

Fungal diversity of *Macrotermes-Termitomyces* nests in Tsavo, Kenya

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1 INTRODUCTION

Fungus-growing termites (subfamily Macrotermitinae, Termitidae) comprise one of three unrelated groups of insects that have evolved obligatory digestive exosymbioses with filamentous fungi. The other two groups are the fungus-growing ants (tribe Attini, Myrmicinae, Hymenoptera) and ambrosia beetles (subfamilies Scolytinae and Platypodinae, Curculionidae, Coleoptera), respectively. In ambrosia beetles fungus-cultivation has evolved independently several times within different lineages, whereas in termites and in ants the transitions to fungus-cultivation have occurred only once, thus resulting in monophyletic fungus-growing clades of host insects (Mueller et al. 2005).

The fungal symbionts of all higher termites belong to the genus *Termitomyces* (Lyophyllaceae, Basidiomycota), which only includes obligately termite-symbiotic species (Aanen et al. 2002; Rouland-Lefevre et al. 2002). The fungal mycelia are grown in sponge-like combs built from termite-digested plant matter in the subterranean galleries of termite nests (Wood and Thomas 1989). The insects actively regulate the microclimate of the fungal galleries in order to maintain favorable conditions for fungal growth (Korb 2003). Plant matter for the fungal combs is collected from the vicinity of the nest by foraging termites. The insects collect plant matter from the environment and deposit it into the fungus combs in the form of partly indigested fecal material. As a reciprocal service, the termites can feed on nitrogen-rich fungal hyphae and/or plant material further decomposed by the fungal symbiont (Hyodo et al. 2003).

The initial domestication of *Termitomyces* by termites is believed to have occurred in the rain forests of Africa at least 30 million years ago (Aanen and Eggleton 2005; Brandl et al. 2007; Nobre et al. 2011b). Since then both the termite hosts and the fungal symbionts have radiated into several lineages, and today the associations vary considerably in levels of reciprocal symbiont specificity (Aanen et al. 2002; Aanen et al. 2007). Several lineages of fungus-growing termites have

specialized to live in semi-arid shrubland and savanna environments, where they have become principal degraders of dead plant matter (Jones 1990; Jouquet et al. 2011). In some dry savanna ecosystems of East Africa up to 90% of all litter decomposition can take place within the subterranean chambers of fungus-growing termites (Buxton 1981).

Fungus-growing termites culture the heterokaryotic mycelium of the fungal symbiont in specialized fungal combs within the galleries of their nests (De Fine Licht et al. 2005). Most *Termitomyces* species can occasionally produce fruiting bodies that release basidiospores into the environment (Johnson et al. 1981; Wood & Thomas 1989; De Fine Licht et al. 2005). Concurrently, most fungus-growing termites seem to rely on horizontal symbiont transmission in the acquisition of compatible fungal symbionts (Johnson et al. 1981; Sieber et al. 1983; Korb & Aanen 2003; De Fine Licht et al. 2006). Soon after dispersal, the firstborn foragers of a newly established termite colony are believed to acquire fungal symbionts from the environment, presumably as ingested basidiospores of compatible *Termitomyces* genotypes. There are two known exceptions: all species of the termite genus *Microtermes* and one species of *Macrotermes* (*M. bellicosus*) rely on uniparental vertical symbiont transmission, i.e., one of the winged alates (king or queen) transports a fungal inoculum from the maternal colony within its gut (Johnson 1981; Korb and Aanen 2003). The genetic composition of fungal symbionts indicates that occasional host-symbiont switching also takes place in the termite species which mainly rely on horizontal transmission of symbionts (Nobre et al., 2011a).

During their evolution the termite-fungus symbioses have effectively colonized most of sub-Saharan Africa and also dispersed into tropical Asia. Among the termite hosts at least five independent dispersal events have occurred into Asia and one genus (*Microtermes*) has reached Madagascar (Aanen and Eggleton 2005; Nobre et al. 2010). Among the fungal symbionts similar range expansions seem to have occurred even more frequently, at least from the African mainland into Madagascar (Nobre et al. 2010; Nobre and Aanen, 2010).

Here we provide a short overview of what is presently known about fungal diversity within the mounds of fungus-growing termites. The focus is on new findings from the Tsavo ecosystem in southern Kenya, where the landscape is peppered by innumerable *Macrotermes* nests dispersed within expanses of dry tropical shrubland (Fig. 1).

2 DIVERSITY OF TERMITOMYCES AND MACROTERMES

While only about 40 species are currently accepted in the genus *Termitomyces* (MycoBank; Index Fungorum; Kirk et al. 2010), the wealth of DNA data accumulated during the past 15 years clearly indicate that their species diversity is much higher. Interestingly, most DNA sequences obtained directly from fungal galleries of termite nests do not correspond with those sequenced from the fruiting bodies of 'classical' *Termitomyces* species. As one consequence, GenBank presently contains numerous fungal ITS sequences obtained from the nests of different *Macrotermes* species identified as 'unnamed *Termitomyces*'. Similar problems are familiar from other fungal groups in which classical taxonomic concepts have been based solely on fungal fruiting-bodies.

While there thus are many unresolved problems in the taxonomy of *Termitomyces*, the DNA data so far accumulated clearly indicates that the *Termitomyces* symbionts of *Macrotermes* species include several species-level operational taxonomic units (OTUs) which all belong to one monophyletic group (Aanen et al. 2002; Osiemo et al. 2010; Vesala et al. unpubl.). Fungal genotypes can now be reliably identified from haploid and dikaryotic hyphae and also from the asexual conidia formed in fungus combs or within termite guts. Several *Termitomyces* genotypes have been repeatedly identified from termite nests, but as previously described, most of them have not been linked to type specimens or other voucher material of classical *Termitomyces* species. Phylogenetic analyses combining DNA data from herbarium specimens and environmental isolates indicate that several undescribed species exist. Furthermore, some classical *Termitomyces* species (e.g. *Termitomyces clypeatus* and *T. microcarpus*) may represent complexes of several cryptic taxa (Aanen et al. 2002; Froslev et al. 2003).

Macrotermes is one of the 11 genera of fungus-growing termites and the ecologically most important genus in many dry savanna areas in East Africa. At least 47 *Macrotermes* species are currently recognized, 13 in Africa and 34 in Asia (Kambhampati & Eggleton 2000). However, as in the symbiotic fungi, several cryptic species are believed to exist at least in Africa (Brandl et al. 2007). Most African *Macrotermes* species live in relatively open savanna and semi-desert environments. However, two closely related species, *M. muelleri* and *M. nobilis*, are restricted to forest habitats in central Africa (Ruelle 1970; Aanen and Eggleton 2005). Interestingly the fungal symbionts of these two species also belong to separate lineages which do not associate with any species of savanna termites. Is this true evidence of coevolution or do only these specific pairs of termites and fungi happen to co-occur in shady forest habitats?

The southernmost *Macrotermes* species in Africa is *M. natalensis*, which seems to only associate with a single *Termitomyces* species (Aanen et al. 2007). Again one may ask whether this is evidence of high symbiont specificity, or is the selected fungal symbiont the only one that can survive in the relatively cool climate of South Africa. Concurrent adaptation via selection of cold tolerant fungal symbionts has been described from leafcutter ants at the northern limits of their distribution in North America (Mueller et al. 2011).

3 PATTERNS OF SYMBIONT SPECIFICITY IN MACROTERMES NESTS IN TSAVO

Symbiosis between the fungus-growing termites and their fungal symbionts is symmetrical in the sense that both the hosts and symbionts consist of single monophyletic lineages that are not known to include any groups that would have reversed to a non-symbiotic state (Aanen et al. 2002; Nobre et al., 2011c). Both lineages have radiated into several clades showing different levels of interaction specificity with each other. These range from strict co-evolutionary relationships, where a certain termite genus always associates with one monophyletic clade of *Termitomyces* symbionts, to more promiscuous relationships, where species of several different termite genera associate with what appear to be the same fungi. The latter type of situation is known from the termite genera *Microtermes*, *Ancistrotermes*, and *Synacanthotermes*, which all seem to share the same *Termitomyces* lineage (Aanen et al. 2002; Aanen et al. 2007).

Experiments by De Fine Licht et al. (2007) revealed that *Macrotermes natalensis* was not able to survive with the fungal symbiont of *Odontotermes badius*. However, when combining the latter termite with the fungal symbiont of the former host, no reduction in survival was detected. These and similar results demonstrate that there is considerable variation in the levels of symbiont specificity between different termite species and different genera. Most patterns of symbiont specificity so far detected have been on the generic level, while different species of the same termite genus are frequently able to share and switch fungal symbionts (Aanen et al. 2007).

Association between *Macrotermes* and *Termitomyces* are characterized by relatively strict co-evolutionary linkages between the hosts and their symbionts. Several studies and all sequence data so far published suggest that all *Macrotermes* species always associate with only one monophyletic group of *Termitomyces* species that does not associate with other groups of termites (Aanen et al. 2002; Rouland-Lefevre et al. 2002; Froslev et al. 2003; Osiemo et al. 2010; Makonde et al. 2013).

However, at least in East Africa several different *Macrotermes* species depend on what appears to represent a common pool of *Termitomyces* symbionts (Osiemo et al 2010; Vesala et al. unpubl.). Our recent studies indicate that at least eight different *Termitomyces* species occur in *Macrotermes* nests in Africa. The full diversity of the fungal symbionts may be much higher, as fungal symbionts with restricted ranges may exist and can only be detected through comprehensive sampling.

Some *Macrotermes*-associated *Termitomyces* species seem to be widely distributed and have for example been identified from termite nests in both equatorial East Africa and South Africa. Their diversity appears to be highest near the equator, which is consistent with the presumed ancestral range and rain forest origin (Aanen and Eggleton 2005). Since their early origin in Africa, some species have successfully dispersed into Asia and presently occur in for example Thailand, Vietnam and Malaysia. A phylogeny constructed from all ITS sequences of *Macrotermes* associated *Termitomyces* available in GenBank indicates that there have been two independent migrations from Africa into Asia (Fig. 2). At least three migrations into Asia and some into Madagascar have taken place in other *Termitomyces* lineages (Aanen & Eggleton 2005; Nobre et al. 2010).

Our studies in Tsavo have revealed that two *Macrotermes* species (*M. cf. subhyalinus* and *M. cf. michaelseni*) and three *Termitomyces* species (sp. A, B and C) are present in the termite mounds of this region (Vesala et al. unpubl.). In full congruence with earlier observations (Aanen et al. 2002; Katoh et al. 2002; Moriya et al. 2005; Aanen et al. 2009; Makonde et al. 2013) only one *Termitomyces* species has always been found in each nest, but even closely adjacent nests of the same termite species in a seemingly uniform environment may house different fungal species (Fig. 3). *Termitomyces* species A dominates in all study sites in Tsavo (Fig. 3). Interestingly, the same fungus has previously been identified in several other studies of fungal diversity in *Macrotermes* nests (De Fine Licht et al. 2005; De Fine Licht et al. 2006; Osiemo et al. 2010; Nobre et al. 2011a, 2011b). In fact, this apparently undescribed *Termitomyces* species seems to be the most common *Termitomyces*-symbiont of *Macrotermes* nests in sub-Saharan Africa (e.g. Senegal, Ivory Coast, Kenya, and South Africa). It can clearly associate with several different hosts (*M. subhyalinus*, *M. michaelseni*, *M. jeanneli*, *M. natalensis*, *M. bellicosus*, and *M. herus*).

Temperature and moisture availability, as reflected by zonal climates are by far the most important factors that affect the global distribution of fungi (Tedersoo et al. 2014). The same factors also largely delimit the overall distribution of termites, which are essentially a tropical group and virtually absent above or below 45°N and 45°S latitudes (Eggleton 2000). In Sub-Saharan Africa distances from the equator range from 0 to 4000 kilometers and consequently also the climates of

different semi-arid habitats within the region are quite different. Against this background the apparently almost pan-African distribution of one *Termitomyces* species is surprising, even if taken into account the relatively stable thermal conditions within the fungal galleries of *Macrotermes* nests.

Termitomyces species C was less frequent in Tsavo, but nevertheless found from several *Macrotermes* nest in many habitats studied (Fig. 3). Also this symbiont has been identified in many previous studies (Rouland-Lefevre et al. 2002; Guedegbe et al. 2009b; Osiemo et al. 2010; Nobre et al. 2011a, 2011b; Makonde et al. 2013). This *Termitomyces* species seems to be widely distributed in sub-Saharan Africa, but only in equatorial regions. Maybe this fungus requires constant warmth or is intolerant of low temperatures. Also it has been found in the nests of several different *Macrotermes* species (*M. subhyalinus*, *M. michaelseni*, *M. jeanneli*, and *M. bellicosus*).

Termitomyces species B was only found from some nests at one site (Fig. 3). Since identical fungi have not previously been reported this fungus may have a more restricted distribution and/or ecology. One can predict that many localized *Termitomyces* species will be found in the future when systematic sampling of *Macrotermes* nests proceeds to new regions and habitat types.

As shown by Figure 3 there was considerable variation in *Termitomyces* diversity between sampled habitats. Some of this variation might be explained by dispersal history and by site specific differences in vegetation cover, soil properties and many other ecological factors. However, at present we can only demonstrate that interesting and potentially significant diversity patterns exist – the experimental work required to explain these patterns has only barely begun.

It is not yet certain whether the fungal galleries of all *Macrotermes* nests always represent true monocultures or whether more than one *Termitomyces* genotype could sometimes exist within the fungal galleries. The general mechanism leading to a single-strain *Termitomyces* monoculture within a termite colony is based on positively frequency-dependent propagation (Aanen 2006; Aanen et al. 2009). In this process the dominance of one *Termitomyces* genotype is continuously reinforced by the biased selection of nursing termites that feed on and presumably also preferentially propagate the most productive *Termitomyces* genotype. Thus, while the first fungal gallery of a young termite colony might at first contain several competing *Termitomyces* genotypes, the most productive symbiont is selected for preferential propagation and becomes dominant. But does this always mean that the other genotypes are totally exterminated from the system – as a total dependence on a monoculture always involves some inherent risks? Second-generation sequencing methods may soon help to reveal whether the fungal combs of *Macrotermes* species can sometimes contain several minor symbionts. Such diversity might even be maintained by the

termite hosts in order to cope with temporal changes in food quality or other environmental factors which could shift the ecological equilibrium within fungal combs.

4 OTHER FUNGI IN TERMITE NESTS

Although *Termitomyces* always dominates a healthy termite colony also other fungi can be identified from the fungus combs. These can include common molds (e.g. *Aspergillus* and *Penicillium* species) and yeasts (e.g. *Candida* and *Pichia* species) that are likely accidentally introduced from the environment by foraging termites. Such fungi seem to generally only be present as spores and not as growing colonies (Thomas 1987a, 1987b; Wood and Thomas 1989; Guedegbe et al. 2009b; Mathew et al. 2012). Also, *Hypocrea* (anamorphs *Trichoderma* spp.) and *Pseudoxyllaria*, both belonging to Sordariomycetes (Ascomycota) have been repeatedly found from fungal combs of different Macrotermitinae genera (Batra and Batra 1966, 1979; Wood and Thomas 1989; Mathew et al. 2012).

In healthy fungus combs the non-*Termitomyces* fungi generally comprise only a very minor portion of the total fungal biomass (Moriya et al. 2005), but in the absence of actively nursing termites the *Termitomyces* symbiont may be rapidly overgrown by such fungi. Species of *Pseudoxyllaria* are particularly aggressive and may rapidly take over fungus gardens (Batra and Batra 1979; Wood and Thomas 1989; Visser et al. 2009). At least 20 species of *Pseudoxyllaria* form a monophyletic group which only includes termite-associated species (Rogers et al. 2005; Ju and Hsieh 2007; Guedegbe et al. 2009a; Visser et al. 2009; Hsieh et al. 2010). However, the different species do not seem to be restricted to live only in the nests of particular termite hosts (Visser et al. 2009).

The precise ecological role of termite-associated *Pseudoxyllaria* species is still insufficiently understood. According to Visser et al. (2011) the ascomycetes and *Termitomyces* compete for the same resources and the ascomycete seems to have developed a reduced level of antagonism towards *Termitomyces* compared to free-living species of Xylariaceae. In any case, the species of termite-associated *Pseudoxyllaria* seem to have specialized to exploit the partly digested plant material of fungus combs. In healthy nests their growth seems to be effectively suppressed, and they seem thus to be latent opportunistic saprotrophs waiting for the moment when the nest is abandoned by termites (Visser et al. 2011).

The mechanisms of how termites control the growth of *Pseudoxylaria* are not precisely known. However, termites, like other fungus-growing insects, must obviously have ways to reduce the growth of unwanted micro-organisms in the fungus gardens. These involve direct actions like mechanical weeding and many indirect effects via controlled temperature, humidity, and CO₂ concentration of fungal chambers, and the moisture content and pH of the comb substrate (Batra and Batra 1979; Thomas 1987c; Wood and Thomas 1989; Mueller et al. 2005; Mathew et al. 2012).

Two peptides with antifungal properties have been identified from the salivary glands of the fungus-growing termite *Pseudacanthotermes spininger* (Lamberty et al. 2001). Mathew et al. (2012) also noticed that some *Bacillus* species isolated from the fungal combs and guts of termite *Odontotermes formosanus* inhibited growth of the potentially harmful fungus *Trichoderma harzianum*, and did not have the same effect on *Termitomyces*. Concurrently, some *Bacillus* strains isolated from *Macrotermes natalensis* nests produce a substance that inhibits the growth of *Pseudoxylaria* and several other fungi isolated from termite combs, but does not affect *Termitomyces* (Um et al. 2013). Analogous insect-fungus-bacteria interactions are well known from the nests of fungus-growing ants, where certain Actinobacteria inhibit the growth of *Escovopsis*, a specialized parasitic ascomycete that lives in the fungus gardens of the ants (Currie et al. 1999). Actinobacterial strains with fungicidal effects have also been isolated from Macrotermitinae nests, but their potential role as defensive agents in the fungal combs has not yet been elucidated (Visser et al. 2012).

5 CONCLUSIONS

The fungal symbionts of all higher termites belong to the basidiomycete genus *Termitomyces*, which only includes obligate termite-symbionts. DNA data indicate that the *Termitomyces* symbionts of all species of the termite genus *Macrotermes* belong to one monophyletic lineage that is not shared by other groups of fungus-growing termites. The symbiont genotypes identified directly from termite nests do not correspond with 'classical' *Termitomyces* species described on the basis of fruiting bodies. This indicates that these fungi only rarely produce fruiting bodies and that their dispersal is thus unlikely to mainly occur via basidiospores. In addition to the *Termitomyces* symbionts also other fungi can occur in termite nests. They are effectively controlled in active nests but can rapidly overtake the fungal combs in the absence of termites.

The mechanisms how pathogenic fungi are suppressed are not well known, but certain bacterial symbionts may play an important role in this process.

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FIGURES



Figure 1. The *Macrotermes*–*Termitomyces* symbiosis. A. *Macrotermes subhyalinus* nest with open ventilation shafts. B. *Macrotermes michaelseni* nest with closed ventilation shafts. C. Termite workers repairing nest wall. D. Fungal comb with nodules (small white spheres). E. Close-up of nodules. F. Two minor soldiers and immature workers on fungal comb. Since immatures and soldiers are unable to feed themselves, workers must feed them. G. Major soldier and nymphs on fungal comb. H. Fungal rhizomorphs (likely *Pseudoxylaria*) growing among dead termite soldiers in recently abandoned *Macrotermes* nest.

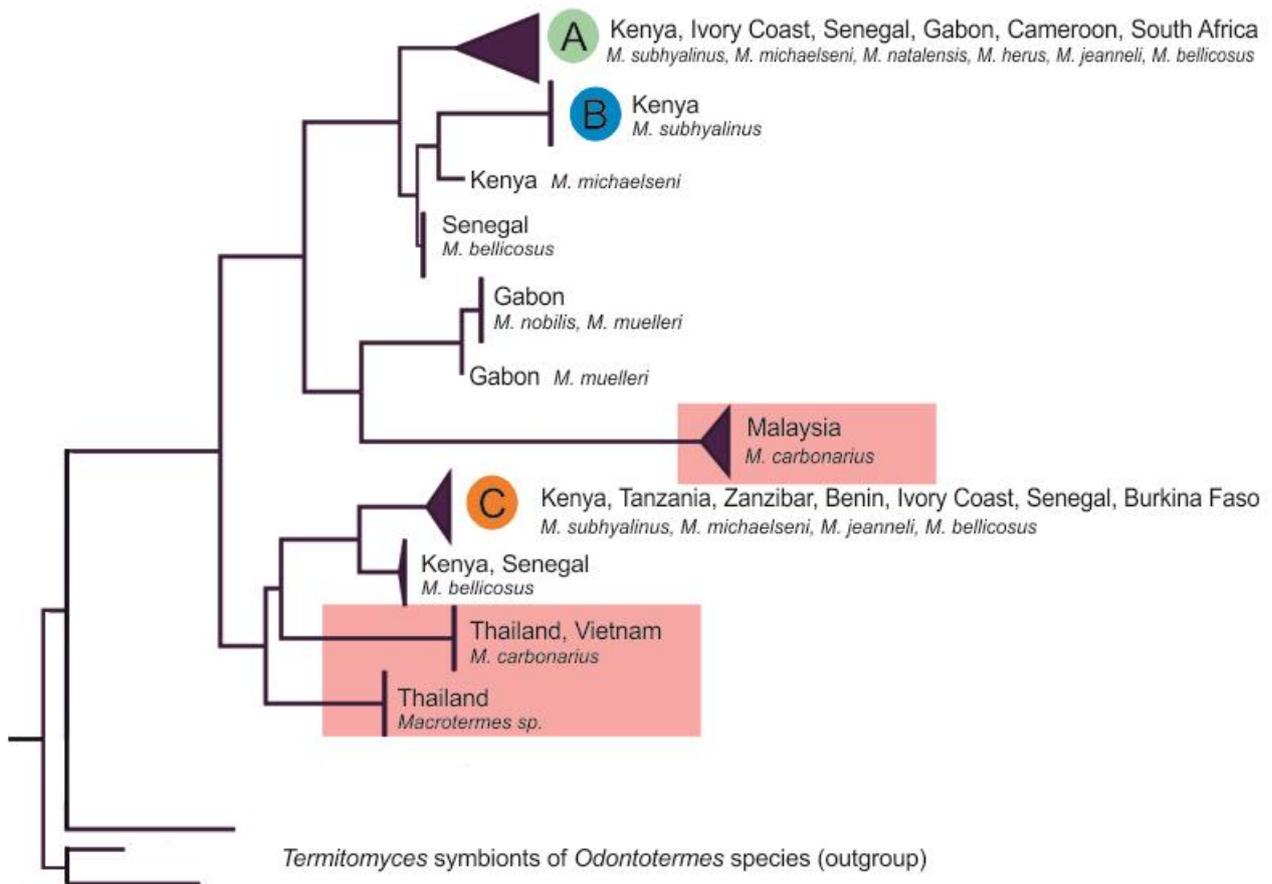


Figure 2. Maximum likelihood tree of *Termitomyces* ITS sequences identified from the nests of *Macrotermes* species. The tree is based on all sequences available in GenBank and new data from the Tsavo ecosystem. Sequences within all lineages show > 99 % similarity, except those of the Malaysian lineage (upper red box) in which the similarity is between 97 and 98 %. The three *Termitomyces* species found from *Macrotermes* nests in Tsavo are marked with colored circles (A–C).

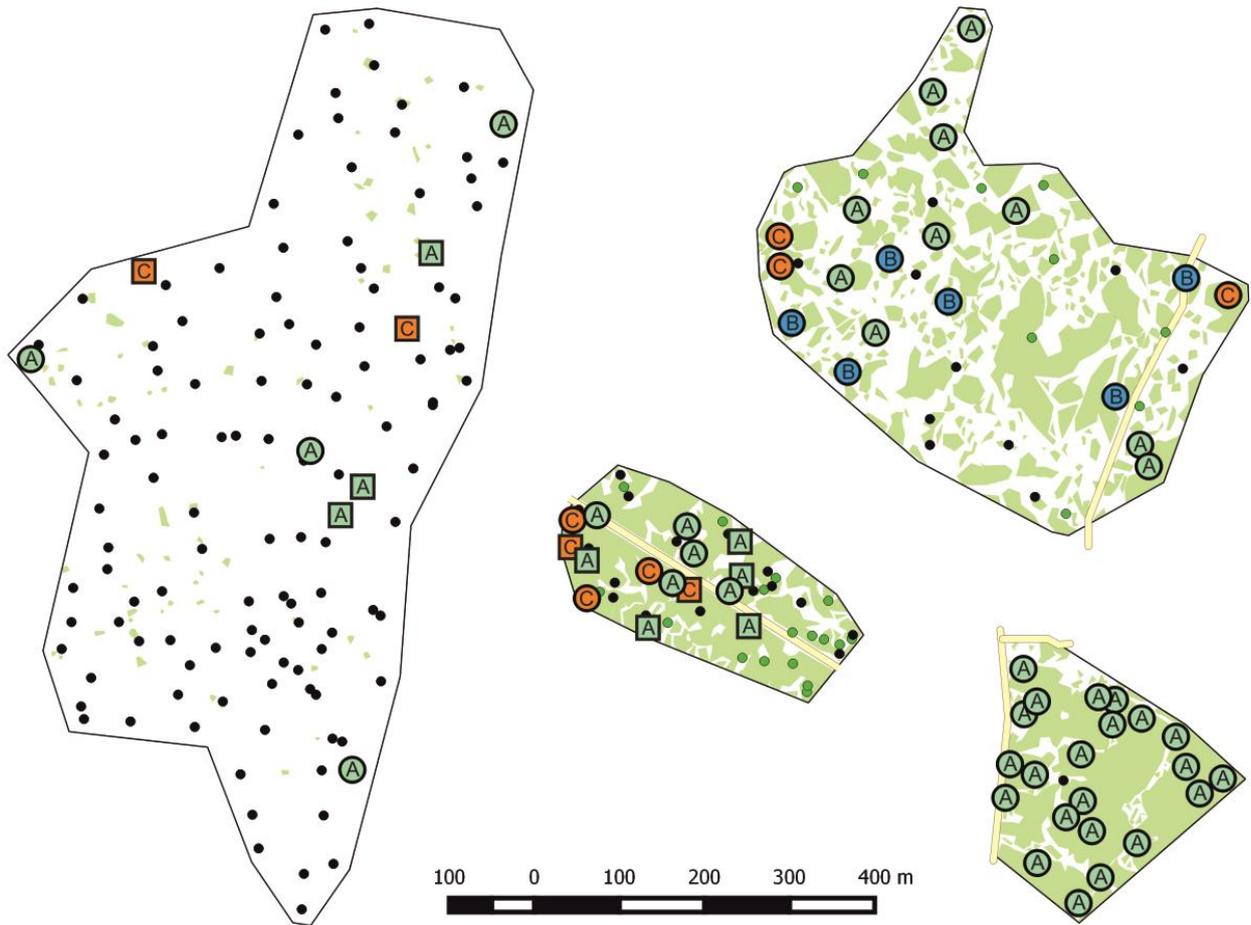


Figure 3. Distribution of three different *Termitomyces* species (A–C) identified from the active nests of *Macrotermes subhyalinus* (circles) and *Macrotermes michaelseni* (squares) at four study areas in Tsavo, Kenya. The black dots show the distribution of inactive (dead) *Macrotermes* nests and the green dots indicate active *Macrotermes* nests that were not sampled for DNA. The green background indicates woody vegetation, while the white background represents grassland or bare ground.