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DIVERSITY AND ECOLOGY OF *TERMITOMYCES* SYMBIONTS IN *MACROTERMES* MOUNDS OF THE TSAVO ECOSYSTEM, KENYA

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DIVERSITY AND ECOLOGY OF *TERMITOMYCES* SYMBIONTS IN *MACROTERMES* MOUNDS OF THE TSAVO ECOSYSTEM, KENYA

Risto Vesala

ACADEMIC DISSERTATION

To be presented for public discussion with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Nylander hall, Botany Unit of the Finnish Museum of Natural History, on the 18th of October, 2019 at 12 o'clock.

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Cover photo: *Macrotermes michaelseni* mound in Taita Hills Wildlife Sanctuary, Kenya (Risto Vesala)

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ABSTRACT

Fungus-growing termites are ecologically important animals in tropical Africa and Asia. Especially in dry savannas, they contribute to local carbon and mineral recycling and alter soil physical properties, thus facilitating the success of many plant species. This, in turn, has indirect impacts also on animals that may e.g. benefit from improved food supply and quality.

The success and ecological significance of fungus-growing termites arise from their exosymbiotic relationship with the fungal genus *Termitomyces*. Termites cultivate fungal symbionts within specialized compost structures in their underground nests where the mycelium assists in degradation of plant matter collected by the termites, thus providing a constant food supply for the large termite colonies. Symbiotic food processing is especially advanced in the termite genus *Macrotermes* which construct large above-ground soil structures – termite mounds – to enhance ventilation of the below-ground nests and to provide a favorable microclimate for fungal growth even in arid savanna environments.

The aim of this thesis was to study interactions between *Macrotermes* termites and their *Termitomyces* symbionts in the semiarid Tsavo Ecosystem in Southern Kenya. We assessed the local diversity of the host insects and their fungal symbionts and produced an up-to-date phylogeny of the fungal symbionts based both on our new results and previously published DNA data. We found that the *Macrotermes–Termitomyces* diversity in the Tsavo Ecosystem involves two host species and three symbiont species that occur in different combinations, and the frequencies of different associations vary over the landscape. Studies on mound architecture and symbiont diversity revealed correlations between the size and type of above-ground mounds and specific host-symbiont combinations. These were linked to architecturally induced differences in nest temperatures, suggesting that different *Termitomyces* species may differ in their ranges of tolerable growth temperatures.

Stable isotope studies provided important new information on the nutritional role of *Termitomyces* for *Macrotermes* colonies. *Termitomyces* promotes the nutrition of the host insects directly, as highly nitrogenous food for queen and young larvae, and indirectly, by decomposing plant matter that is eaten by workers, soldiers, and developing alates. Thereby, the fungal symbiont does not have a single universal role in the nutrition of a termite colony, but instead, different termite castes depend on the symbiosis in different ways. The isotopic imbalance of nitrogen also implied that, although the nutrition of fungus-growing termites is facilitated by the fungal symbionts, also bacterial nitrogen fixing may provide an essential complementary nitrogen source for termite colonies.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Vesala, R., Niskanen, T., Liimatainen, K., Boga, H., Pellikka, P., & Rikkinen, J. (2017). Diversity of fungus-growing termites (*Macrotermes*) and their fungal symbionts (*Termitomyces*) in the semiarid Tsavo Ecosystem, Kenya. Biotropica, 49(3), 402–412.

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The publications are referred to in the text by their roman numerals.

The contribution of the author to the publications:

I Risto Vesala designed the sampling together with Jouko Rikkinen and Petri Pellikka, excavated the termite mounds together with field assistants, performed DNA extractions and PCR, processed and analyzed the sequence data together with Tuula Niskanen and Kare Liimatainen, and wrote the first version of the manuscript.

II Risto Vesala designed the experimental setup together with Jouko Rikkinen, Petri Pellikka and Petri Rönnholm, conducted field work together with Anu Hakkarainen and Anni Harjuntausta, performed DNA extractions and PCR, analyzed the data, wrote the first version of the manuscript, and is the corresponding author.

III Risto Vesala planned the sampling together with Jouko Rikkinen and Laura Arppe, performed the field work together with Jouko Rikkinen and Laura Arppe, prepared samples for the isotope analysis together with Laura Arppe, analyzed and interpreted the data together with the other authors, wrote the first version of the manuscript, and is the corresponding author.

1 INTRODUCTION

1.1 TERMITE DIVERSITY

Termites (order Blattodea, formerly Isoptera) are eusocial insects: they live in colonies that typically consist of sexual reproductives (queen, king, and at times winged alates) and sterile castes including workers and soldiers (Eggleton 2011). Termite colonies most often have one reproductive royal pair that produces all the sexual and sterile individuals of the colony, although in some species the presence of several queens (polygyny) or kings (polyandry) is also relatively common (Thorne 1984; Atkinson and Adams 1997; Brandl et al. 2001; Brandl et al. 2004; Hacker et al. 2005). The primary reproductives typically live as long as the colony (in some cases more than ten years) while the original royal couple can occasionally be replaced by new reproductives in some species (Sieber and Darlington 1982; Thorne 1984; Korb et al. 2015).

The origin and phylogenetic position of termites have been under active research during the last decades and it now seems clear that termites form a monophyletic group within the order Blattodea (includes termites and cockroaches) under which they are currently treated as an epifamily Termitoidae (e.g. Lo et al. 2000; Inward et al. 2007; Eggleton et al. 2007; Xiao et al. 2012; Djernæs et al. 2015; Legendre et al. 2015; Evangelista et al. 2019). Termites include more than 2600 species that are presently classified in nine families and 281 genera (Kambhampati and Eggleton 2000; Engel et al. 2009). Termites are common everywhere in the tropics with the highest diversity in lowland rain forests of Africa (Jones and Eggleton 2011). There is also considerable diversity in African savannas where termites are among the most abundant animal groups (Jones and Eggleton 2011; Jouquet et al. 2011). Only a few termite species live at temperate regions including USA and southern parts of Europe (Jones and Eggleton 2011).

Termites are either herbivores that consume plant material (either wooden or herbaceous tissues, feeding groups I and II by Donovan et al. 2001) or detritivores that consume decomposed soil organic matter (humus and soil feeders, corresponding to feeding groups III and IV). Termite life styles range from ‘one-piece’ termites (colonies live in their food source) to species that construct complex nest systems with extensive underground tunnel networks that allow termites to collect food from a large area (Abe 1987; Noirot and Darlington 2000; Eggleton and Tayasu 2001).

Phylogenetically basal termite taxa are referred to as ‘lower termites’ (Legendre et al. 2008; Engel et al. 2009). An important feature that characterizes this group are the presence of endosymbiotic gut flagellates that degrade lignocellulose of the ingested plant material, thus assisting termites to feed on plant matter (Cleveland 1923; Yamin and Trager 1979; Brune and Ohkuma 2011; Brune 2014; Bignell 2016). All the lower termites belong to

feeding group I and mostly consume dead wood or in some cases dry grass (Donovan et al. 2001). Living plant matter is only consumed by relatively few species of termites (Collins 1983; Waller and La Fage 1987).

The second group, the so called 'higher termites', lack eukaryotic gut symbionts but instead have a rich bacterial flora in the hindgut (Brune and Ohkuma 2011; Bignell 2016). Higher termites are evolutionarily advanced and comprise a monophyletic family Termitidae (Inward et al. 2007; Legendre et al. 2008; Engel et al. 2009). This lineage contains a majority of extant termite diversity and includes numerous ecological keystone species (Engel et al. 2009). Higher termites belong to feeding groups II, III and IV (Donovan et al., 2001), and may thus consume either dead wood, herbaceous plant matter or grass (II), or soil organic matter at different stages of decay (III and IV, Donovan et al. 2001). One special lineage within the higher termites are the fungus-growing termites that have evolved to cultivate basidiomycetous fungi and to utilize these fungal symbionts in the degradation of plant cell wall compounds.

1.2 FUNGUS-GROWING TERMITES

Fungus-growing termites (subfamily Macrotermitinae) are an ecologically specialized group within the Termitidae (Aanen et al. 2002). Their distribution is restricted to the Old World tropics with the highest diversity in rain forests and savannas of equatorial Africa (Jones and Eggleton 2011). Fungus-growing termites establish highly obligatory symbioses with species of the fungal genus *Termitomyces* Heim (Agaricales, Lyophyllaceae). The fungal symbionts are cultivated in specialized fungal chambers (Figure 1A) within termite nests (Wood and Thomas 1989; Rouland-Lefèvre and Bignell 2001).

The principal benefits of fungus-cultivation for termites are in colony nutrition (Rouland et al. 1991; Hyodo et al. 2000, 2003; Rouland-Lefèvre and Bignell 2001; Nobre and Aanen 2012). Termites provide a constant food supply for the fungus which, as a reciprocal service, assists in the degradation of recalcitrant plant cell wall components by producing enzymes that act in lignocellulose degradation (Martin and Martin 1978; Martin and Martin 1979; Rouland et al. 1988a, 1991; Nobre and Aanen 2012; Poulsen et al. 2014; da Costa et al. 2018). This, in turn, allows termites to effectively exploit partly decayed plant matter (Rouland et al. 1991, Hyodo et al. 2000). As *Termitomyces* mycelium per se is highly nitrogenous, it most likely also serves as an additional food source for the termites (Matsumoto 1976; Collins 1983). However, the overall significance of the fungus as a direct food source for the colonies is still largely unconfirmed, as the nutritional utilization of fungal hyphae has so far only been demonstrated for some of the termite species (Hyodo et al. 2003).

Division of labor between castes in food processing has been studied in the termite genera *Macrotermes* and *Odontotermes* (Batra and Batra 1979; Sieber

and Leuthold 1981; Badertscher et al. 1983; Gerber et al. 1988; Hinze and Leuthold 1999; Hinze et al. 2002; Li et al. 2015). In all studied species the food processing chain starts from termite foragers that typically are old major workers (Badertscher et al. 1983; Hinze and Leuthold 1999). The foragers collect plant litter from the environment and transport it to the nest where the material is eaten by younger workers (Sieber and Leuthold 1981; Badertscher et al. 1983). The homogenized and partly decayed plant biomass is soon defecated into sponge-like compost structures (fungus combs, Figure 1B) where it becomes substrate for the *Termitomyces* mycelium growing within the combs (Sieber and Leuthold 1981; Rouland-Lefèvre and Bignell 2001). In addition to foraged plant material, young workers consume spherical fungal structures (nodules, Figure 1C) produced in matured parts of fungus combs (Sieber and Leuthold 1981; Leuthold et al. 1989). Nodules contain fungal conidia that effectively inoculates the newly added plant material (Leuthold et al. 1989). Nodules contain also fungal enzymes that degrade lignin and cellulose synergistically with enzymes produced both by the termites and their gut bacteria (Abo-Khatwa 1978; Martin and Martin 1978; Veivers et al. 1991; Nobre and Aanen 2012; Poulsen et al. 2014; Li et al. 2017; da Costa et al. 2018). Finally, the old stagnant parts of the fungus combs are eaten by old workers who also ingest soil and plant litter during foraging (Sieber and Leuthold 1981; Badertscher et al. 1983). All the other termite castes are fed by the workers either with solid comb material or liquid food excreted presumably from their labial glands (Sieber and Leuthold 1981; Badertscher et al. 1983 ; Hinze et al. 2002).

While the symbiotic *Termitomyces* plays a vital central role in the ecology of a termite colony, the fungus itself is totally dependent on care of the termite workers. In addition to providing a constant food supply, termites prevent the growth of bacteria and competing fungi in fungus combs via mechanical weeding (Wood and Thomas 1989) and by applying antibiotic compounds produced by the termites or their gut-symbiotic bacteria (Lamberty et al. 2001; Mathew et al. 2012; Um et al. 2013). Many species of fungus-growing termites also construct soil structures that effectively regulate nest interior climates and enhance ventilation, thus creating favorable conditions for the fungal growth (see section 1.4).

1.3 HOST-SYMBIONT INTERACTIONS

Phylogenetical analyses have indicated that the cultivation of fungi within termites has evolved only once and no later returns to a non-symbiotic lifestyle have occurred within the group (Aanen et al. 2002; Rouland-Lefèvre et al. 2002; Frøslev et al. 2003). Concurrently, the genus *Termitomyces* currently only includes termite symbiotic species (Aanen et al. 2002; Rouland-Lefèvre et al. 2002). Based on molecular dating and fossil records, the initial domestication of *Termitomyces* by Macrotermitinae seems to have occurred

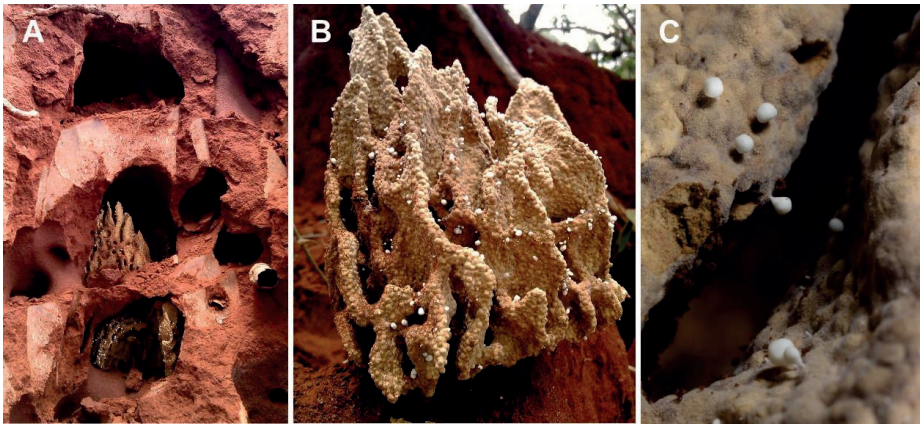

Figure 1 **A.** Fungal chambers in an opened *Macrotermes* mound. **B.** Fungus comb taken out from the chamber. **C.** Fungal nodules produced by the *Termitomyces* in mature parts of a fungus comb. Photos: Risto Vesala (A, B) and Jouko Rikkinen (C).

ca. 30 MA in African rain forests (Aanen and Eggleton 2005; Brandl et al. 2007; Nobre et al. 2011; Roberts et al. 2016). The current diversity of fungus-growing termites include ca. 330 species in 10 genera (Kambhampati and Eggleton 2000; Aanen et al. 2002). The diversity of *Termitomyces* is still poorly known. Some 40 fungal species have been described within the genus (Kirk et al. 2008), but DNA sequences have recently indicated a much higher diversity (e.g. Aanen et al. 2002; Frøslev et al. 2003; Osiemo et al. 2010 ; Makonde et al. 2013). Gap between the described species and DNA data is largely due to the fact that many species do not produce basidiomata or produce them only rarely (Johnson et al. 1981; Frøslev et al. 2003, Koné et al. 2011).

Most genera of fungus-growing termites (e.g. *Macrotermes*, *Odontotermes*, *Acanthotermes*) show relatively high levels of symbiont specificity (Aanen et al. 2002, 2007). For example all *Termitomyces* symbionts cultured by the species of the genus *Macrotermes* form a monophyletic group that is not cultivated by any other genera of Macrotermitinae (Aanen et al. 2002; Nobre et al. 2011). On the contrary, termite genera *Microtermes*, *Ancistrotermes* and *Synacanthotermes* have been found to share a single *Termitomyces* lineage (Aanen et al. 2002). Different levels of specificity have also been observed within single genera: for example South African *Macrotermes natalensis* (Haviland) seems to always associate with one specific *Termitomyces* lineage whereas several other *Macrotermes* species (e.g. *M. subhyalinus* Rambur and *M. bellicosus* Smeathman) typically cultivate several distinct lineages within the *Macrotermes* associated *Termitomyces* clade (Aanen et al. 2007; Nobre et al. 2011).

Only one *Termitomyces* genotype is always found from a single termite colony, or at least this has been the case in all termite colonies studied so far (Aanen et al. 2002, 2009; Katoh et al. 2002; Makonde et al. 2013). The fungal monoculture of a fungus comb seems to be established and maintained by frequency dependent propagation during food processing (Aanen et al. 2009). As the dominant *Termitomyces* genotype produces the highest yield of fungal nodules, termite workers ingesting nodules (including abundantly fungal conidia) tend to automatically favor this genotype to inoculate newly added plant matter, which eventually leads to the predominance of one fungal lineage within the whole comb (Aanen 2006; Aanen et al. 2009). However, it remains unclear why and how a particular *Termitomyces* genotype is initially selected for cultivation in newly founded termite colonies (Nobre and Aanen 2012).

Symbiont transmission between termite generations appears to be horizontal in most cases, meaning that the hosts and the fungal symbionts disperse separately, and the initial *Termitomyces* inoculum (presumably haploid spores) are obtained from the nest environment by the first generation of foraging workers (Johnson et al. 1981; Sieber 1983; Korb and Aanen 2003; Nobre et al. 2011). However, as many *Termitomyces* species have never been observed to produce fruiting bodies and sexual spores, the actual origin of the fungal inoculum remains unknown in many cases (Johnson et al. 1981; Korb and Aanen 2003). Horizontal transmission is believed to be the ancestral mode of symbiont dispersal in termite-fungus symbioses, whereas vertical transmission (i.e. inoculum obtained and transported by alates from their parental colonies) has evolved independently in two termite lineages: in *Macrotermes bellicosus* where the fungal inoculum is carried by dispersing males, and in the genus *Microtermes* where the inoculum is carried by dispersing females (Korb and Aanen 2003; Nobre et al. 2010). Vertical transmission may be especially beneficial in long-distance dispersal and probably explains why *Microtermes* is the only genus of fungus-growing termites that has been able to colonize Madagascar (Nobre et al. 2010). However, occasional symbiont switching and sexual recombination seem to take place also in termite species that largely rely on vertical symbiont transmission (Aanen et al. 2002, 2007; Nobre and Aanen 2010; Nobre et al. 2010, 2011).

1.4 MOUND ARCHITECTURE

The conspicuous termite mounds that characterize many African savanna landscapes are mostly built by the species of the genus *Macrotermes* (Korb 2011). Also some species of *Odontotermes* and *Pseudacanthotermes* build above-ground mounds, which however are typically quite modest compared to those built by *Macrotermes* (Darlington 1994; Turner 1994; Darlington 1997). Mound building as such is not restricted to fungus-growing termites: for example some *Amitermes* species construct the large ‘compass mounds’ in

northern Australia (Korb 2003a). However, the architecture and purpose of such mounds differ considerably from those built by the Macrotermitinae, which principally construct mounds to provide favorable growth conditions for their *Termitomyces* symbionts (Korb 2011).

In some areas of Central Africa soil structures built by the *Macrotermes* termites can reach heights of up to 10 meters and have basal diameters of up to 15 meters (Pullan 1979). However, such massive 'termite hills' typically evolve through repeated cycles of mound building and erosion during hundreds or even thousands of years, and termite activity typically only occurs in the topmost parts (Pullan 1979; Erens et al. 2015). Mounds built by the East, West and South African *Macrotermes* species tend to be smaller, with typical dimensions up to a few meters. Although recolonization of old and abandoned mound sites occurs, majority of the existing mound structure is typically built by the most recent termite colony. Pomeroy (1976) found that the most active development of *M. bellicosus* mounds in Uganda took on average three years after which growth rates slowed down as the colonies achieved maturity. The erosion of large abandoned *Macrotermes* mounds may, in turn, typically take 20–25 years (Pomeroy 1976; Lepage 1984).

Fungus-growing termites build above-ground mounds to regulate temperature and humidity in their nests and to facilitate gas exchange (Turner 2001; Korb 2003b, 2011). Effective control of nest interior climate is crucial for the symbiotic *Termitomyces* which grows best at temperatures of ca. 29–31 °C and needs constant humidity that is typically maintained at the level of 98–99 % (Lüscher 1961; Collins 1977; Thomas 1981). The ambient temperature and humidity regimes of arid and semi-arid savannas are thus far from optimal for the symbiotic fungi. The thick walls of large termite mounds buffer temperature fluctuations and prevent evaporation. Large termite colonies also produce large quantities of respiratory gases, including up to 1500 liters of carbon dioxide per day (Darlington et al. 1997), which must be disposed of and continually replaced with fresh air. Mound cavities induce air circulation within the nests which, in turn, promotes gas exchange between nest interior and ambient air (Weir 1973; Korb 2011; King et al. 2015; Ocko et al. 2017).

Architectural details of mound structure are species specific to a certain degree, and the inhabitant termites can often be identified based on the basis of mound structure (Korb 2011). For example, two closely related Kenyan *Macrotermes* species are morphologically almost identical, but build different types of mounds: the mounds of *M. subhyalinus* (Rambur) have several large ventilation shafts that open to the mound surface, whereas the mounds of *M. michaelseni* (Sjöstedt) lack open shafts (Arshad 1981; Darlington 1984a; Darlington 1985; Bagine et al. 1994). Instead, the topmost parts of *M. michaelseni* mounds are porous enabling the easy passage of respiratory gases and fresh air through the walls (Turner 2001). The physical basis of the two types of mounds are completely different with the ventilation of open mounds type being wind-induced and that of closed mounds relying on solar-induced

within-mound air circulation (Weir 1973; Korb 2011; Ocko et al. 2017). The closed mounds of the West African *M. bellicosus* (Smeathman) are functionally similar to those of *M. michaelseni* but their nests are typically situated higher in relation to the soil surface than in mounds of *M. michaelseni* (Korb 2011). Finally, open mounds built by the Kenyan *M. jeanneli* (Grassé) can be easily distinguished from the mounds of *M. subhyalinus* as they typically have a tall central chimney with a single large ventilation shaft opening to the top (Darlington et al. 1992, 1997; Korb 2011).

In addition to architectural differences in mounds of different termite species, also mounds built by a single termite species may differ in architecture when built in different habitats, thus reflecting complicated equilibria between above-ground mound structure, nest thermoregulation and gas exchange, which can be differently balanced depending on local temperature and wind conditions, vegetation, and other external factors (Korb and Linsenmair 1998a, 1998b, 1999, 2000a, 2000b; Korb 2003b, 2011).

1.5 ROLE IN ECOSYSTEM

Conditions in arid and semi-arid savannas are highly disadvantageous for saprotrophic fungi, especially during the dry season. However, the innovation of fungus cultivation in architecturally advanced nests has allowed Macrotermitinae and their *Termitomyces* symbionts to effectively occupy this harsh ecological niche (Collins 1983; Jones 1990; Aanen and Eggleton 2005; Jouquet et al. 2011). As a result, fungus-growing termites and their fungal symbionts are now major litter decomposers in many dry regions in tropical Africa (Collins 1983; Jones 1990; Schuurman 2005; Jouquet et al. 2011). For example in the drier parts of the Tsavo Ecosystem (Kenya) fungus-growing termites and their symbionts may take care of up to 90 % of wood litter decomposition (Buxton 1981). In the relatively humid Southern Guinea savanna of Nigeria their contribution was more modest but it was still estimated to account for 60 % of wood and 24 % of total annual litter decomposition (Collins 1981). In dry tropical forests fungus-growing termites were found to mineralize 7.5–11.2 % of all carbon stored in the annual above-ground litterfall (Yamada et al. 2005). Similar magnitudes were reported from humid West African savannas where CO_2 emissions of *Ancistrotermes cavithorax* and *Odontotermes pauperans* colonies represented 11.3 % of the annual above-ground production of organic carbon that was not mineralized by fire (Konaté et al. 2003).

Fungus-growing termites can utilize a wide selection of grasses and both leaf litter and woody tissues of trees and shrubs as their main food source, and the selection of food often depend on the spatial and temporal availability of different types of plant matter (Lepage 1981; Boutton et al. 1983; Lepage et al. 1993; Dangerfield and Schuurman 2000). To obtain a sufficient amount of nitrogen especially from nutrient-poor wood, termite colonies need to process

large amounts of plant material, and excessive carbon is released to the atmosphere mostly as CO2 (Collins 1983; Jones 1990; Higashi et al. 1992). While a significant proportion of the mineral nutrients obtained from the plant matter may return to circulation via predation of foraging workers and swarming alates, major amounts of nitrogen, phosphorus and other nutrients are concentrated into termite nests in the form of living biomass, fecal material and termite saliva used in wall construction (Jouquet et al. 2011). As mound walls erode during seasonal rains, these minerals are flushed onto mound outwash pediments where they are again readily available for grasses and other plants (Arshad 1982; Dangerfield et al. 1998).

In addition to their contribution to element cycles and nutrient translocation, fungus-growing termites also affect to physical soil properties by carrying subsoil material with high clay content to the ground surface (Arshad 1981; Arshad 1982; Dangerfield et al. 1998; Jouquet et al. 2002a; Jouquet et al. 2011; Abe et al. 2012). Termites have been shown to alter clay mineralogical properties although the actual mechanisms of this process remain poorly known (Jouquet et al. 2002b; Jouquet et al. 2011). Obviously termite nests affect the local water balance of arid savannas especially during the dry season, as the humidity inside active nests remains constantly high (Lüscher 1961; Turner 2006). Innumerable foraging tunnels around the nest areas also increase soil porosity and facilitate the infiltration of surface water (Darlington 1982; Jouquet et al. 2011). Due to all these effects, large termite mounds tend to represent 'islands of fertility' especially in nutrient-poor savanna ecosystems where nitrogen and phosphorus deficiency and other challenging soil conditions strictly limits plant cover (Sileshi et al. 2010; Jouquet et al. 2011).

By altering soil conditions in many different ways, the fungus growing termites act as true 'ecosystem engineers', and significantly influence the structure of plant communities by increasing species and functional diversity around their nests (Arshad 1982; Dangerfield et al. 1998; Davies et al. 2014; Joseph et al. 2014; Davies et al. 2016a). This generates and maintains spatial heterogeneity and vegetation patchiness so characteristic of many vegetation types in African savannas (Sileshi et al. 2010; Erpenbach et al. 2012; Okullo and Moe 2012). This, in turn, has many indirect consequences that affect many animals including large mammals: termite nest areas can often provide high quality food for both grazers and browsers (Holdo and McDowell 2004; Davies et al. 2016b), provide nesting and perching facilities for birds (Joseph et al. 2011), and maintain appropriate microclimates for several different animals (Joseph et al. 2016; Joseph et al. 2018). Such effects of termite mounds may also decrease the overall vulnerability of dry ecosystems to drought and even protect them against desertification (Bonachela et al. 2014).

2 OBJECTIVES OF THE THESIS

The main objective of my thesis was to explore *Termitomyces* diversity in East African *Macrotermes* mounds, to study environmental and ecological interactions behind the observed diversity patterns, and to provide new precise information on the nutritional role of the fungal symbionts for the fungus-growing termites. I used state-of-the-art tools and methods from several disciplines, including molecular phylogenetics, geoinformatics and stable isotope techniques.

In the first article (I) we mapped and sampled termite mounds from several study areas representing different semi-arid savanna habitat types within Taita-Taveta County in Southern Kenya. The principal aims were to study interactions between the host termites and their symbionts in different environments and to fulfill a major gap in knowledge regarding the overall diversity and relative abundance of different *Termitomyces* species at the habitat and landscape level. As even the preliminary analyses soon indicated that the relative abundances of different *Termitomyces* species were not constant but varied within the landscape, the logical next step was to try to identify some ecological factors that might explain the observed patterns.

In the second article (II) we studied interactions between mound ambient and nest temperatures and the *Termitomyces* symbionts cultivated by the two dominant *Macrotermes* species (*M. subhyalinus* and *M. michaelseni*) in the Tsavo Ecosystem. As mound architecture was known to be involved in regulation of nest microclimates, we hypothesized that, if the different *Termitomyces* species would have different thermal requirements for optimal growth, some relationship might exist between mound architecture and the species identity of the cultivated fungus. We also wanted to get more precise information of how differences in mound building activities affect the nest interior temperatures of our target species. The mound architecture of numerous termite colonies was modeled by using 3D photogrammetry and the nest interior temperatures were measured using long-term data logging.

In the third manuscript (III) we focused on another important factor with obvious links to symbiont diversity: food selection and processing within termite colonies. The original idea was to use carbon and nitrogen stable isotopes to determine how termite colonies with different fungi utilize different food sources in the environment. However, as many important details in symbiotic food processing and colony nutrition are still poorly understood, we decided to first elucidate major principles of the element cycles that take place within individual termite colonies. Thus, the primary aims were (1) to reveal how the fungal degradation within termite nests affects to the compositions of carbon and nitrogen stable isotopes, and (2) to obtain more detailed information about the nutritional role of the symbiotic fungus for different termite castes and age-groups present within *Macrotermes* colonies.

3 MATERIALS AND METHODS

3.1 STUDY AREA

Field research was conducted in the dry savannas, shrublands and woodlands surrounding the Taita Hills and Mount Kasigau in Taita-Taveta County, southern Kenya (Figure 2). The study area is situated between the Tsavo West and Tsavo East National Parks. The annual mean temperature in the area is ca. +23 °C with March being typically the warmest (+25 °C) and July the coolest (+20 °C) month of the year (Figure 3 in article II). Annual precipitation is ca. 600 mm with most of the rainfall concentrating on two rainy seasons: 'long rains' on March–May and 'short rains' on November–December. However, the rains are typically erratic and periods of prolonged drought are common.

Most of the studied termite colonies were located at eight study sites representing semi-arid savanna habitats all slightly different with respect to vegetation type and land use intensity (Figure 2 and Table 1 in article I). Some termite mounds studied in article II were sampled also outside these sites, but all the colonies were located within the maximum distance of 80 km from each other.

3.2 SAMPLING OF TERMITE COLONIES

To obtain biological material for DNA analysis (articles I and II) nests were excavated using a pickaxe and shovel until the first fungal chambers were reached. Several termites representing different castes and *Termitomyces* nodules were collected from the chambers with tweezers and preserved immediately in absolute ethanol. In case of 16 colonies (article I) parallel specimen sets were collected from opposite sides of the mounds to confirm that the cultivated fungus was genetically uniform in all chambers within the nest.

In addition to DNA specimens, materials for stable isotope analysis was obtained from four termite colonies (manuscript III). Three of these colonies situated at the Kasigau Road study site representing *Acacia-Commiphora* woodland with negligible grass cover present during the sampling (October 2018). One of the colonies was sampled nine months earlier (January 2018) at the Mbula study site representing bushland savanna with abundant shrubs and small trees and also with some grass in the field layer. Fungus combs, fungal nodules and different castes of termites (minor/major workers and soldiers, larvae representing different instars, king and queen) were collected from fungal chambers, queen chambers and from nursery areas of each studied colony. Food storages, fecal material, immature alates (nymphs) and

presoldiers were also collected when found. In order to compare stable isotope values of nest interior materials to those of the surrounding vegetation, plant specimens (including grasses and woody tissues/leaves of trees/shrubs) were collected from the surroundings of the sampled mounds. All biological specimens were dried within 24 hours of collection using a mushroom drier (+40 °C, overnight).

3.3 DNA METHODS AND PHYLOGENETICAL ANALYSIS

Ribosomal ITS1-5.8S-ITS2 DNA region was amplified from fungal nodules and mitochondrial cytochrome c oxidase subunit 1 coding gene (COI) from termites by using direct PCR method (Thermo Scientific, Phire Animal Tissue Direct PCR Kit for the termite and Phire Plant Direct PCR Kit for the fungal samples). We used either universal primer ITS1 (article I) or *Termitomyces* specific primer ITS1FT (article II) in combination with ITS4 (White et al. 1990; Aanen et al. 2007) for the fungal specimens. Termite specific COI primers TL1862 and TH2877 (Aanen et al. 2002) were used for the host insects. PCR program included initial denaturing (98°C for 5 min), 40 amplifying cycles (98° for 5 s, 55°C for 5 s, 72° for 20 s) and the final elongation (72°C for 1 min). Exo I/FastAP protocol (Thermo Scientific, Werle et al., 1994) was used to purify the PCR products prior sequencing, after which they were sequenced in two directions in FIMM Sequencing Unit (Helsinki). The two directions were aligned and manually checked using CodonCode Aligner 6.0.2 for Windows. Because of the ITS polymorphisms typically occurring in DNA extracted from heterokaryotic *Termitomyces* nodules (de Fine Licht et al. 2005) alignment of some fungal sequences was laborious. In such cases, either the two ITS haplotypes were deduced and corrected manually, or the fungal species was identified from the chromatograms by using two species specific marker sites within ITS1 (Figure 1 in article I). A total of 83 *Macrotermes* COI sequences and 104 *Termitomyces* ITS sequences from different termite colonies have been deposited in GenBank (accession numbers KY197485–KY197625 for COI and KY197626–KY197709 and MK275596–MK275616 for ITS sequences).

For the phylogenetical analysis of the article I all *Termitomyces* ITS sequences published in UNITE and NCBI GenBank (prior 2016) were downloaded using PlutoF workbench. To produce an up-to-date phylogeny of *Termitomyces* based on all published ITS sequences, and to find all those sequences that belong to the *Macrotermes* associated *Termitomyces* clade, the previously published sequences, three representatives of our new sequences, and three *Lyophyllum* spp. ITS sequences from GenBank (outgroup) were aligned in SeaView (version 4.5.4.) with MUSCLE. Maximum-likelihood phylogeny was produced in RAxML (version 8) using model GTR-CAT and 1000 bootstraps.

The group of *Macrotermes* associated *Termitomyces* was studied more detailed in a separate analysis. All sequences from the previous analysis that clustered within this group, twelve of our own new sequences, and three *Termitomyces* ITS sequences that clustered to the *Odontotermes*-associated clade (outgroup) were aligned (MUSCLE) in SeaView, and the maximum-likelihood analysis was run in RAxML using model GTR-GAMMA with 1000 bootstraps.

To study genetic diversity of the sampled host termites a total of 141 COI sequences obtained from the insect specimens were aligned in SeaView using MUSCLE and the maximum-likelihood analysis was run in RAxML using model GTR-GAMMA with 1000 bootstraps.

3.4 MOUND ARCHITECTURE AND TEMPERATURE MEASUREMENTS

In article II we recorded mound sizes in the field by measuring heights and widths from a total of 164 termite mounds including both *M. subhyalinus* and *M. michaelseni* colonies. Many of the measurements were done already during the sampling for the article I. Fourteen different-sized *M. subhyalinus* mounds were documented in high detail by producing photogrammetrical 3D models. Models were based on 50–120 photographs taken from the distance of 2–5 meters from the mounds with Nikon d5000 equipped with a 35 mm fixed focal length lens. Images were combined into 3D models using Agisoft PhotoScan software and the scales were determined based on a 45 cm long scale bar included in each of the acquired photographs. Mound volumes and surface areas were computed from the models by using Geomagic Qualify 11 software. Ventilation shafts (their number, size and mutual distances) were investigated by using Fiji ImageJ (v. 1.51) software from directly up to down orientated orthophotographs that were produced from the 3D models. In addition to the external mound dimensions, underground nests were measured in case of two small 'miniature mounds' of *M. subhyalinus* to get a general idea of the nest sizes. These colonies were completely excavated, and the maximum width and height of the nest interior were determined.

Temperature data logging of termite nests was performed during two independent time periods: January – August 2015 in two *M. subhyalinus* and two *M. michaelseni* mounds (first measurement campaign), and March 2016 – April 2017 in 14 different-sized *M. subhyalinus* mounds (second measurement campaign; Figure 1 and Table 1 in article II). The 14 mounds studied during the second campaign were the same that were 3D modeled. Temperatures were always measured from fungal chambers by using temperature data loggers (iButton Thermochron DS1922L, Maxim). To insert the temperature sensors each studied mound was opened by digging from the mound base until the first active fungus chambers were exposed. After installation the sensors in one of these chambers, the mound wall was repaired

and filled to its original level. In addition, a single data logger was installed in a tree (at height of ca. 2 m, covered with light-impermeable shields) at each study site to measure the local air temperature in the immediate proximity of the studied termite colonies. During campaign 1 the data loggers were programmed to record temperature at each full hour, and during the second campaign at three hour intervals (00:00, 03:00, 06:00, etc.).

3.5 ANALYSIS OF CARBON AND NITROGEN STABLE ISOTOPES

Dried specimens were pretreated in laboratory, including e.g. preparation and exploration of gut contents of workers under a stereo microscope (separation of first and second gut passages), division of queens into three sub-specimens (whole, abdomen, head/thorax), and clean-up of specimens containing mineral soil (food storages, final feces). Samples were then homogenized either manually by using a mortar and pestle (fungus combs, termites, nodules, final fecal material) or cryo-milled with liquid N2 cooling (plant specimens).

Homogenized samples were weighed in tin cups and the contents of carbon and nitrogen and the stable isotopic compositions were measured at the Laboratory of Chronology of Finnish Museum of Natural History (Helsinki) using NC2500 elemental analyzer coupled to Thermo Scientific Delta V Plus isotope ratio mass spectrometer. The raw isotope data was normalized with a multi-point calibration using certified isotopic reference materials (USGS-40, USGS-41, IAEA-N1, IAEA-N2, IAEA-CH3 and IAEA-CH7). Parallel analyses of subsamples placed consecutively within the analytical sequence yielded always reproducibility of ≤ 0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ values. Measurements of quality control reference materials over the entire analytical period indicate an internal precision of ≤ 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$.

3.6 DATA ANALYSIS AND STATISTICS

The maps in articles I and II were produced by using QGIS (2.8.1. Wien) or ArGIS (10.3.1.) software. Wood coverage and habitat heterogeneity (article I) was evaluated based on Google Earth satellite images using OpenLayers Plugin (QGIS Development team 2016). Graphs of the articles II and III were produced in R Studio (1.9.153). All graphics including phylograms, maps and charts were finalized in CorelDRAW Graphics Suite 2017.

Statistical analysis applied in article II were performed in R Studio (1.0.153). The effect of different mound architectural variables on nest interior temperatures was studied using generalized least squares models (GLS) in package nlme (Pinheiro et al. 2017). Weather data included in GLS models was obtained from the Maktau weather station of the Taita Research Station. To

evaluate correspondence of temperatures measured at the weather station and at the two study sites we used simple linear regression. Distribution of different *Termitomyces* species in different-sized mounds was studied by using one-way ANOVA.

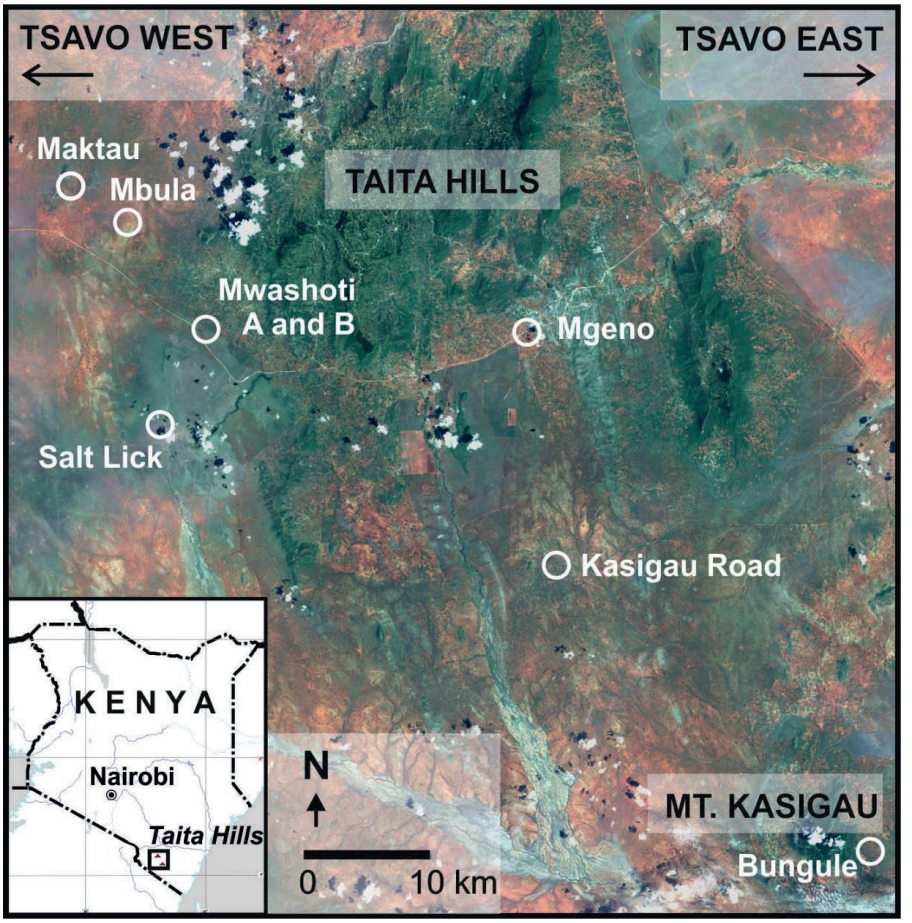

Figure 2 **Figure 2.** Research area around the Taita Hills and Mt. Kasigau and the exact locations of the eight study sites introduced in the first article (I).

4 MAIN RESULTS AND DISCUSSION

4.1 DIVERSITY OF *TERMITOMYCES* SYMBIONTS

A total of 104 complete *Termitomyces* ITS sequences originating from different *Macrotermes* colonies were obtained during the study (GenBank accession numbers KY197626–KY197709 and MK275596–MK275616). In order to compare our sequences with previously published sequences and to obtain an updated view of currently known diversity of *Macrotermes* associated *Termitomyces*, we performed a maximum likelihood analysis of all good quality *Termitomyces* ITS sequences available in GenBank (article I). In congruence with previous studies (Aanen et al. 2002; Rouland-Lefèvre et al. 2002; Frøslev et al. 2003; Osiemo et al. 2010; Nobre et al. 2011), the symbionts of the termite genus *Macrotermes* formed a well supported monophyletic group (Figure 3A).

The results of the more concise analysis concentrating solely on *Macrotermes* associated *Termitomyces*, together with comparisons with the Species Hypothesis (SH) groups of UNITE, suggested that the clade included 9 or 14 fungal species depending on the selected cut-off level (97 % or 98.5 %, respectively). The global diversity in the genus *Macrotermes* includes 47 host termite species (Kambhampati and Eggleton 2000), although the real number is probably higher, as cryptic species are known to exist (Brandl et al. 2007). However, *Termitomyces* ITS sequences currently available in GenBank represent symbionts from only eight different *Macrotermes* hosts. This implies that also diversity of *Macrotermes* associated *Termitomyces* may well be higher than is presently known.

Our novel ITS sequences represented three different fungal species that were tentatively named as *Termitomyces* sp. A, B and C in article I (Figure 3B). Two species (A and C) clustered together with several other ITS sequences obtained from different *Macrotermes* hosts originating from different parts of Africa (Figure 3 in article I). ITS sequences almost identical to our *Termitomyces* sp. A had been previously found from the western and southern parts of Africa, whereas the sequences clustering with *Termitomyces* sp. C originated from both East and West Africa but the geographical distribution was seemingly restricted to the equatorial areas (Figure 3B). In contrast, *Termitomyces* sp. B did not cluster with any previously published ITS sequences, suggesting that the overall distribution of this fungus may be limited.

We sampled *Termitomyces* species A and C from the nests of *M. subhyalinus* and *M. michaelseni*, whereas species B was only found in association with *M. subhyalinus*. Based on GenBank information, especially *Termitomyces* species A seems to be commonly cultivated also by several other termite species including *M. bellicosus*, *M. natalensis* and *M. jeanneli*

(Figure 3B). The wide diversity of observed host termites and large geographical range suggests that this symbiont is a generalist and highly adaptable to different environments and ecological settings.

Our maximum likelihood analysis of the *Macrotermes* associated *Termitomyces* demonstrated also that *M. muelleri*, a termite species that inhabits rain forests of Central Africa (Ruelle 1970; Aanen and Eggleton 2005), interestingly seems to have its own symbiont lineage that is not shared with any of the termite species occurring in savanna habitats (Figure 3B). Data also suggests that, within the group of *Macrotermes* associated *Termitomyces*, at least two independent out of Africa migrations have occurred into tropical Asia (Figure 3B). *Termitomyces* lineages originating from *Macrotermes* nests in Thailand, Vietnam or Malaysia were not shared with any of the African species, suggesting that both migration events have occurred in the relatively distant past.

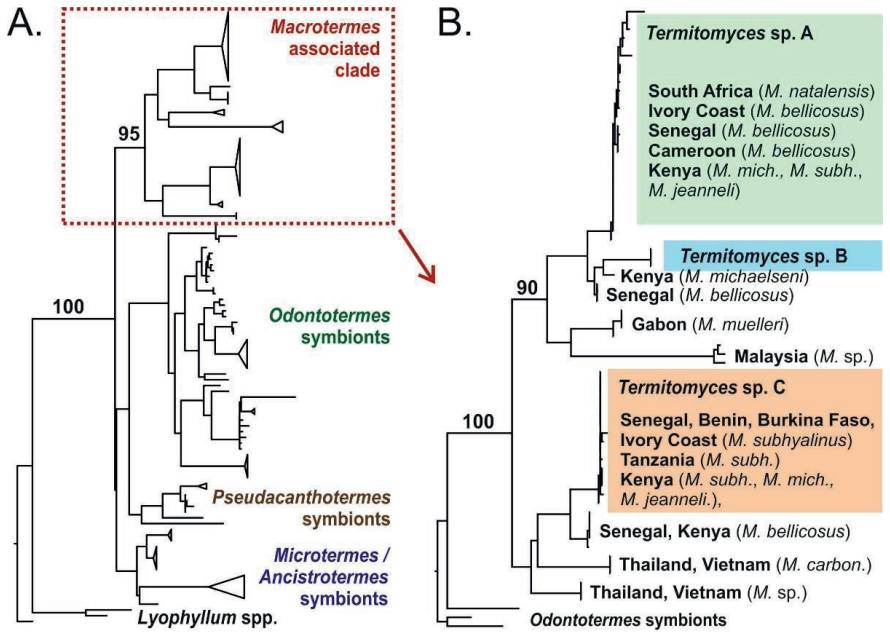

Figure 3 Figure 3. Maximum likelihood phylogeny of all *Termitomyces* ITS sequences published in GenBank prior 2016 (A) and more detailed phylogeny of *Termitomyces* ITS sequences in *Macrotermes* associated clade with sample information (country and host termite when available) provided in GenBank (B). See article I for more detailed results and accession numbers for the sequences used in the analysis.

4.2 *MACROTERMES* AND *TERMITOMYCES* DIVERSITY IN THE TSAVO ECOSYSTEM

The maximum likelihood analysis of termite COI sequences from a total of 141 termite colonies (article I) showed that two closely related but genetically distinct *Macrotermes* species occurred in our study area (Figure 4A). The more common COI lineage was largely restricted to open mounds (Figure 4C) characteristic for East African *M. subhyalinus* (Darlington 1984a), while the other lineage mostly inhabited closed mounds (Figure 4B) typically built by *M. michaelseni* (Darlington 1985; Schuurman and Dangerfield 1996; Turner 2000). As in only in four cases the 'wrong termite' was identified from a specific mound type (Figure 4A) we felt quite confident that the more common COI lineage represented *M. subhyalinus* Rambur 1842 and the other COI lineage *M. michaelseni* Sjöstedt 1914.

Three *Termitomyces* species were found from a total of 172 nests analyzed during the study (articles I and II). Consistently with all previous studies (Aanen et al. 2002, 2009; Katoh et al. 2002; Makonde et al. 2013), only one fungal genotype was always found when several samples were analyzed from opposite sides of the same mounds. Species *Termitomyces* A was the most common fungal symbiont and it was identified from 131 termite mounds, representing 76 % of analyzed colonies. *Termitomyces* C was the second most common species and occurred in 33 nests (19 %), whereas species B was relatively rare and was only found from eight termite colonies (5 %).

Both *Macrotermes* and *Termitomyces* diversities varied between the different study sites representing different savanna habitats. Termite species *M. michaelseni* occurred only at three of the eight sites included in article I, whereas the more common *M. subhyalinus* was present in all studied habitats (Figure 2 in article I). Fungal diversity was highest at the Maktau study site where all three *Termitomyces* species co-occurred with relatively equal abundances. In contrast, the Bungule study site had very low symbiont diversity with *Termitomyces* sp. A being present in all 22 analyzed termite colonies. The most common *Termitomyces* species A was the dominant fungal symbiont at all study sites. Considering its dominance also at the global scale (see section 4.1.), *Termitomyces* species A seems to be quite pre-eminent in most African environments when compared with all other fungal symbionts associated with *Macrotermes* species.

Ecological reasons behind the obvious success of the *Termitomyces* species A in the Tsavo Ecosystem may include its tolerance for a rather wide range of different growth temperatures, as suggested by the results of the article II (see section 4.3.). Another explanation could involve enzymatic differences between the fungal symbionts that, in turn, might favor the use of different food sources (e.g. herbaceous vs. lignified plant tissues). The high *Termitomyces* diversity observed at the Maktau study site could be explained by this, as it clearly was the most heterogeneous landscape analyzed with respect to different food sources available. Potential enzymatic differences bet-

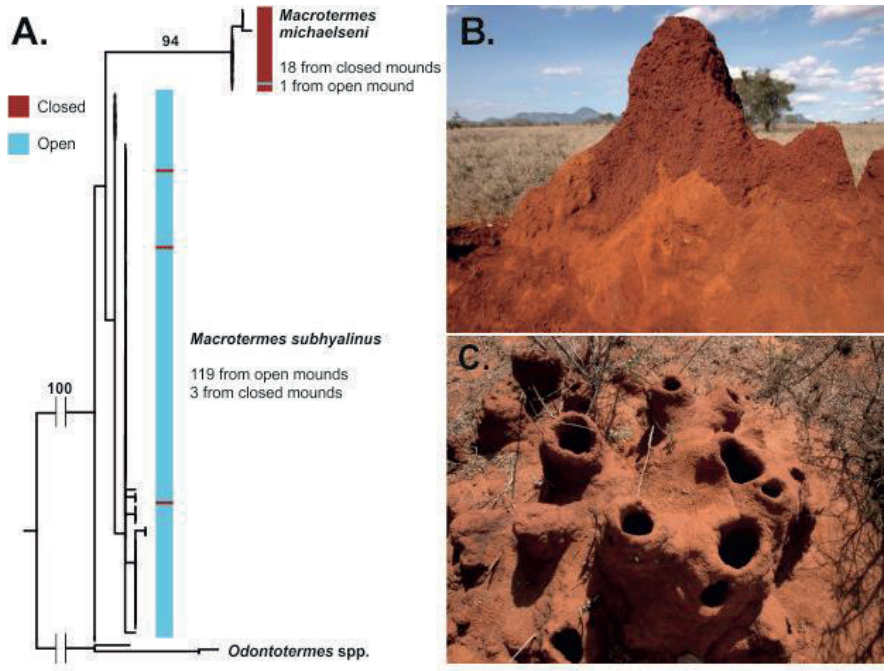

Figure 4 **Figure 4.** Maximum likelihood analysis of *Macrotermes* COI sequences originating from 141 different termite mounds (A) representing either closed (*Macrotermes michaelseni*) mounds (B) or open (*Macrotermes subhyalinus*) mounds with several large open ventilation shafts (C).

ween the different *Termitomyces* species have not yet been studied, and therefore this hypothesis remains to be tested in future studies.

4.3 NEST THERMOREGULATION AND SYMBIONT DIVERSITY

Results of the article II demonstrated that mound architecture has a major influence on the temperatures experienced within the fungal chambers of termite nests. During the cool period in June and July the temperatures within closed *Macrotermes michaelseni* mounds were consistently higher than those in open *M. subhyalinus* mounds (Figure 4 in article II). The wind-induced gas exchange of open mounds (Weir 1973; Korb 2011) clearly cools the nest interior more effectively than the solar-induced within-nest air circulation of closed mounds (Ocko et al. 2017). This may be disadvantageous for *M. subhyalinus* populations that occur in open locations and in regions that

experience relatively low temperatures during the cool season. On the other hand, the open ventilation type gives effective protection against overheating during hot weather. Species specific differences in nest thermoregulation may thus explain why *M. michaelseni* is more common at relatively high elevations, whereas *M. subhyalinus* is the dominant mound builder in constantly hot lowland habitats (Bagine et al. 1994; Pomeroy 2005).

When open *M. subhyalinus* mounds were studied more detail, we found that small 'miniature mounds', having reduced ventilation shafts and diameters of less than 1.5 meters, constantly had much higher temperatures than large mounds with many wide ventilation shafts (Figure 5 in article II). The difference was most pronounced during the coolest season from June to August when diurnal mean temperatures in miniature mounds were typically more than 3 °C higher than in large open mounds.

DNA sequencing of the fungal symbionts revealed that the three *Termitomyces* species were distributed unequally among different-sized *M. subhyalinus* mounds. In these open mounds *Termitomyces* sp. C was largely confined to small mounds whereas *Termitomyces* species A occurred equally in both small and large mounds (Figure 6A in article II). A concurrent pattern was not observed in closed *M. michaelseni* mounds in which the distribution of neither *Termitomyces* species correlated with mound size (Figure 6B in article II). As large *M. subhyalinus* mounds maintained occasionally much lower nest temperatures than any other mound types, and on the other hand, *Termitomyces* species C was absent from such mounds, this fungus seems to be thermophilic and is probably intolerant of the cool nest temperatures experienced in large open mounds during the coolest season. Low temperatures might reduce fungal growth or lower its enzyme activity, which in turn, could effectively jeopardize the food processing of termite hosts, thus predisposing the colony to starvation.

It remains unclear, whether termites can actively adjust their mound building behavior to meet the specific thermal needs of fungal symbionts. Such a process would allow the *M. subhyalinus* hosts of *Termitomyces* species C to actively limit mound size and build only narrow ventilation shafts in order to maintain the constantly high nest temperatures required by their thermophilic symbiont. This might have a considerable negative feedback effect via poor nest ventilation, as the amount of air moving through ventilation shafts is known to correlate positively with the size of above-ground mound structures (Weir 1973). From this perspective the architecture of open miniature mounds must be disadvantageous by default, and may set strict limits for colony productivity and growth. In any case, because of the obvious trade-off between mound ventilation and nest temperature maintenance, the total absence of *Termitomyces* species C from large *M. subhyalinus* mounds may be explained by the fact that termite colonies with this symbiont do not have the option of building effectively ventilated, i.e., large mounds.

Regardless of the exact mechanisms generating the observed interaction patterns between mound architecture and symbiont diversity, our study areas

in Tsavo seem to represent a suboptimal habitat for the *Termitomyces* species C during the coolest months of the year. *Termitomyces* sp. C may be adapted to more low-lying equatorial regions where temperatures remain constantly hot. It is also possible that this fungus is primarily adapted to live in the constantly warm closed mounds of *M. michaelseni*, and an association with *M. subhyalinus* would always represent a kind of misstep for the species. This could also mean that for *M. subhyalinus*, at least in the Tsavo Ecosystem, establishing an association with *Termitomyces* sp. C would be much less advantageous than establishing a relationship with either of the two other fungal symbionts.

4.4 FOOD SELECTION

The results of stable isotope analyses, provided important information about litter utilization of *Macrotermes michaelseni* and *M. subhyalinus*. Carbon stable isotopes have been widely used to study food habits of herbivores in East African savannas (Tieszen et al. 1979; Boutton et al. 1983). The approach is based on the fact that C4 photosynthetic savanna grasses accumulate higher proportions of heavy carbon (^{13}C) in their tissues than C3 photosynthetic trees and shrubs, leading to much higher $\delta^{13}C$ values in grasses than in woody plants (Smith and Epstein 1971; Smith and Brown 1973). This is also reflected to the carbon isotope compositions of herbivorous animals that are typically ^{13}C enriched by approximately 1 ‰ compared to their diets (DeNiro and Epstein 1978).

The low $\delta^{13}C$ values obtained from termite food storages and fungus combs analyzed in manuscript III indicate that the studied termite colonies fed on plant material derived mostly from trees and shrubs. This was expected as all the analyzed colonies were from woody vegetation types with limited grass availability (especially in Kasigau Road). However, both *M. subhyalinus* and *M. michaelseni* also occur in neighboring grassland areas (Figure 2 in article I), where the colonies consume exclusively grasses (Vesala et al. unpublished). Thus, our results confirm the previous observations that both grasses and woody plants are present in diets of *M. michaelseni* with their proportions depending on local availability (Boutton et al. 1983). Although the exact food preferences of *M. subhyalinus* and *M. michaelseni* are not well-known, both species have been regarded primarily as grass-litter feeders (Collins 1983; Darlington 1984a, 1984b; Darlington and Dransfield 1987). This needs to be re-evaluated as the species can obviously also survive on tree/shrub based diets.

The nitrogen isotope values ($\delta^{15}N$) obtained from the analyzed food storages and fungus combs ranged roughly from 5 to 7 ‰, which corresponded more closely to the $\delta^{15}N$ values of leaves than those of wood (Table 1 and Figure 3 in manuscript III). Thus, majority of the plant matter in fungus combs seemed to have originated from leaf litter which makes sense because of the

much higher nitrogen contents in leaf tissues than in wood (see e.g. Table 1 in manuscript III). The $\delta^{15}N$ values of the three colonies sampled at Kasigau Road were much lower than those in the single colony at Mbula, suggesting higher leaf litter consumption at the later site. However, variation in $\delta^{15}N$ values in plant specimens was found to be relatively high (Table 1 in manuscript III), reflecting the fact that nitrogen isotope composition of plants is affected by several factors, including e.g. mechanisms of nitrogen uptake and assimilation, symbiotic interactions, and the form and abundance of nitrogen in soils (Handley and Raven 1992; Evans 2001).

Termitomyces diversity was not assessed in manuscript III. However, fungal symbionts might well affect to the food selection and utilization of *Macrotermes* colonies. Assuming that different *Termitomyces* species would have acquired enzymatic traits that allow them to specialize on the degradation of divergent types of plant matter, the cultivation of different fungal species by neighboring termite colonies could allow the colonies to primarily utilize different food sources. This, in turn, could effectively reduce the competition between different termite colonies, and might even allow a higher number of termite colonies, with partly overlapping foraging territories, to exist per given area. In future studies, dietary data should be collected that would allow effective comparison between *Macrotermes* colonies that rely on different fungal symbionts.

4.5 THE ROLE OF *TERMITOMYCES* IN TERMITE NUTRITION

The average nitrogen content of plant specimens collected around the studied termite colonies ranged from 1.0 % (wood tissues) to 2.8 % (leaves of trees/shrubs) (Table 1 in manuscript III). The highest nitrogen contents (up to 4.8 %) were found from *Acacia tortilis* leaves. The nitrogen contents of fungus combs (ca. 2.5 % nitrogen) were in the same range as those of plant material, without consistent differences between fresh and old comb parts. Conversely, a multifold increase in nitrogen contents was detected from fungus combs to *Termitomyces* nodules (7–9 % nitrogen). Comparable nitrogen contents have been reported from fungus combs and *Termitomyces* nodules of several different termite species (Matsumoto 1976; Abo-Khatwa 1977; Rohrmann and Rossman 1980; Collins 1983).

The high nitrogen content of fungal nodules compared to the fungus combs indicates that nitrogen is actively transported within the fungal mycelium and predominantly allocated into nodules. Although such a protein rich food source is of obvious potential importance for the colony, it has been thought that species of *Macrotermes* would predominantly feed on plant material pretreated by the fungus (Hyodo et al. 2000, 2003). Thus, consumption of fungal nodules would be mostly due to translocation of fungal lignocellulolytic enzymes that are known to act synergistically with termite and bacterial

enzymes within termite guts and fresh parts of fungus combs (Abo-Khatwa 1978; Martin and Martin 1978; Martin and Martin 1979; Rouland et al. 1988a; Rouland et al. 1988b; Rouland et al. 1991; Nobre and Aanen 2012; Poulsen et al. 2014, da Costa et al. 2018). However, focusing only on termite workers, which were exclusively analyzed by Hyodo et al. (2003), may produce a strongly biased view of the dietary patterns of entire termite colonies. Eggs and larvae constitute a large proportion of *Macrotermes* populations (Darlington 1984b; Darlington and Dransfield 1987) and developing instars that constantly accumulate new biomass obviously need more proteins than adult workers. Also the queen, due to her constant and massive egg production of up to 18 000 eggs per day (Kaib et al. 2001), needs to be constantly supplied with large amounts of food with a high nitrogen content.

The results of our carbon stable isotope analysis (manuscript III) clearly confirmed that different termite castes and age-groups within the *Macrotermes* colonies get their nourishment from different sources, some from *Termitomyces* mycelium and/or nodules, and others mainly from partly decomposed plant matter. The queen and larvae were mostly fed with fungal material, whereas the adult workers and soldiers, and surprisingly also nymphs (developing alates), utilized fungus combs as their main source of food. This challenges the general idea that the fungal symbiont would have a universal main role, i.e. lignocellulose degradation, in nutrition of *Macrotermes* colonies (Hyodo et al. 2000, 2003). Also *Termitomyces* nodules probably have several functions in colony food processing, which either are not mutually exclusive: 1. source of fungal conidia that inoculate fresh plant material and stabilizes fungal monocultures (Leuthold et al. 1989; Aanen 2006; Aanen et al. 2009), 2. source of enzymes that enhance plant degradation (e.g. Martin and Martin 1978; Rouland et al. 1991), and 3. source of nitrogen-rich food for maintaining colony reproduction and growth (manuscript III).

There was a dramatic difference in the distribution of nitrogen stable isotopes between *Macrotermes* queens and kings. Kings always had the highest and the queens the lowest $\delta^{15}N$ values among all termites analyzed from the same colony (Figure 3 in manuscript III). This can only be explained by the fact that nitrogen incorporated in their tissues originates largely from different sources. The mechanisms that accumulate heavy nitrogen (relatively enriched in ^{15}N) in kings and light nitrogen (relatively depleted in ^{15}N) in queens are currently unknown, but feasible explanations may involve uric acid recycling and/or fixation of atmospheric N_2.

Uric acid is typically accumulated in abdominal fat body tissues of old termite workers, from where it is recycled by utilizing uricolytic hindgut bacteria, presumably mostly via cannibalism or necrophagy (Potrikus and Breznak 1980a, 1980b, 1981; Collins 1983; Slaytor and Chappell 1994). Tayasu et al. (2002) found that abdominal uric acid content correlates with ^{15}N depletion of *Macrotermes* workers, suggesting that ^{15}N is discriminated during uric acid synthesis in termites. Thus, assuming that uric acid would be offered to queen, continuous consumption of ^{15}N depleted uric acid

accumulated in old workers (i.e. cannibalism of offspring) might act to decrease the $\delta^{15}N$ values of the queens. At least direct consumption of uric acid would require uricolytic bacteria to occur within queen digestive tract, which needs to be unraveled in future studies.

The other potential explanation involves symbiotic nitrogen fixation, which is known to occur in the guts of many termites (Brune and Ohkuma 2011). It has been recently shown, that also *Macrotermes* species harbor diazotrophic bacteria, capable to fix atmospheric nitrogen, although their functionality in vivo remains to be resolved (Sapountzis et al. 2016). Atmospheric N2 shows $\delta^{15}N$ values of ca. 0 ‰ (Peterson and Fry 1987). Thus, constant input of N_2 in termite–fungus food web would generally decrease the $\delta^{15}N$ values from the level of colony food sources (5–7 ‰, manuscript III). The highly ^{15}N depleted queens, together with the observed absence of ^{15}N depletion in analyzed workers' guts, suggests that, if atmospheric nitrogen is indeed fixed within termite mounds, it could take place in the gut or some specific tissues of the queen. Such a process would make ecological sense, as the nitrogen demand of physogastric *Macrotermes* queens appears to be higher than could be gained from the available dietary sources. The existence of a diazotrophic microflora in the queen would be highly advantageous for the whole termite colony, as the queen presents a central node in within-colony nitrogen cycle (Figure 1 in manuscript III). The phenomenon could be especially significant for explaining the extremely high reproduction rates of *Macrotermes* colonies (Kaib et al. 2001).

5 CONCLUSIONS AND FUTURE PROSPECTS

During this thesis project we studied the ecology of *Termitomyces* species and their *Macrotermes* hosts on several different levels ranging from phylogenetical and co-evolutionary questions to carbon and nitrogen cycling within termite mounds. This holistic approach provided answers for several intriguing questions from several different subject areas. On the other hand, an equal number new questions arose and were left unanswered. This underlines the fact that many fundamental aspects in ecology of fungus-growing termites and their symbionts still remain poorly known.

In article I we showed that local *Termitomyces* communities occupying termite mounds in East African savannas typically consist of a few fungal species that seemingly share their niche. Shared host termites, identical dwellings (fungus combs), and very similar microhabitats within underground nest chambers, raise many questions regarding the evolutionary origins of such narrow but obviously well-established symbiont diversity. What have been the mechanisms of speciation that have generated the extant patterns, with a handful of apparently sympatric species colonizing these savanna landscapes? And what are the mechanisms that maintain this diversity and prevents *Termitomyces* A from outcompeting the other two fungi?

The evolutionary questions could be addressed in controlled laboratory conditions by exposing *Termitomyces* pure cultures to variable conditions, which might include e.g. cultivation in different temperatures and with variable carbon sources. Enzymatic and genomic studies especially focusing on comparisons of different *Termitomyces* species would provide another applicable approach. Large-scale samplings in other regions of Africa could also produce new ideas, and provide a more comprehensive understanding of large-scale diversity and host-symbiont patterns.

The results of article II indicate that behavioral differences in mound building may affect local *Termitomyces* diversity. The key determinant for fungal diversity may lie in the ability of termites to maintain nest microclimates appropriate for specific symbionts. Different architectural solutions may result in different outcomes that may favor different *Termitomyces* species. On the other hand, identical architecture may lead to different nest microclimates in different environments. Comparable temperature time series from structurally different termite mounds and from different habitats and climates are needed to better understand the complicated interactions between mound architecture and nest thermoregulation. *Termitomyces* symbionts should not be forgotten in subsequent studies, as architectural variation in part controls their living conditions.

The results of the manuscript III provided new precise information on the nutritional role of *Termitomyces* for *Macrotermes* colonies. The most important conclusion was that the role of the symbiotic fungus in colony nutrition is pluralistic: termites colonies utilize the fungus both directly (by eating mycelium) and indirectly (by eating plant material decayed by the fungal enzymes). However, the direct utilization of *Termitomyces* obviously is largely restricted to the reproductive individuals and larvae. Especially the ambiguous nitrogen isotope patterns need further studies. These could include e.g. stable isotope analysis of nitrogenous gas emissions of termite mounds. Compound specific isotope analysis of amino acids might also provide an applicable tool to elucidate caste-specific diets and within-nest nutrient fluxes more detailed. One interesting research question is related to the potential fixation of atmospheric nitrogen within *Macrotermes* queens, which definitely deserves further studies.

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