastern Africa is still fragmentary. The fungus species *T. microcarpus* was observed within nests of *Odontotermes badius* (Sands, 1956) and *O. montanus* (Sieber, 1983) from Kenya. Two additional fungal symbionts *T. eurhizus* and *T. globulus* were reported for *O. badius* in Kenya but with circumstantial evidence (Wood and Thomas, 1989). Otieno (1964, 1969) described six species (*T. narobiensis*, *T. magoyensis*, *T. biyii*, *T. rabuorii*, *T. tylerianus* and *T. badius*) from Kenya and *T. schimperi* from Ethiopia. This study has not only, but also

added to the knowledge of the diversity of *Termitomyces* in Kenya but also their relationship with their host termites.

The fungal symbionts of the termite host genera *Macrotermes, Pseudacanthotermes* and *Odontotermes* were found to represent separate phylogenetic groups in Kenya with strong sequence divergence between them. No symbiont was shared among these host genera indicating high host specificity at the genus level. This host genus specificity was supported by a randomization procedure, since host genera were not randomly distributed across the reconstructed tree. The observed pattern of host genus specificity is in accordance with previous studies (Aanen *et al.*, 2002, 2007; Rouland-Lefèvre *et al.*, 2002), However, there were also some exceptions with the same fungal lineage being cultivated by different termite genera as previously reported by Aanen *et al.* (2002), Rouland-Lefèvre *et al.* (2002) and Taprab *et al.* (2002).

The extensive data on host genus specificity of *Termitomyces* fungi by now suggest that this might be a general trait for fungus-growing termites and that the symbiotic relationship between fungal symbionts and their termite host genus is very stable and fairly strong. This suggests that the symbionts have differentiated after acquisition by the ancestors of their host termites and have coevolved with their host termites to form a stable symbiosis at least in some termite genera (Aanen *et al.*, 2002; Rouland-Lefèvre *et al.*, 2002).

At the species level the host-symbiont relationships seemed to be more complex. Within the genus *Pseudacanthotermes* the two species included in this analysis (*P. spiniger* and *P. militaris)* cultivated the same lineage of fungal symbionts (III) with only low sequence divergence between samples despite the large distances between sample sites (from Kenya to Congo). Deep sequence divergence between fungal symbionts was realized within the *Termitomyces* clades of host genera *Macrotermes* and *Odontotermes,* indicating that a diversity of fungal lineages harbored within these termite genera. For example, at least four genetically distant lineages were cultivated among the investigated *Macrotermes* colonies. Single lineages that probably represented different species of *Termitomyces* symbionts were shared among different species of *Macrotermes* (e.g. I-d). Furthermore, divergent lineages of fungal symbionts were cultivated by the same species even at the same locality, e.g. within neighboring nests of *M. michaelseni* from Kajiado (I-c, I-d). Altogether the results indicated a low interaction specificity at the host species level with frequent exchanges of symbionts between termite species, although the host species were not completely random distributed across the phylogenetic tree of fungi. This complex pattern at the host species level is consistent with previous studies (Aanen *et al*., 2002, 2007; Katoh *et al*., 2002) and could be possibly attributed to the mode of symbiont transmission to be discussed later. An additional explanation could be the level of specialization of the *Termitomyces* fungi. Most of the fungal symbionts are thought to be generalists rather than specialists with respect to their hosts (Rouland-Lefèvre, 2000; Taprab *et al.,* 2002). It has been reported that enzymatic production of the fungal symbionts as well as their digestive or nutritional role for the host varies according to their actual hosts and/or environmental factors (Hyodo *et al.,* 2003). This possibly could be a proximate explanation for finding similar fungal sequences

associated with different host termite species. One example is the occurrence of the same fungal lineages (I-c and I-d) within the nests of *Macrotermes subhyalinus* and *M. michaelseni*. It was mentioned before that the strategy for digestion of the first species agree with the acquired enzyme hypothesis whereas the latter do not (Rouland-Lefèvre, 2000). But it seems unlikely that these fundamentally different strategies of a digestive mutualism occur if they cultivate the same fungi. However, it was shown that some generalist fungi produce different enzymes in relation to the provided substrate or the environmental conditions of the fungus-comb (Rouland-Lefèvre, 2000) and therefore may play different roles in the digestion processes of different host species.

The same fungal lineages were found to occur across steep environmental gradients, for example lineages I-d within *M. michaelseni* from Thika and Kajiado (semi arid areas) and *M. subhyalinus* from Magadi (a much more arid area). It was concluded that *M. michaelseni* harbored different fungal lineages within the same environment. Therefore, the association of similar fungi with several host species is not the result of an allopatric distribution of lineages between climatic regions. Identical ITS sequences of *Termitomyces* within different geographic zones were also found in previous studies (Rouland-Lefèvre *et al.*, 2002) and this seems to be a common pattern. As the spores of the fungi are wind-dispersed frequent dispersal over long distances is possible and this might explain the occurrence of similar lineages in far distant localities. During their growth, the fungi are largely independent from the environment outside the termite mound, as the termite hosts provide stable and

predictable environmental conditions within the nests even in localities far away from each other.

The fungi associated with *Macrotermes bellicosus* and at least five species of *Microtermes* have been known to be transmitted vertically from generation to generation through ingestion of asexual spores by reproductive males and females respectively (Johnson, 1981; Johnson *et al.*, 1981). This mode of symbiont transmission (Aanen *et al.*, 2002) should lead to a very strong and specific association between the fungi and their hosts particularly with regard to the absence of basidiocarps in these symbionts. It has been argued by some authors that some degree of horizontal transmission must occur as presumably vertically transmitted symbionts were shared between species (Aanen *et al.*, 2002, 2007). This finding was also the case in our study where *M. bellicosus* from Kapenguria shared the same fungal lineage (I-b) with *M. herus* from Marigat. These finding indicates some symbiont sharing between these termite species. This means that horizontal symbiont transmission happens between conspecific colonies of *M. bellicosus*. It has been hypothesized that termite species with a vertical transmission mode might prevent the fruiting of their fungal symbionts actively by continuous harvesting of the fruiting bodies (Korb and Aanen, 2003; Aanen and Boomsma, 2006). Nevertheless, there could be rare events where basidiocarps emerge during or shortly after colony decline with the release of basidiospores to the environment. Such spores then could be picked up by foraging workers of other termite species leading to the observed patterns of shared symbionts between species with potentially different modes of symbiont transmission. This might not explain the occurrence of similar fungal symbionts in different species of

Microtermes in South Africa (Aanen *et al.*, 2007) unless vertical transmission of symbionts is not a general mode within the entire genus *Microtermes*. At the moment, there is no evidence so far from population genetics that vertical symbiont transmission frequently occurs in the field. Instead it was shown for *Macrotermes natalensis*, a supposed further candidate for vertical transmission that the genetic population structure reflects quite clearly a heterothallic life cycle with sexual reproduction although no basidiocarps are known (De Fine Licht *et al.*, 2005, 2006). Further studies on the population genetics of host species with experimentally proven vertical symbiont transmission are necessary to answer the question whether the asexual fungal spores in the crop of the reproductives are only a kind of 'assurance' if foraging workers are not successful in the acquisition of spores from the environment.

Horizontal transmission is considered to be the ancestral mode of symbiont transmission between generations (Aanen *et al.*, 2002) and seems to be the norm in most of the fungus-growing termites (Johnson *et al.*, 1981; Siebers, 1983; Katoh *et al.*, 2002; Korb and Aanen, 2003; De Fine Licht *et al.*, 2006). It is widely accepted that some species' incipient colonies acquire the fungi from sexual spores collected from the vicinity of the nest by the first foraging workers few weeks after nest foundation (Sands, 1960; Siebers, 1983). These spores originate from fruiting bodies (basidiocarps) that arise from mature termite colonies. In the case where same symbiont lineages occurred within the same locality by different host species, the fungi might have been exchanged horizontally between these termite species with intergenerational transfer by acquisition of compatible spores from the environment, depending on their availability at that time of colony establishment. This seems to

have been the case for *Termitomyces* lineage I-d in *Macrotermes michaelseni* and *M. subhyalinus* from Kajiado or for the same fungus which was cultivated by *M. herus* and *M. jeanneli* from Marigat. During fieldworks in Kajiado Sieber (1983) found no basidiocarps on nests of *M. michaelseni* although young field colonies were developing successfully at this time. One of his suggestions was that foraging workers acquire the basidiospores from *Termitomyces* mushrooms of nearby congeneric *M. subhyalinus* nests, and *vice versa*. These results support this suggestion as the same fungal lineage was cultivated in both species at Kajiado sample site. Another example for possible horizontal symbiont transmission between congeneric species has been reported for *Macrotermes natalensis* in South Africa. As mentioned above no basidiocarps are formed by the fungal symbiont although horizontal transmission is evident by population genetic data (De Fine Licht *et al.*, 2006). Aanen and Boomsma, 2006) hypothesized that *M. natalensis* 'parasitizes' on other sympatric *Macrotermes* species where symbionts produce fruiting bodies although no congeneric species were investigated. Interestingly, the isolated fungus of this termite species belonged to *Macrotermes* symbiont lineage I-d together with *M. subhyalinus*, *M. michaelseni*, *M. jeanneli* and *M. herus*. These results hence support the idea of the authors because of the sympatric occurrence of *M. subhyalinus* and *M. michaelseni* with *M. natalensis* in South Africa, even if the fungal sequences in this study came from a far distant region.

Whereas the cultivation of a single strain or even a single clone of *Termitomyces* by inoculation of newly formed fungus-comb with asexual spores in a mature colony is reasonably understood (Leuthold *et al.*, 1989; Aanen, 2006), little is known about the very first steps of fungus-cultivation in incipient colonies. The mode of symbiont

choice by termite species with horizontal symbiont transmission still remains unknown but is one of the key questions towards the understanding of the evolutionary pathway causing this unique mutualistic symbiosis. Many mechanisms are conceivable: (i) an active choice by morphological or chemical recognition cues of the basidiospores could occur by the first foraging workers; (ii) during the first steps of cultivation at the primordial fungus-comb, one fungal strain could be actively promoted by positive discrimination of specific crop characteristics like nodule size, nodule numbers or growth rates (Aanen, 2006) and weeding out competing strains (Aanen and Boomsma, 2005); (iii) a different resistance of basidiospores during their gut passage before inoculation of the fungus-comb or differing resistance against termite saliva could be responsible for the occurrence of specific fungi in specific termite hosts. It has been suggested earlier that termite saliva inhibits the germination and growth of 'weed'-fungi (Thomas, 1987); (iv) the fungus-comb has been characterized as a very selective environment (Wood and Thomas, 1989), thus a selection by specific conditions at the fungus-comb (e.g. substrate, chemical conditions) where only specific basidiospores could germinate is also possible; (v) an indirect linkage could be generated by the synchronization of the ripeness of the basidiocarps in mature colonies with the emergence of the first foraging workers in incipient conspecific or congeneric colonies, as proposed by Johnson *et al.* (1981). Such timing in combination with differing developmental rates in other fungus-host pairs, as observed for *Ancistrotermes cavithorax*, *Macrotermes subhyalinus* and *Odontotermes pauperans* in Nigeria (Johnson *et al.*, 1981) and this could strengthen a certain degree of host specificity. However, in other termite species the fruiting of *Termitomyces* coincides rather with the nuptial flight of the reproductives at the beginning of the rainy season than with the emergence of the first workers (Johnson *et*

al., 1981; Darlington, 1994). Experimental studies at the first steps of fungus cultivation in newly established colonies accompanied by genetic and chemical analyses are needed to clarify the partner selection and maintenance in the termite-fungus symbiosis.

3.6 Conclusion

This is the first study to investigate the phylogenetic relationships of fungal symbionts of fungus-growing termites in Eastern Africa and to give a general overview of their diversity patterns in relation to their hosts in this region. High amount of sequence divergence realized in the ITS sequences among different recognized clades revealed the existence of at least eight different *Termitomyces* species cultivated by the host termites. Many more species are expected with regard to the small number of sampled colonies. The fungal lineages were well distributed across Kenya and are not restricted to a particular locality or biome. The study revealed high interaction specificity between the termites and their fungal symbionts at host genus level, implying some degree of co-evolution. However, the lower fungal symbiont diversity compared to the host diversity blurred their phylogenetic relationships at the host species level by frequent host switches.

CHAPTER FOUR

4.0 ASSESSMENT OF TERMITE BIODIVERSITY IN KAKAMEGA FOREST (KENYA) USING DNA BARCODES

4.1 Introduction

The level of overall biodiversity is thought to be highest in tropical forest ecosystems (Marshell, 1992). However, these ecosystems are among the most threatened by changes in land use (Harcourt, 1992). Conservationists and other biologists are concerned with monitoring and conserve\ation of this diversity for a number of reasons. These include the preservation of potentially valuable organisms and genes, the maintenance of ecosystem stability and the protection of environments from the consequence of forest clearance on global climate change (Groombridge, 1992). Species level is recognized as the major unit of biodiversity and as a key element for measurement and description of biodiversity (Purvis and Hector, (2000). In the face of increasing man-made changes in ecosystems, which implies extinction of species, there is an urgent need to do assessment of the diversity of threatened biota. The tropical habitats on our planet are known as the most species rich and many of the so-called “hot spots” of biodiversity are located in or around the tropical belt. This forest is heavily affected by increasing human-induced disturbances. One of this “hot spots” is the Kakamega forest in Kenya, which is the only primary rain forest in the country and distinguished by a high number of endemic species. This forest is the most eastern extension of the Congo forest block reaching western Kenya and is threatened by the increasing human population.

The termite diversity in Kakamega forest is poorly known despite them being large and hyper diverse group of arthropods (Abe, 2000). Termites are extremely important components of tropical ecosystems (Lee and Wood, 1971; Wood and Sands, 1978; Swift *et al.*, 1979; Wilson, 1990), having been described as "ecosystem engineers" due to their important role in providing soil ecosystem services such as, distribution, protection and stabilisation of organic matter, increasing microsite heterogeneity, the genesis of soil microaggregates and porosity, humification, the release of immobilized Nitrogen and Phosphorus, the improvement of drainage and aeration, and increasing the exchangeable cations (Lavelle *et al*., 1997; Holt and Lepage, 2000; Donovan *et al*., 2001). They have a primary role as decomposers of organic material and through this contribute significantly to carbon fluxes (Collin, 1981, 1983; Matsumoto and Abe, 1997). They are also a biotic constituent (Higashi *et al.* 1992), thus forming the basis for a large food web (Deligne *et al*. 1981). Their importance is reflected in their abundance, and can make up to 10% of animal biomass and up to 95% of soil insect biomass (Abe *et al*., 2000; Eggleton, 1996). Since the destruction of natural habitats by clearing and cultivation reduces termite diversity, (Bandeira, 2003; Eggleton, 1996), it will also negatively affect termite influenced processes and the functioning of an ecosystem. Global surveys have shown that termite species diversity is greatest in closed-canopy of tropical rain forest, where much of the group's radiation has occurred (Eggleton, 1996). The family termitidae contribute over 90% of the species (Eggleton, 2000). Not only does it constitute the majority of species, but also exhibits the widest range of ecological and behavioral diversity, including many traits fundamental to the evolution of the Isoptera, such as soil-feeding and fungus-growing. The ecological importance of termites, together with their uniquely derived social

structures and cooperative behaviour (Darlington, 1994) makes it appropriate for us to investigate their true diversity in tropical rain forests.

In order to accurately measure biodiversity, species definition is vital, unfortunately there are no standardized operational criteria to define species (Sites and Marshall, 2004). Recently, there has been considerable interest in the use of haplotype phylogenies from DNA sequence data to infer species boundaries (Avise and Ball 1990; Baum and Donoghue 1995; Graybeal, 1995). Currently, biodiversity assessment is mainly based on morphological description of species, which is a complex and non-neutral marker. Especially for hyperdiverse insects and other invertebrates, morphological taxonomy is not sufficient (Smith *et al*., 2005; Floyd *et al*., 2002). Besides, morphological taxonomy normally leads to underestimation of biodiversity (Gomez *et al*., 2006). The taxonomy as well as the identification of termites to the species level mostly rely on characters of soldiers which are difficult to identify. However, workers are the most frequently sampled caste during ecological surveys. Recently, it was suggested that DNA-sequences (barcoding) can be used for inventories of such hyper-diverse taxa as well as taxa which are difficult to identify. To date, only one such study has been conducted for ants in Madagascar (Smith *et al*., 2006) to examine if barcodes were an effective surrogate for morphospecies morphological and molecular diversity. With today's technology for production of molecular sequences, cryptic speciations have been reported across all phyla and there seems to be a frequent bias associated with morphological taxonomy. There are several inherent advantages of molecular identification, (1) Molecular data can be obtained from single specimens, often without compromising subsequent morphological identification; (2) morphologically indistinguishable taxa can be

separated; (3) all stages and morphs of taxa are accessible; and (4) a single technique is applicable to all taxa.

Using molecular divergence thresholds, Molecular Operational Taxonomic Units (MOTUs) (Sites and Marshall, 2003; DeSalle and Amato, 2004) can be determined under DNA barcoding system as described by Herbert (2003). Although DNA barcoding is still controversial (Moritz and Cisero, 2004; Will and Rubinoff, 2004; Waugh, 2007), it appears to be a useful tool to delimit species and recognize cryptic species in many taxa (Herbert *et al*., 2004; Vences *et al*., 2005; Ward *et al*., 2005; Stahls and Savolainen, 2008; Rach *et al*., 2008; Tavares and Baker 2008), especially in highly diverse groups of invertebrates where morphological taxonomy is limiting (Floyd *et al*., 2002; Ball *et al*., 2005; Smith *et al*., 2005; Hajibabaei *et al*., 2006; Smith *et al*., 2006; Rach *et al*., 2008; Stahls and Savolainen, 2008). The protein-coding COII gene of mitochondial DNA together with other genes have been used for many phylognenetic studies of termites, resulting to a lot of sequences of COII gene appearing in the GenBank (Kambhampati *et al*., 1996; Miura, 1998; Kambhampati and Eggleton, 2000; Lo *et al*., 2000; Thompson *et al*., 2000). This is the reason why COII gene was suggested for this study.

To barcode species, it is necessary to define threshold values that ideally provide a sharp distinction between intraspecific and interspecific divergence values. Hebert *et al*. (2004) proposed a standard sequence threshold of 10 times the mean intra-specific variations to delimit animal species. The major problem with defining this threshold value arises when there is a wide range of overlap between intra- and interspecific divergence values. Another possibility is using a tree-based species identification

technique which relies on clustering as an indication for identification. Most studies use tree-based identification tools based on Neighbor-Joining (NJ) trees (Floyd *et al*., 2002; Hebert *et al*., 2003a; Tautz *et al*., 2003; Barrett and Hebert, 2005) due to its computational efficiency. Although this method is well suited for grouping closely related sequences, other methods such as Maximum Parsimony, Maximum Likelihood or Bayesian inference of phylogeny are best in constructing phylogenetic trees. Nevertheless, both methods in combination with morphological data have been used successfully to give barcodes in various organisms. In this study, the approach of species identification with a NJ tree profile as first proposed by Wiemers and Fiedler (2007) was used.

The current study aimed at defining a molecular threshold using sequence divergences for termite species delimitation (Molecular Operational Taxonomic Units-MOTUs) and estimating the number of termite species in Kakamega forest by barcoding and subsequent comparisons with morphospecies. The sequences will form a DNA sequence database to provide alternative set of characters to assist in inferring termite species boundaries in future taxonomic studies to facilitate and complement conventional morphological taxonomic studies.

4.2 Materials and Methods

4.2.1 Sampling and Identification of termites

Termites were sampled along a transect across five types of human land-use: primary forest, disturbed forest, secondary forest, grasslands, and farmlands. This was done to

assess the regional pool of termite species in Kakamega forest, Kenya. Specimens were identified to their respective genera immediately upon collection and preserved in absolute ethanol. The termites were roughly identified morphologically to genera level by Godfrey H. Kagezi (Makerere University) and there after up to species level by J.P.E.C. Darlington of Cambridge University, UK. The specimen which could not be identified, due to the absence of soldiers were grouped using special features, mostly in regard to their size.

4.2.2 Extraction and Sequencing of termite DNA

DNA was extracted from ethanol-preserved specimens using the DNeasy® Tissue Kit (Qiagen). Each termite specimen was washed with distilled water in a petri dish to remove ethanol. Whole specimen or head and thorax or all six legs, depending on the size of the termite was used for DNA extraction. In some cases the presence of some minerals or organic matter in the gut was found to inhibit PCR amplification, particularly in soil-feeding termites, so the abdomen or gut was subsequently removed from specimens before extraction. Soldiers or alates were preferentially used for DNA extraction because their large muscles gave more DNA.

PCR was performed in 20 µl of PCR mix composed of 4.0 µl, 10× PCR-buffer, 3.0 µl of dNTP mix (2mM of each dNTP), 1 µl of each primer 0.4 µM of each (10mM) primer (A-Leu-modified: forward: 5´ CAG ATA AGT GCA TTG GAT TT, B-Lys: reverse 5´ GTT TAA GAG ACC AGT ACT TG), 0.2 µl of *Taq* polymerase ($5U\mu l^{-1}$), 6.8 µl of PCR water and 4.0 µl of DNA ($10ng\mu l^{-1}$) template. These primer sets had been used in previous phylogenetic studies (Inward *et al*., 2007). Temperature profile

for DNA amplification include, initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 sec, an annealing step at 45°C for 1 min, an extension step at 65°C for 3 min and a final elongation at 72°C for 10 min then 4°C soak. Amplifications were performed in a Mastercycler (Eppendorf, Hamburg, Germany). The successful amplifications were checked by gel electrophoresis. Samples containing clean single bands were purified for sequencing. Purification was done using QIAquick® PCR Purification Kit (Qiagen). The purified PCR products were sequenced in both directions by one of two commercial companies, GENterprise GmbH, Mainz, Germany or Scientific Research and Development GmbH, Bad Homburg, Germany.

4.2.3 Sequence alignment

Sequences with a length of approximately 750bp were obtained from sequencing COII gene. BLAST search was done at NCBI website http://www.ncbi.nlm.nih.gov/blast/ to test for similarity of the query sequences to termite database of COII gene in the GenBank. Although the current BLAST search involves sequences submitted for phylogenetic studies, the searches were successful in identifying conspecific termite sequences from the hits as the most similar to the query sequence. Sequences shorter than ~600bp or ambiguous were not used for alignment and subsequent analyses. Primer ends were removed and sequences pruned to a maximum size of 681 bp equivalent triplet codons for this protein coding gene. Pairwise alignments of the sequences obtained were performed with Clustal W (Larkin *et al*. 2007), with default parameters in BioEdit 7.0.9.0 (Hall, 1999) and then adjusted deletion positions manually especially for the gaps wrongly created by Clustal W. Sequences with gaps which could not be divided by three were detected as possible pseudogenes and deleted from the data set. The sequences were translated into amino-acid sequences

using invertebrate mitochondrial genetic code in MEGA 4.0 (Kumar *et al*., 2001). Those with numerous stop codons were omitted from the data set. Identical haplotypes were also omitted from the data set too but their numbers indicated in brackets in the tree that was reconstructed.

4.2.4 Defining COII sequence divergence threshold for termite species delimitation

To define sequence divergence threshold for termite species delimitation, all GenBank sequences of the COII gene from NCBI website (up to March 2008) were analysed. Only sequences meeting a priori defined criteria of length, position, similarity and taxonomy were analysed. A 681 bp-fragment of the COII gene of the termite mitochondrial DNA was used. The sequences were sorted into 7 family alignments and were aligned to a reference sequence of a complete termite mitochondrial genome (*Reticulitermes santonensis* (NC_009499).

Pairwise genetic distances using Kimura's two parameter (K2P) substitution model (Kimura 1980) substitution model were computed in MEGA 4.0. A Neighbour-Joining (NJ) tree rooted with *Cryptocercus relictus* (AB005908) using the distances was constructed. The sequence divergences calculated were intraspecific divergences (S), intrageneric divergences (G) intrafamilial divergences (F) and among subfamilies within Termitidae (iSF). Their frequency distributions were determined. The threshold values were obtained by determining the percentage of samples (*y*-axis) of each distribution that were below intraspecific distribution or above interspecific distribution over a range of threshold (*x*-axis). This percentage was then considered to be the chance of success for each threshold to discriminate the samples from a distribution. The best threshold (compromise) was found where intra and interspecific

success curves intersected. The performance of this threshold was finally obtained by the percentage of any samples that would have been correctly sorted using it. Weak and strong overlap situations are possible. Two fully overlapping or very close distributions leads to a success between 50 and 60%. Overlapping but nonetheless differentiated distributions produces a success between 60 and 80%. Weakly overlapping distributions leads to a success between 80 and 90%. Different or entirely disjoint distributions produces a success superior to 95% (Lefébure *et al*., 2006).

4.2.5 Termite species delimitation using sequence threshold on a NJ tree profile

K2P distance model was used to compute pairwise sequence divergence estimates among all individuals of the data set in MEGA 4.0. To visualize the results, NJ tree was constructed of the divergences using K2P distances. The MOTU delineation approach relied on sequence divergence on the NJ trees using the defined molecular threshold for termite species delimitation obtained after screening all COII sequences which have recently appeared in the GenBank. The mean sequence divergence of 0.1018 and threshold value of 0.056 were used to calculate the numbers of MOTUs across the linearized NJ tree. The values for the mean and threshold were divided by two and a line was drawn vertically through the tree. Every branch which crossed the measure line was indicated a single MOTU. Denomination of MOTUs was based on most similar GenBank sequences after doing a BLAST search.

4.2.6 Termite species richness (diversity), evenness and relative abundance

To estimate the real number of species from the morphological and molecular approach, an extrapolation method published by Chao and implemented in the vegan-package of R, was used.

The alpha diversity was measured using the Simpson and Shannon indices as follows, Shannon Index (H) = -Σpi ln (pi), Simpson Index (D) = Σpi^2 and Evenness (E) = H/log(S). pi is the proportion of total number of species made up of the ith species and S is the total number of species. The species occurrences and their relative abundances were ranked using rank abundance curves.

4.3 Results

4.3.1 Sampling results of termites

A total of 240 specimen were collected, and were morphologically summarised to belong to at least 11 genera and 16 morphospecies (Table 4.1) as identified by J.P.E.C. Darlington of Cambridge University, UK. After sequencing of the COII gene of the termites, there were 68 identical haplotypes detected in the sequences. 90 sequences were therefore used for analyses after omitting the haplotypes. The omitted haplotype numbers are indicated in brackets in the NJ tree (Figure. 4.3).

Table 4.1 Termites in Kakamega forest, Kenya, as morphologically identified by Darlington J.P.E.C. Identical haplotypes were omitted from the data. The corresponding numbers are either field coded or accession numbers of the GenBank sequences included in the NJ tree.

AB109523	Microtermes obesi
AB304488	Microtermes
AB304489	Odontotermes
AB304490	Pseudacanthotermes spiniger
AB304497	Macrotermesherus
AM041	Microtermes
AM043	Soil feeder
AM045	Odontotermes
DQ442059	Aderitotermes
DQ442066	Amitermes
DQ442076	Anoplotermes
DQ442084	Astalotermes murcus
DQ442086	Astalotermes
DQ442090	Basidentitermes
DQ442121	Eburnitermes grassei
DQ442150	Labritermes buttelreepeni
DQ442171	Nasutitermes
DQ442191	Nasutitermes
DQ442228	Promirotermes
K0003A	Odontotermes
K0005A	Basidontitermes
K0009A	Basidentitermes
K0015A	Nasutitermes
K0018A	Odontotermes
K011A	Soil feeder no soldiers
K417B	Microtermes
K421B	Odontotermes large sp black fungus
K422B	Odontotermes large
K424B	Odontotermes large
K426	longbodied probably Macrocerotermes
K428A	Microtermes workers only
K429B	Soil feeder
K433A	Pseudacanthotermes spiniger
K435	small Soil feeder
K437B	Odontotermes large sp
K439aB	Basidentitermes
K439bA	Promirotermes
K440B	Pseudacanthotermes spininger
K442B	Soil feeder
K444B	Microtermes w
K445	Soil feeder
K446B	No soldiers
K448B	probably Microtermes
K449B	Microtermes
K450B	Pseudacanthotermes spiniger
K455B	Odontotermes many worker soldiers
K461A	Pseudacanthotermes spiniger
K462B	Odontotermes large sp
K466A	Odontotermes large sp
K467A	Soil feeder
K469B	Pseudacanthotermes spiniger
K470A	Odontotermes small
K482A	Microtermes
K483A	Pseudacanthotermes spiniger
K484A	Odontotermes probably many workers large
K486B	Pseudacanthotermes spininger
K487A	Odontotermes large sp
K488A	Odontotermes large sp
K491B	Cubitermes many soldiers
K493A	Soil feeder
K494B	Odontotermes large sp
K495aB	mixed probably Odontotermes
K499	Odontotermes small like 470
K529A	Nasutitermes
K531B	Basidentitermes
K533bB	Macrotermes herus
K535aA	Pseudacanthotermes spiniger
K535bA	Pseudacanthotermes spiniger
K536A	Microtermes
K537b	Macrotermes herus
K538b	Cubitermes
K547A	Odontotermes large sp
K549B	Microtermes
K550	Odontotermes large sp
K551A	Soil feeder two spp
K558	Odontotermes large sp
K561	Odontotermeslarge sp many workers
K564A	Odontotermes large sp two soldiers
K565	Odontotermes large sp
K566	Odontotermes large sp many workers
K574A	Microtermes long heads many soldiers
K577B	Amitermes
K578A	Amitermes
K580	Pseudacanthotermes spiniger
K582	Microtermes
K583	Soil feeder including 2 alates
K586	Soil feeder
K589	Soil feeder problably soldierless
K590	Soil feeder
K591	Soil-feeding workers
K595A	Basidentitermes
K597A	Basidentitermes
K599	Soil feeder no soldiers
K602	Cubitermes
K603A	Basidentitermes
K604A	MacrotermesSPPseudaspiniger
K605A	Foraminitermes 1soldier workers
K607	Microtermes long-headed
K608	Microtermes
K609	Soil feeder no soldiers
K610	Basidentitermes
K619A	Pseudacanthotermes militaris
K621A	Soil feeder
K625	Cubitermes

4.3.2 Sequencing and sequence attributes

Full-length PCR products of about 750 bp fragment was obtained from the amplification. The right sized amplified fragment suggests that NUMTs (nuclear DNA sequences originating from mitochondrial DNA sequences) were not sequenced. Only a few sequences had stop codons which is consistent that all amplified sequences are functional mitochondrial COII sequences. The sequences from samples collected from Kakamega forest belonged to the family Termitidae, subfamilies Macrotermitinae, Termitinae and Nasutitermitinae.

4.3.3 A COII sequence divergence threshold to delimit termite species

The results of genetic distances of the mean (+SE), median, minimum, maximum values together with the number of taxa used for species (S), genera (G), family (F), and for the Termitidae intra subfamily (iSF) are shown in Table 4.1. For the families Mastotermitidae, Hodotermitidae and Serritermitidae the sequences were not enough to analyse distances for each taxonomic level. The threshold values to discriminate species within Kalotermitinae, Rhinotermitidae, Termitidae and in Isoptera were 0.06, 0.05, 0.06 and 0.06 with success of 99.5%, 88.1%, 91.7% and 89.9% respectively; while values to discriminate genera were 0.21, 0.12, 0.14 and 0.14 with success of 81.9%, 97.7%, 93.5% and 91.2% respectively. The high success rates of over 80% in all the distributions among families, most of which were above 90%, showed weak overlaps and disjoint distributions (Lefébure et al., 2006). The threshold values for species discrimination were always smaller than genera, which were also smaller than for family following a pattern of (S<G<F) for most of the well sequenced families. The COII gene sequence divergence increased with the taxonomic level (Fig.4.1).To discriminate species collections of Kakamega forest, which all fall within family

Termitidae, a threshold of 0.056 substitutions per site was used to differentiate intra- from interspecific divergences with a success of 92% (Fig 4.2). This indicates more or less disjoint distributions.

Table 4.2 Mean (+SE) and median, of intraspecific (S) intrageneric (G) and intrafamilial (F) pairwise genetic distances (K2P) of termite sequences as well as the threshold values with percentage success of their distribution within seven termite families. The numbers (n) of sequences are also given.

Termite families	taxonomic level	n	mean	SE	median	threshold	success [%]
Mastotermitidae	S	2	0.0130	0.0045			
	G						
	F						
Hodotermitidae	S	2	0.0622	0.0101			
	G						
	F						
Termopsidae	S	12	0.0726	0.0405	0.0743		
	G	17	0.1490	0.0374	0.1769		
	F	20	0.2846	0.0135	0.2781		
Kalotermitidae	S	18	0.0373	0.0203	0.0142	0.06	99.5
	G	43	0.1815	0.0041	0.1902	0.21	81.9
	F	44	0.2277	0.0026	0.2279		
Serritermitidae	S						
	G						
	F	2	0.2987	0.0240			
Rhinotermitidae	S	291	0.0245	0.0042	0.0137	0.05	88.1
	G	320	0.0782	0.0011	0.0816	0.12	97.7
	F	370	0.2266	0.0068	0.2356		
Termitidae	S	166	0.0207	0.0030	0.0161	0.06	91.7
	G	352	0.1018	0.0010	0.1085	0.14	93.5
	F	410	0.1719	0.0003	0.1718		
overall	S	473	0.0252	0.0031	0.0148	0.06	89.9
	G	732	0.0999	0.0010	0.0972	0.14	91.2
	F	846	0.1730	0.0003	0.1722		

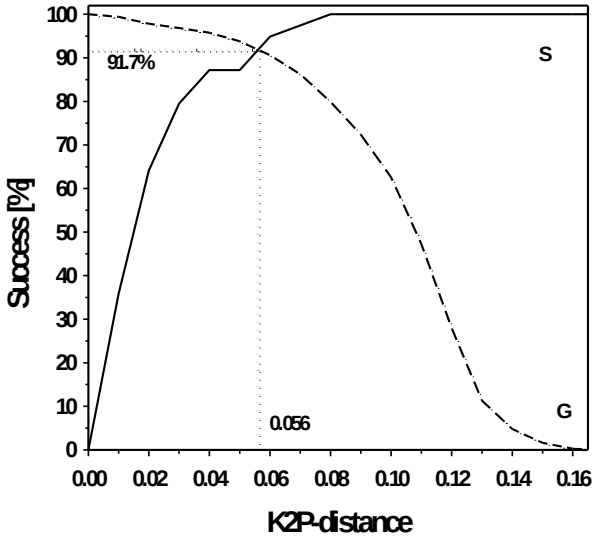

Figure 4.1 Analysis of the overlap between distributions of intraspecific (S) and intrageneric (G) genetic distances for the COII gene sequences of the Termitidae

4.3.4 MOTU delineation using sequence threshold on a NJ tree profile

The species obtained could be discriminated on NJ tree based on K2P sequence divergence using molecular threshold. These clades were defined as MOTU I to XXII based on the defined threshold value for the termites (0.056) and 18 MOTUs based on mean value (Figure 4.2). The number of MOTUs clustered in the NJ tree varied depending on the chosen threshold value. While considering all theoretical K2P distance thresholds for termite species delimitation, the MOTUs increased with decreasing thresholds (Figure 4.3). The results of morphological determination by J.P.E.C. Darlington did not always correspond with delineation using the threshold based MOTUs. Some single morphospecies were recognized as two separate MOTUs e.g within genera *Basidentitermes* with 100% bootstrap support, whereas using the mean value, only one MOTU was recognised. *Pseudacanthotermes* group with two morphospecies was recognised as three MOTUs by the defined threshold value and as one MOTU using mean value. The soil feeders, whose soldier caste were not sampled,

could not be classified to any genus or species using morphological features. It was possible to delimit these specimens to the most probable taxon by using BLAST search to find the most similar determined GenBank sequence. They all formed separate MOTUs on NJ tree (Figure 4.2). Similary, within genus *Microtermes* with two morphospecies, three distinct MOTUs were distinguished by the defined threshold, and the same number using the mean. When using different threoritical threshold values the same group was recognized as different MOTUs. For example, using theoritical threshold of 0.025, five MOTUs were recognised, all supported with 99% bootstrap values. On the other hand, within genus *Odontotermes* with three morphospecies, the group clustered in one MOTU using both the defined threshold and the mean. Whereas using theoritical threshold of 0.025, the same group was recognised as five separate clusters. Morphologically distinguished *Odontotermes* species as "large species" form two separate clusters, but the "small species" clustered together. Similary, the *Cubitermes* group with one morphospecies was recognised as two separate MOTUs with 100% bootstrap support, using theoritical threshold value of 0.025 as one MOTU using mean value. Some MOTU clusters corroborated well with morphospecies, for example *Macrotermes*, *Promirotermes* and *Amitermes* among others.

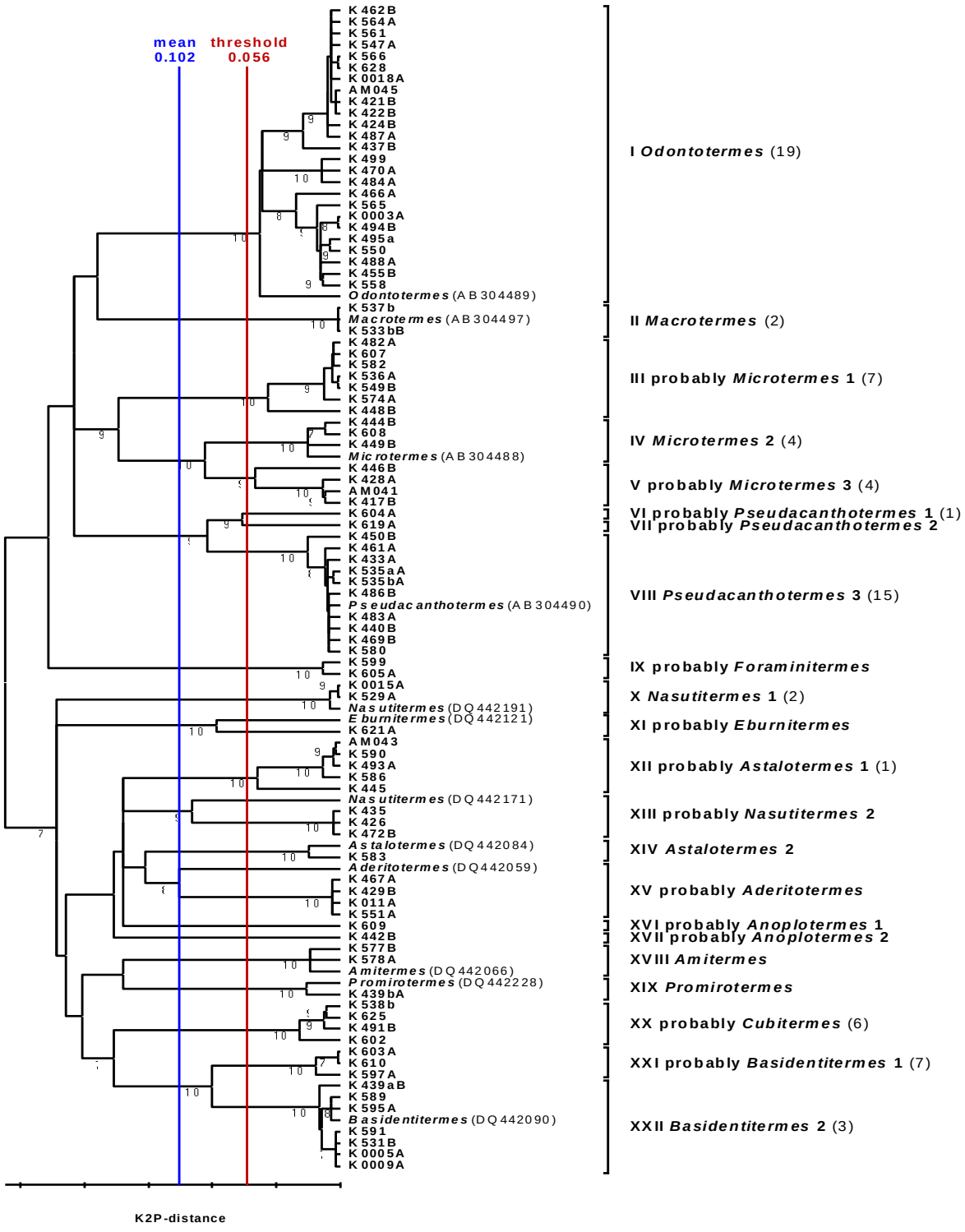

Figure 4.2 Linearized NJ tree of pairwise sequence divergences of termites from Kakamega Forest. The two lines indicate the values on which the MOTU delineation were based. The blue line belongs to the mean value (0.102), which defines 18 MOTUs. The red line belongs to the threshold value (0.056), which defines 22 MOTUs. Numbers of excluded identical haplotypes in brackets. Bootstrap support values are indicated on the nodes.

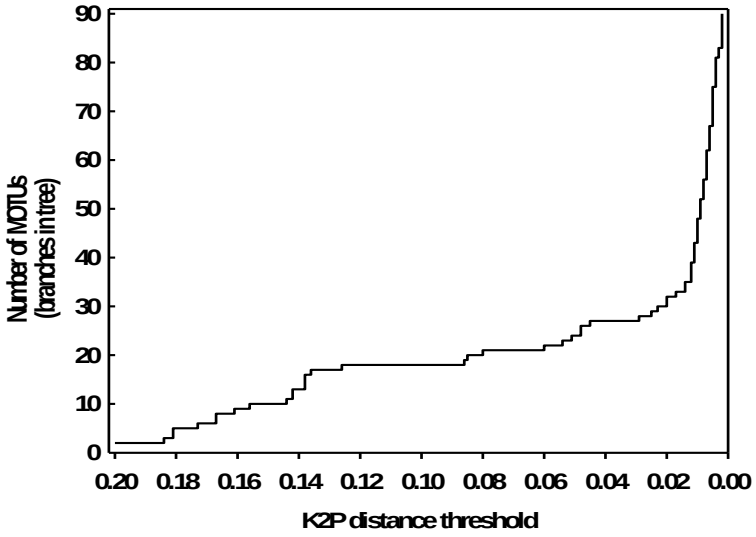

Figure 4.3 Respective number of MOTUs considering all theoretical K2P distance thresholds for termite species delimitation.

4.3.5 Termite species richness (diversity), evenness and relative abundance

The estimated observed species numbers were 16, 18 and 22 for morphospecies, MOTUs using means and the defined threshold respectively (Figure 4.2). Morphospecies estimate was less than estimates by the MOTUs. Estimates of species numbers of the termites as extrapolated by Chao were higher than observed numbers (Figure 4.4).

Termite species of *Odontotermes* (41% morphospecies and 28% MOTUs), *Pseudacanthotermes* (24% morphospecies and 17% MOTUs) and *Microtermes* (10% morphospecies and 9% MOTUs) were the most common in the forest (Figure 4.5). *Promirotermes* and *Foraminitermes* were ranked as less abundant species in the forest with less than 1% relative abundance.

Simpson and Shannon indices (2.45 and 7.73 respectively) revealed higher alpha diversity of the termite species for the MOTUs than for morphospecies (1.85 and 4.08 respectively). Species evenness for MOTUs was higher (0.80) than morphospecies (0.67). Simpson index showed higher alpha diversity than Shannon index (Table 4.2). The rank abundance curve for MOTU threshold showed a more even gradient as compared to morphospecies curve (Figure 4.5).

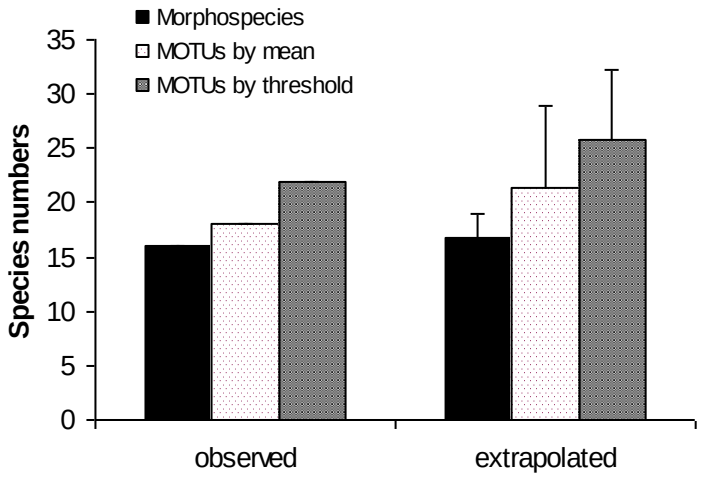

Figure 4.4 Observed and extrapolated numbers (+SE) of termite species in Kakamega forest using morphological and molecular approaches.

Table 4.3 Alpha diversity indices and evenness of termite species in Kakamega forest**.**

	Shannon Index	Simpson Index	Evenness
Morphospecies	1.85	4.08	0.67
Threshold	2.45	7.73	0.80
Mean	2.27	6.89	0.78

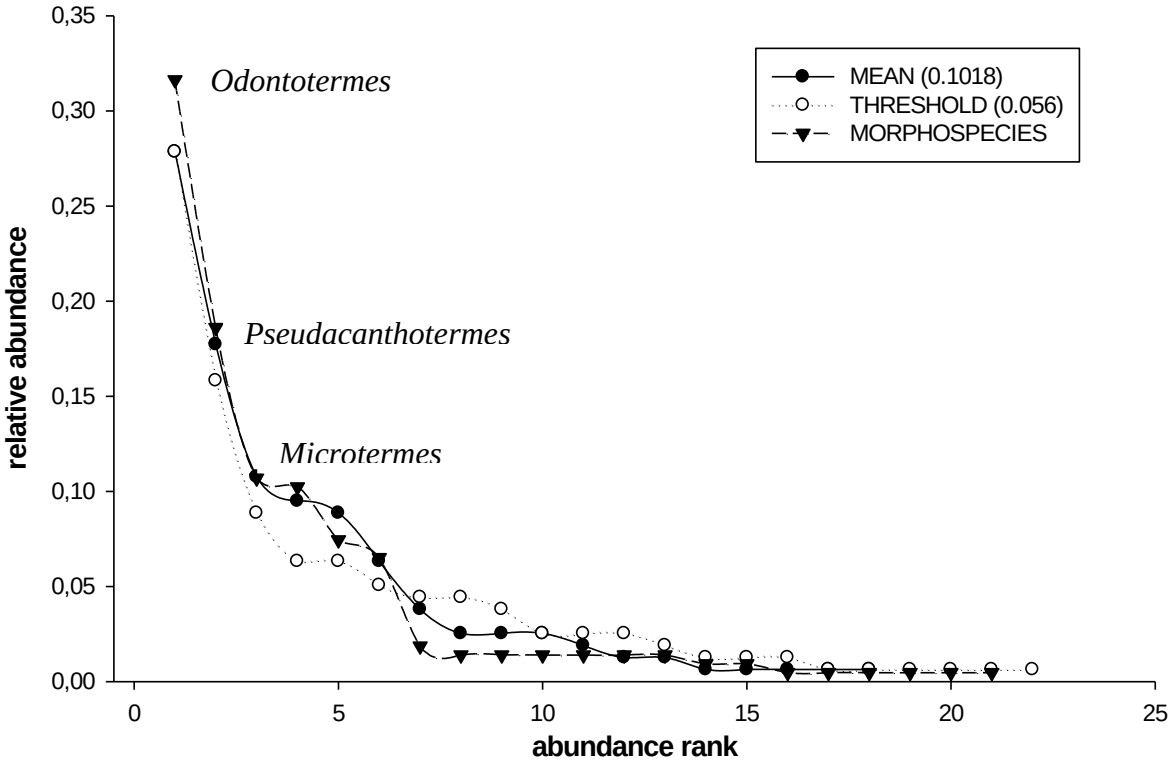

Figure 4.5 Rank abundance curves of morphospecies and MOTUs (mean and threshold) values. Only the three dominant termite species i.e. morphospecies and MOTUs (rank 1-3) are labelled.

4.4 Discussion

It was revealed in this study that large numbers of termite specimens which can be overwhelming to morphological taxonomists can quickly be distinguished by sequencing. The overall threshold value of 0.058, with success rate of 91.2% using intra and interspecific divergences, was defined for termite species delimitation for whole group of Isoptera, using all available COII sequences in GenBank. Different thresholds were defined for different termite families. The high success rates of over 80% in all the distributions among families, most of which were above 90%, showed weak overlaps and disjoint distributions. Out of all the termite species which were found in Kakamega forest, majority of which were within family Termitidae, 91.7% of the species could successfully be discriminated by using a threshold of 0.056. This showed that the intra and interspecific sequence divergences were not overlapping. These thresholds can be used with a very high success to help in the delimitation of the termite species. Alternatively the overall threshold within whole group of Isoptera could also be used. The means and threshold values for species discrimination were always smaller than for genera, which were also smaller than for family following a pattern of Species<Genera<Family for most of the well-sequenced families. This increase with the taxonomic level suggested morphological taxonomy was in agreement with DNA evolution.

Species delineation from sequence divergence has been applied mainly to very small organisms, such as prokaryotes or soil nematodes, in which morphological discrimination is difficult or impossible (Floyd *et al*., 2002; Gregory and DeSalle, 2005) In the case of nematodes, MOTUs have been assigned based solely on sequence

divergence (Blaxter, 2004) because there is no better way of classifying these organisms to date. While the observation of large inter- and low intra-species variation promises easy identification of described species and the discovery of many cryptic species (Hebert *et al*., 2003a; 2004; 2004*b*), there is concern regarding variability in the use of different threshold values in different organisms and even within one group. In this study different theoritical thresholds could delimit species differently. Although cytochrome *b* and *cox1*gene (Hebert *et al*. 2004b) have been used and suggested for barcoding, for this case, COII could distinguish these termite species using the best threshold found from analysis of all existing GenBank sequences for the simple reason that it is the most sequenced gene so far for the termites.

The assignment of samples to morphotypes did not always correspond to the molecular clusters (MOTUs) of COII gene sequencing data of the termite species on a NJ tree. Some MOTU clusters corroborated well with morphospecies in MOTU delineation using the sequence divergence threshold e.g all species in subfamily Termitinae except species within *Basidentitermes*, *Cubitermes* and soil feeders clustered as several MOTUs.Within subfamily Macrotermitinae, only *Macrotermes herus* corroborated with morphological identity. Consequently, there were less morphotype species as compared to MOTU species numbers as delimited by the defined threshold and mean values. This revealed some morphologically cryptic species. As expected, the number of MOTUs increased with decreasing threshold values. This is consistent with the findings of Smith *et al*. (2005) who used different MOTU levels for ant diversity assessment in Madagascar. Other findings showing cryptic speciation in other groups of taxa has been reported by several authours (Floyd *et al.*, 2002; Stahls and Savolainen, 2008; Rach *et al.*, 2008; Hajibabaei *et al.*, 2006;

Smith *et al.,* 2006; Ball *et al.,* 2005). These authours have shown that DNA barcodes can reveal cryptic species, thus consequently revealling hidden diversity of hyperdiverse organisms like termites. For example, within the genus *Odontotermes* which has been known to be problematic in identification (Darlington, 2008), it is obvious that molecular methods can reveal some cryptic species. Also within the genus *Microtermes*, it is obviously possible that there are many more species than what could be known by morphological determination. These results highlight the advantage that molecular based species delimitation revealed some morphological cryptic species and consequently giving a more comprehensive picture of termite diversity.

Another important finding of this study is that termites without sampled soldiers were assigned to their respective phylogenetic clusters. They could not be determined by morphology and so were able to be assigned to their respective MOTUs. Most of them which were soil feeders formed distinct clusters in the tree, hence appeared as separate species. Termites without soldier caste are normally difficult to determine leading to underestimation of termite diversity (Darlington, 2008). Futhermore, these results highlight the advantage that any morph of the termites collected from field surveys could be used for identification. Some samples which had only alate caste were correctly placed to their respective phylogenetic clusters without necessarily using soldiers which are usually difficult to find in an ecological survey. In addition, samples which were morphologically determined with a lot of uncertainty were also placed in their respective phylogenetic clusters.

Higher termite diversity was realized by using sequence-based thresholds than morphospecies. This implies that MOTU based species delimitation revealed the actual diversity of termite species in Kakamega forest. Similar termite diversity studies have been done elsewhere in tropical rain forests using Shannon index and Evenness (Bandeira *et al*., 2003) but limited to only morphospecies. Other studies were conducted in Kibale forest, Uganda (Darlington *et al.,* 1997) and small reminant forests in Shimba Hills, Kenya (Darlington *et al.,* 2001). In Uganda 11 species were collected while in Shimba Hills, Kenya 27 species were collected, all morphospecies. *Cubitermes umbratus* was recorded as the commonest species in Shimba Hills. In this study, species of *Odontotermes, Pseudacanthotermes* and *Microtermes* were most abundant in Kakamega forest. In Kakamega forest, at least 16 morphospecies were collected and 22 MOTUs by sequence-based biodiversity assessment. The extrapolation termite species numbers using Chao estimate were higher (17 and 26 for morphospecies and MOTUs respectively) than the above observed numbers. This shows that species diversity is generally underestimated by morphological determination and a sequence-based diversity estimates would be necessary even for the already assessed termite biodiversity elsewhere. This study is consistent with other studies on DNA barcoding especially in large and hyperdiverse insect orders, which have recorded higher biodiversity using DNA barcodes barcodes (Floyd et *al.,* 2002; Smith *et al.,* 2005; Hajibabaei *et al.,* 2006; Smith *et al.,* 2006; Rach *et al.,* 2008; Stahls and Savolainen, 2008).

4.5 Conclusion

As expected, termite diversity in Kakamega forest based on DNA barcoding was higher than based on morphological identification. A more comprehensive assessment of termite species in Kakamega forest was given. Although DNA barcoding may not be much useful without classical taxonomy data (Will and Rubinoff, 2004), it has been proved as a tool to give a more comprehensive view on termite biodiversity in Kakamega forest. These results have proven the reliability of DNA barcoding for a rapid biodiversity assessment of the termites using any available morph of termite specimen collected during field surveys. The study also highlights the advantage that molecular based species delimitation had revealed some morphological cryptic species.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General Discussion

The knowledge of symbiosis between fungal symbionts and their host has been scanty because of the taxonomic problem of *Termitomyces*. Their taxonomy relies mostly on basidiocarps, but these symbionts never fruit. This is the first study in Kenya which investigated the phylogenetic relationship of the fungal symbionts of the fungus-growing termites. At least eight *Termitomyces* lineages were found in Kenya, which probably represented different species. Genetic characterization of *Termitomyces* by direct sequencing of fungal nodules collected directly from the nest was possible without necessarily having to cultivate them. All the strains analysed across the termite host genera, species and in different localities in Kenya were found to be *Termitomyces*-related basidiomycetes. This was confirmed by comparing the sequences obtained in this study with those in the GenBank. Unfortunately, because of the use of fungal nodules from the fungus-comb, the species affiliation of the sequenced fungi in this study was not possible. The knowledge about the diversity of *Termitomyces* in Eastern Africa is still fragmentary. The fungus species *T. microcarpus* was observed within nests of *Odontotermes badius* (Sands, 1956) and *O. montanus* (Sieber, 1983) from Kenya. Two additional fungal symbionts *T. eurhizus* and *T. globulus* were reported for *O. badius* in Kenya but with circumstantial evidence (Wood and Thomas, 1989). Otieno (1964, 1969) described six species (*T. narobiensis*,

T. magoyensis, *T. biyii*, *T. rabuorii*, *T. tylerianus* and *T. badius*) from Kenya and *T. schimperi* from Ethiopia. This study has not only, but also added to the knowledge of the diversity of *Termitomyces* in Kenya but also their relationship with their host termites.

Symbionts of *Macrotermes, Pseudacanthotermes, Odontotermes* and *Microtermes* genera formed separate clusters in the cladogram of the reconstructed phylogenetic trees. This was revealed by strong sequence divergence among these major clades. No symbiont was shared among these groups showing high interaction specificity at genera level. Each genus cultivateed its own lineage of symbiont which was different from the the ones cultivated by other host termite genera. This host genus specificity was supported by a randomization procedure, since host genera were not randomly distributed across the reconstructed tree. The observed pattern of host genus specificity was in accordance with previous studies (Aanen *et al.*, 2002, 2007; Rouland-Lefèvre *et al.*, 2002). However, there are also findings that have reported some exceptions with the same fungal lineage being cultivated by different termite genera (Aanen *et al.*, 2002; Rouland-Lefèvre *et al.*, 2002; Taprab *et al.*, 2002). This implies that the symbionts have differentiated after acquisition by the ancestors of their host termites and have coevolved with their host termites to form a stable symbiosis with a specific genus of termites.

Deep sequence divergence between fungal symbionts was realized within the *Termitomyces* clades of host genera *Macrotermes* and *Odontotermes,* indicating a diversity of fungal lineages harbored within these termite genera. Single lineages that probably represented different species of *Termitomyces* symbionts were shared among

different species of *Macrotermes. Pseudacanthotermes spiniger* and *Pseudacanthotermes militaris* cultivated same lineage of *Termitomyces.* Furthermore, different lineages of fungal symbionts were cultivated by the same species even at the same locality, e.g. within neighboring nests of *M. michaelseni* from Kajiado. Altogether these results indicate low interaction specificity at the host species level with frequent exchanges of symbionts between termite species, although the host species were not completely random distributed across the phylogenetic tree of fungi. The termite host and symbiont relationship at species level seems complicated probably because of the methods the termite host uses to acquire symbionts during colony establishment and possibly attributed to the mode of symbiont transmission. This pattern at the host species level is consistent with previous studies (Aanen *et al*., 2002, 2007; Katoh *et al*., 2002).

Some symbionts are thought to be generalists rather than specialists with respect to their hosts. Rouland-Lefèvre, 2000 pointed out that these kind of symbionts are able to produce numerous different enzymes according to the substrate available. They are associated with several hosts and are considered not to form strong associations with their host termites. This possibly could be proximate explanation for finding similar fungal sequences associated with different host termite species. In addition, Aanen *et al*. (2002) suggested that many of *Termitomyces* symbionts are shared by different termite species since there are fewer species of fungus than there are species of host termite (Heim, 1977). On the other hand, some symbionts are considered to be specialists rather than generalists. Specialists are thought to have an easier way to strong mutual coevolution (Rouland-Lefèvre, 2000; De Fine Licht *et al.*, 2005). For example, *M. bellicosus* and *Microtermes* are known to cultivate a single lineage of

Termitomyces strains, which are not shared with other Macrotermitinae species. In this study, *M. herus* cultivated a different *Termitomyces* lineage which was also shared with *M. bellicosus*. Similarly, *Microtermes* species also shared the same fungal symbionts with species of *Ancistrotermes* and *Odontotermes.* These findings possibly indicate a recent symbiont exchange between these termite lineages. Symbionts which are selectively cultivated by a particular host species are considered to produce a single enzyme, thus associated with a single termite host besides being considered to form strong association with their host (Rouland-Lefèvre, 2000). They have been reported to have a vertical symbiont transmission. Although horizontal symbiont transmission has been shown experimentally to be the norm in several species of Macrotermitinae and is consistent with the frequent formation of sexual fruiting bodies in many fungal symbionts, symbionts of *M. bellicosus* and *Microtermes* have been reported to be transmitted vertically from generation to generation via males and females respectively (Johnson *et al.*, 1981). This has been the case with gut symbiotic protists in lower termites and termite gut bacteria basically via proctodeal trophallaxis, although the coevolutionary process cannot also be explained by a purely vertical transmission (Hyodo *et al.*, 2003).

To assess the diversity of termites in Kakamega forest, a COII sequence divergence threshold value of 0.056 was used to delimit these termites at species level, with success rate of 91.7% using intra and interspecific distances. Similarly, 0.058 was defined to delimit the whole group of Isoptera with a success rate of 89.9%. The high success rates of over 80% in all the distributions among families, most of which were above 90%, showed weak overlaps and disjoint distributions.

The assignment of samples to morphotypes did not always correspond to the molecular clusters (MOTUs) of COII gene sequencing data of the termite species on a NJ tree. But some MOTU clusters corroborated well with morphospecies in MOTU delineation using the sequence divergence threshold. Within subfamily Macrotermitinae, only *M. herus* converged with morphological identity. Consequently, there were less morphotype species as compared to MOTU species numbers as delimited by the defined threshold and mean values. This revealed some morphological cryptic species. And as expected, the number of MOTUs increased with decreasing threshold values. This was consistent with the findings of Smith *et al*., (2005) who used different MOTU level for ant diversity assessment in Madagascar. Other findings showing cryptic speciation in other groups of taxa has been reported by several authours (Floyd *et al.,* 2002; Stahls and Savolainen, 2008; Rach *et al.,* 2008; Hajibabaei *et al.,* 2006; Smith *et al.,* 2006; Ball *et al.,* 2005). These authours have shown that DNA barcodes can reveal cryptic species, thus consequently revealling hidden diversity of hyperdiverse organisms like termites. These results highlight the advantage that molecular based species delimitation revealed some morphological cryptic species and consequently giving a more comprehensive picture of the diversity.

The termites without sampled soldiers, which are normally difficult to determine could be assigned to their respective phylogenetic clusters. Futhermore, these results highlight the advantage that any morph of the termites collected from field surveys could be used for identification. Some samples which had only alate caste were

correctly placed to their respective phylogenetic clusters without necessarily using soldiers which are usually difficult to find in an ecological survey. In addition, samples which were morphologically determined with a lot of uncertainty were also placed in their respective phylogenetic clusters.

Higher termite diversity was realized by using both sequence-based thresholds than morphospecies. This implies that MOTU based species delimitation revealed the actual diversity of termite species in Kakamega forest. Similar termite diversity studies have been done elsewhere in tropical rain forests using Shannon index and Evenness (Bandeira *et al*., 2003) but limited to only morphospecies. Other studies were conducted in Kibale forest, Uganda (Darlington *et al.,* 1997) and small remunant forests in Shimba Hills, Kenya (Darlington *et al.,* 2001). In Uganda 11 species were collected while in Shimba Hills, Kenya 27 species were collected, all morphospecies. *Cubitermes umbratus* was recorded as the commonest species in Shimba Hills where species of *Odontotermes, Pseudacanthotermes* and *Microtermes* were most abundant in Kakamega forest. In Kakamega forest, at least 16 morphospecies were collected and 22 MOTUs by sequence-based biodiversity assessment. The extrapolation termite species numbers using Chao estimate were higher (17 and 26 for morphospecies and MOTUs respectively) than the above observed numbers. This shows that species diversity is generally underestimated by morphological determination and a sequence-based diversity estimates would be necessary even for the already assessed termite biodiversity elsewhere. This study is consistent with other studies on DNA barcoding especially in large and hyperdiverse insect orders, which have recorded higher biodiversity using DNA barcodes (Floyd et *al.,* 2002; Smith *et al.*, 2005; Hajibabaei *et al.,* 2006; Smith *et al.,* 2006; Rach *et al.,* 2008; Stahls and Savolainen, 2008).

5.2 Conclusions

(i) High interaction specificity between the termites and their fungal symbionts at host genus level was revealed in this study, implying some degree of co-evolution.

(ii) There was low interaction specificity between the termites and their fungal symbionts at host species level, indicating frequent host switches.

(iii) High amount of sequence divergence realized in the ITS sequences among different recognized clades reveals the existence of at least eight different *Termitomyces* species cultivated by the host termites.

(iv) The fungal strains are well distributed across Kenya and were not restricted to a particular locality or biome. Therefore their association with their host termites is not site specific.

(v) Termite diversity in Kakamega forest based on DNA barcoding was higher than their morphological determination. This highlight the advantage that molecular based species delimitation revealed some morphological cryptic species, consequently hidden diversity.

(vi)These results have proven the reliability of DNA barcoding for a rapid biodiversity assessment of the termites using any available morph of termite specimen collected during field surveys.

5.3 Recommendations

(i) More studies need to be done across host termite species and cutting across higher number of colonies not only to assess the complicated termite- symbiont relationship at species level, but also to assess the actual diversity of the *Termitomyces* symbionts in Kenya.

(ii) Termite species diversity in similar tropical forests and other geographical regions need to be done with DNA barcoding approach. This will not only give their rapid assessment but also the sequences will form a database to provide alternative set of characters to assist in inferring termite species boundaries in future taxonomic studies.

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