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High diversity and low host-specificity of *Termitomyces* symbionts cultivated by *Microtermes* spp. indicate frequent symbiont exchange

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ABSTRACT

Fungus-growing termites (subfamily Macrotermitinae) live in an obligate mutualistic symbiosis with species of the fungal genus *Termitomyces* (Basidiomycota). Although the species that build large mounds are the most conspicuous, termites of the genus *Microtermes* construct large underground networks of tunnels connecting many fungus gardens. They are also the only entire genus within the Macrotermitinae in which vertical transmission of the fungal symbiont has evolved. To study patterns of genetic diversity in species of the genus *Microtermes* and their *Termitomyces* symbionts, we sampled at three different locations in South Africa and sequenced COI for the termites and ITS for the fungi. We discovered high genetic diversity in both termites and fungal symbionts but very low interaction specificity. This implies that frequent horizontal exchange of fungal symbionts occurs between species, despite vertical transmission across generations. We also estimated colony size based on termite haplotype and fungal genotype combinations and found indications that colonies may extend over large areas.

1. Introduction

Termites (order Isoptera) are a large and diverse insect group. They play an important role in ecosystem functioning as decomposers, especially in the tropics (Jouquet et al., 2011). In one extant group of termites, the family Termitidae (also called 'higher termites'), several highly specialised feeding types have evolved, such as soil feeding (Inward et al., 2007). Within the Termitidae, all species of the subfamily Macrotermitinae have evolved a unique feeding habit; they live in an obligate symbiosis with species of the basidiomycete fungus *Termitomyces* (Johnson et al., 1981; Wood and Thomas, 1989). The origin of the symbiosis has been estimated at 30 million years ago in the rain forests of Africa (Aanen and Eggleton, 2005; Roberts et al., 2016). The fungus-growing termites have a single origin and their symbiotic fungus *Termitomyces* (Basidiomycota, Lyophyllaceae) as well, so that there has been a single domestication only (Aanen et al., 2002). The distribution of fungus-growing termites ranges from sub-Saharan Africa, including Madagascar, to Asia (Wood and Thomas, 1989; Nobre et al., 2010). Currently there are 10 described genera in the Macrotermitinae of which six occur in southern Africa (Eggleton, 2000; Uys, 2002). The most conspicuous fungus-growing termites construct large epigeal mounds but other genera have subterranean colonies in which they farm their fungus. The termites grow their fungus on a special substrate called the fungus comb, which they build from primary feces consisting of plant material inoculated with asexual spores. They harvest the asexual fruit bodies which serve as a protein-rich food source, a source of additional digestive enzymes, inoculum for new fungus combs and a source of the essential amino acid tryptophan (Martin and Martin, 1978; Leuthold et al., 1989; Nobre and Aanen, 2012; Chiu et al., 2019).

Previous studies have shown that there is some host specificity between termite and fungus at the generic level but not at the species level (Aanen et al. 2002, 2007; Osiemo et al., 2010). One of the factors influencing host-specificity is symbiont transmission. In the Macrotermitinae horizontal transmission is the main transmission mode; basidiospores produced by the sexual fruit bodies (mushrooms) are used to inoculate the fungus gardens of newly founded colonies (Johnson et al., 1981; Korb and Aanen, 2003; de Fine Licht et al., 2006). However, in the genus *Microtermes* and in a single species of the genus *Macrotermes*, *Macrotermes bellicosus*, vertical transmission has evolved. In both cases vertical transmission is uniparental, but in opposite ways: in *Microtermes* the

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female reproductives, and in *M. bellicosus* the male reproductives, carry asexual fungal spores in the gut to inoculate a new fungus garden (Johnson, 1981; Johnson et al., 1981; Nobre et al., 2011a). Theory predicts that vertical transmission should reduce host-symbiont conflicts and increase host-specificity (Frank, 1996). Consistent with vertical symbiont transmission, fruit bodies have never been observed for *Microtermes* (Johnson et al., 1981; Darlington, 1994). If transmission is strictly vertical, the fungus will only be clonally propagated, and in the long term genetic variation in *Microtermes* symbionts will be reduced.

However, previous studies on genetic variation of *Microtermes* symbionts have given conflicting results (Aanen et al., 2002, 2007). *Microtermes* species from western Africa all share one single genotype of a single species of *Termitomyces* and this genotype was also found in species of the genera *Ancistrotermes* and *Synacanthotermes* (Aanen et al., 2002). In contrast, in South Africa a big pool of genetically diverse symbionts was associated with species of the genus *Microtermes* (Aanen et al. 2002, 2007). However, sampling in these studies has been limited and only on large spatial scales so it remains to be studied how these different patterns can be explained.

In the present study we have extensively sampled fungus gardens of species of the genus *Microtermes* on smaller spatial scales in South Africa to study the population genetics of *Termitomyces* and how variation is distributed between species and colonies. We sampled at three different field sites and collected sequence data for both termite host and fungal symbiont. By studying genetic diversity of both host and symbiont we estimated the number of different colonies inhabiting the mounds of *Macrotermes natalensis* or *Odontotermes* spp. We also estimated the average size of a colony and the potential spatial area of a colony. Furthermore we looked for any patterns of host-specificity and whether horizontal transmission of the symbiont occurs.

2. Material and methods

Fungus-growing termites of the genus *Microtermes* have a very inconspicuous lifestyle; for example, they do not construct large epigeal mounds as species of some other genera such as *Macrotermes* do, but instead make large subterranean networks of tunnels connecting many different small fungus gardens (Uys, 2002). There is a documented case where two foraging *Microtermes najdensis* termites from the same colony have been collected 42 m apart, indicating that colonies may stretch over long distances (Pearce et al., 1990). Knowledge is lacking on the exact size of colonies (in both horizontal and vertical extent), the number of fungus-gardens per colony, colony density and species richness on a local scale. This may be due to sampling bias towards the fungus-growing termite species with conspicuous mounds which are easier to find. Due to the lack of taxonomically informative characteristics it is extremely hard to identify *Microtermes* termites to the species, which may be another reason why they have been overlooked in many ecological studies (Hausberger et al., 2011). *Microtermes* often inhabits the mounds of other fungus-growing termites such as *Macrotermes natalensis* and *Odontotermes* spp. (Uys, 2002)(Aanen, van de Peppel Pers. observations). Because of the high concentration of *Microtermes* fungus gardens in a relatively small surface area, we chose these mounds as our study sites for collecting *Microtermes* fungus gardens and termites (Fig. 1).

2.1. Sampling locations

Termites and fungus combs were sampled in South Africa between the 14th and 28th of January 2016 at two different sites in Pretoria [Experimental farm (EF) and Plant Protection Research (ARC)] and at one site in Mookgophong (MO) (Fig. 2). Termites and fungus comb were sampled when encountered during the excavation of *Macrotermes natalensis* and *Odontotermes* spp. mounds. A total of 11 mounds were sampled; five mounds (4x *M. natalensis*, 1x *Odontotermes* sp.) at EF, three mounds (2x *M. natalensis*, 1x *Odontotermes* sp.) at ARC and another three at MO (3x *M. natalensis*). Fungal cultures were made on the collection day or the day after from comb material. Three nodules were placed on a malt yeast extract agar (MYA; per litre demi water: 20 g malt extract, 2 g yeast extract, 15 g agar) plate using sterile forceps. Pieces of each fungus comb were stored in 2 ml tubes with pure ethanol. Since termites from the genus *Microtermes* are hard to identify on the basis of morphology, termites were only identified at the genus level (Korb et al., 2019). Termites were stored in 1.5 ml Eppendorf tubes with pure ethanol on the collection day. Samples were kept at 4 °C prior to DNA extraction.

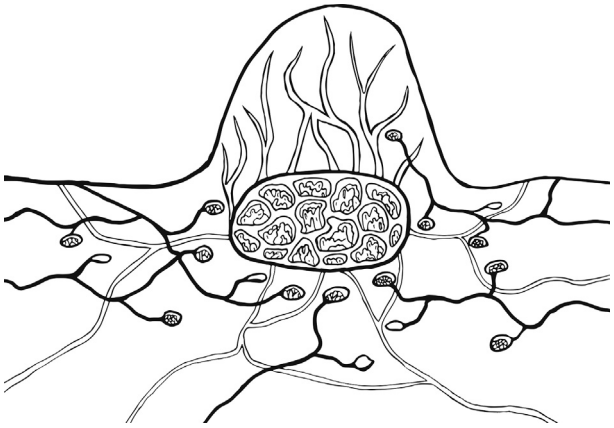

Fig. 1. Schematic overview of the organization of five *Microtermes* colonies (solid black lines) around a mound of *M. natalensis*.

In total 164 fungus comb samples and 134 termite samples were collected. For the final analysis, we only used the collections for which we had both a termite and a fungal symbiont sample.

2.2. DNA isolation, PCR and sequencing

For termite DNA isolations the head of a single termite was used. Initial DNA isolations on whole termites did not yield DNA of sufficiently high quality for polymerase chain reactions (PCRs), probably due to minerals and soil particles in the gut. For fungal DNA isolations, a small piece of mycelium (0.5 g) from a culture was used. All DNA isolations were done following a cetyltrimethylammonium bromide (CTAB) protocol (Nieuwenhuis et al., 2019). Partial sequences of the mitochondrial cytochrome oxidase I (COI) gene were obtained for the termites using a standard PCR reaction using the termite specific primer pair TL1862 and TH2877 (Aanen et al. 2002, 2007). Sequencing was done using only the forward primer and in some cases using both forward and reverse primer if the forward sequence was too short. For the *Termitomyces* symbionts the nuclear ribosomal region containing the internal transcribed spacer 1 (ITS1), 5.8S and the internal transcribed spacer 2 (ITS2) was amplified using a standard PCR reaction using the fungal specific primer ITS1f and the general reverse primer ITS4 (White et al., 1990; Gardes and Bruns, 1993). Primer sequences, the recipe for the PCR master mix and the PCR programs can be found in Supplementary material 1. Sequencing was done by Eurofins Genomics (Ebersberg, Germany). Forward and reverse sequences were assembled using CLC genomics workbench 8.

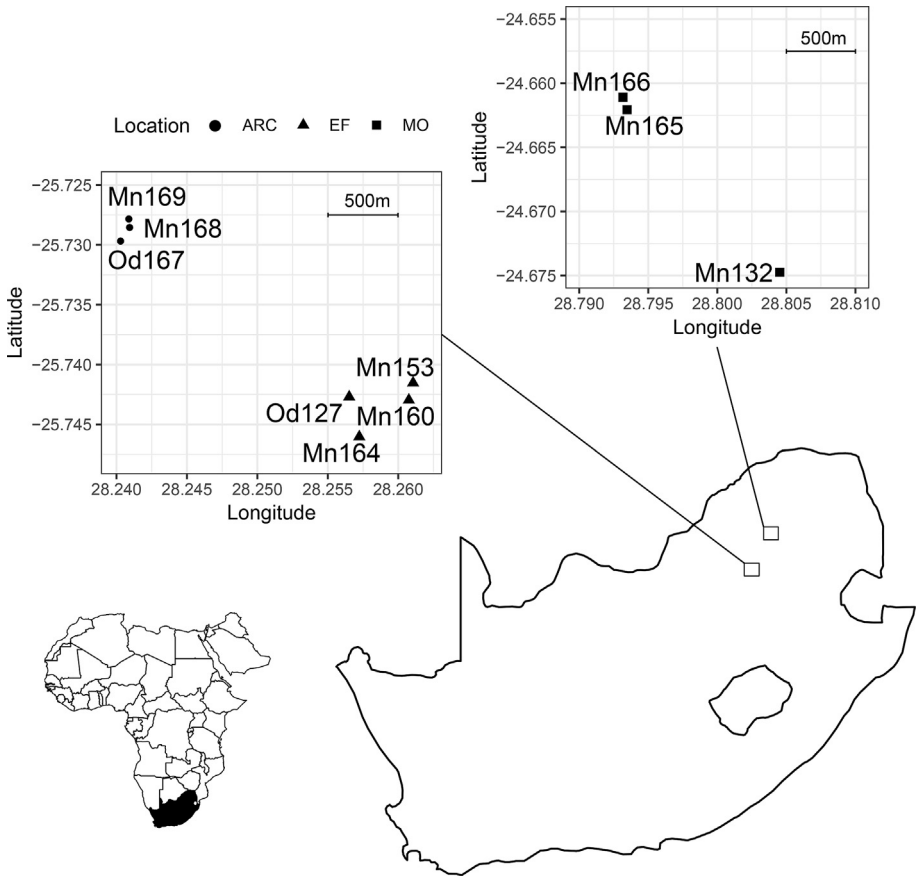

Fig. 2. Sampling locations within South Africa [Experimental farm (EF), Plant Protection Research (ARC) and Mookgophong (MO)] and mounds sampled.

2.3. Alignments, phylogenetic analysis and haplotype analysis

Chromatograms were checked in CLC genomics workbench 8. Since fungal heterokaryons contain two genetically different nuclei, they can be heterozygous for the analysed sequences and the chromatograms from fungal sequences were checked for double peaks. These double peaks were assigned with the correct letter according to the standard International Union of Pure and Applied Chemistry (IUPAC) notation for DNA. In the samples for which length polymorphisms occurred full sequences were generated by combining the forward and reverse sequence, in this case always the longest variant of the two variants was used. In two cases, two length polymorphisms were found and parts of the sequence were not recovered. The nucleotides that could not be recovered were replaced by question marks in the alignment. Because of these double length polymorphisms, both these sequences were treated as unique fungal genotypes (genotype 1Cx and genotype 1Dx).

Additional sequences from previous studies were extracted from GenBank and were added to the alignment (Supplementary Tables 1 and 2). Sequences were aligned using online MAFFT Multiple Sequence Alignment software version 7 using the default settings (Katoh and Standley, 2013, Katoh et al., 2019). Bayesian trees were reconstructed for both alignments and the posterior probability (Bayesian) estimates were calculated using Mr. Bayes 3.2.6. (Ronquist et al., 2012). using a general time-reversible model with gamma distribution (GTR + G), 30,000,000 generations and a sampling frequency of 4.000. The first 25% of the samples were discarded (burninfrac = 0.25). For each dataset the 50% majority rule consensus tree was constructed using the post burn-in samples from the posterior distribution of trees. The analyses were run on the CIPRES science gateway (Miller et al., 2010).

Genotype numbers were assigned to each sample corresponding to the main clades that were recovered from the phylogenetic analysis. A letter was added for each unique genotype within those clades if multiple genotypes were present. For further analysis only pairs of termite haplotypes and fungal genotypes were used; all single types were removed from the dataset. Two haplotype/genotype pairs were also excluded from the dataset because they were the only representative of a mound (mounds: Mn168 and Mn169). The final dataset used for the analysis consisted of 85 pairs of termite haplotypes and fungal genotypes (Supplementary Table 3). Identical combinations of termite and fungal type at one mound were considered to be from the same *Microtermes* colony. This is based on the assumption that the termites cultivate their fungal symbiont in a monoculture (Aanen et al., 2002; Katoh et al., 2002; Shinzato et al., 2005). We did not identify the species either for the termites nor for the *Termitomyces* symbionts, so we will refer to each clade by their haplotype or genotype number.

3. Results

3.1. Diversity

A total of 96 termite sequences and 98 fungal sequences were obtained and used for phylogenetic reconstruction. Phylogenetic analysis of the termite samples revealed three small clades and one large clade which contained 83.3% of all the samples (Fig. 3). In two of the four clades there was genetic variation within the clade. A similar pattern was found in the fungal tree where four main clades were recovered with the most dominant clade containing 61.2% of

the samples and further genetic variation was present in three of the four clades (Fig. 4).

Diversity was highest at location MO for both termites (Shannon index: 1.82) and fungus (Shannon index: 2.42). Seven of the eight different termite haplotypes and 14 of the 22 fungal genotypes were present at this location (Fig. 5). At location EF termite diversity was the lowest (Shannon index: 0.39) but fungal diversity was relatively high (Shannon index: 1.7). Low diversity for both termites and fungus was found at location ARC (Shannon index: termites 0.5 and fungus 0.5). Here only two termite haplotypes and two fungal genotypes were present.

3.2. Colony size estimation and host-specificity patterns

In total, 87 pairs for which both a fungal sequence and a termite sequence could be obtained were used for this analysis. The number of unique haplotype/genotype combinations found was 29. We considered that multiple counts of one haplotype/genotype combination at one mound were from the same colony. The largest potential colonies were found at mound Od127 and Od167 both consisting of at least eight fungus gardens. Overall the average number of sampled fungus gardens belonging to one colony was 2.36 per mound. Colony diversity was generally very high with on average 4.5 colonies per mound and up to ten different colonies per mound.

4. Discussion

4.1. Diversity

In general we found termite and fungal symbiont diversity to be highest at locations EF and MO. The diversity at ARC may in reality be higher, however, because of our limited sampling at this location. The results of this study are consistent with those of previous studies based on more limited sampling. Aanen et al. (2007) found four species of *Microtermes* and six different CO1 haplotypes in South Africa. In the present study we found two exact matches to these haplotypes and another six new ones. On the fungal side Aanen et al. (2007) found six different ITS genotypes, of which we found four in the present study. We found another 16 additional fungal genotypes. The discrepancy between host (eight haplotypes) and symbiont (20 genotypes) can partially be explained by the heterokaryotic nature of the fungus. Whereas the mitochondrial COI used for the termites is a haploid marker, we used the nuclear ITS marker for the fungus. Since *Termitomyces* has two nuclear types and each can have a different ITS sequence, there is more opportunity for unique genotypes. An additional possible explanation is that the resolution for both markers is different.

4.2. Colony size

We estimated the size of one colony on the basis of the number of fungus gardens and also considered the actual physical span of the colony in three dimensions. To test whether fungus gardens belong to the same colony we determined whether sample pairs with identical CO1 and ITS sequences were found in the same mound or also shared between mounds. For two sites (MO and ARC), we only found mound specific colonies and no shared colonies between mounds, but at site EF, we found colonies shared between different mounds. In total we found five cases in which colonies were shared between different mounds (Fig. 6; 1A1D shared among four mounds, 1A1A shared among two mounds, 1A1B, shared among two mounds, 1A2A shared among two mounds and 1C1A shared among two mounds). If we take this into account for calculating the average colony size for this location, this means that the average number of fungus gardens per colony is 4.9 instead of three. It also affects the size of the largest colony we found, which had eight fungus gardens, but will be 13 if we allow overlapping colonies between mounds. This could mean that the size of a *Microtermes* colony extends between different mounds, or, alternatively, that our resolution is insufficient to distinguish all colonies. On average we found 2.36 fungus gardens per mound of a unique combination. There were two cases with up to eight identical pairs at a single mound and 13 if we count overlapping colonies between mounds. Based on these numbers it is hard to estimate how many fungus gardens make up one *Microtermes* colony. An indication for a large colony size is the low number of royal chambers encountered during excavation, we only

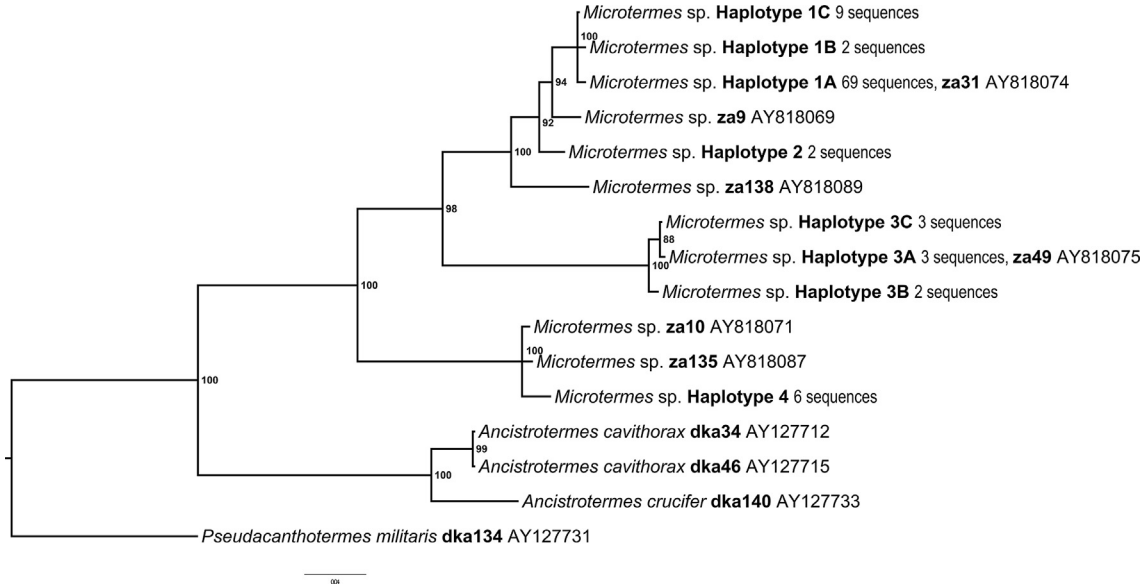

Fig. 3. Majority rule consensus tree of unique termite COI sequences. The number of identical sequences found in this study for each haplotype is indicated at the tip label. Node labels indicate posterior probabilities.

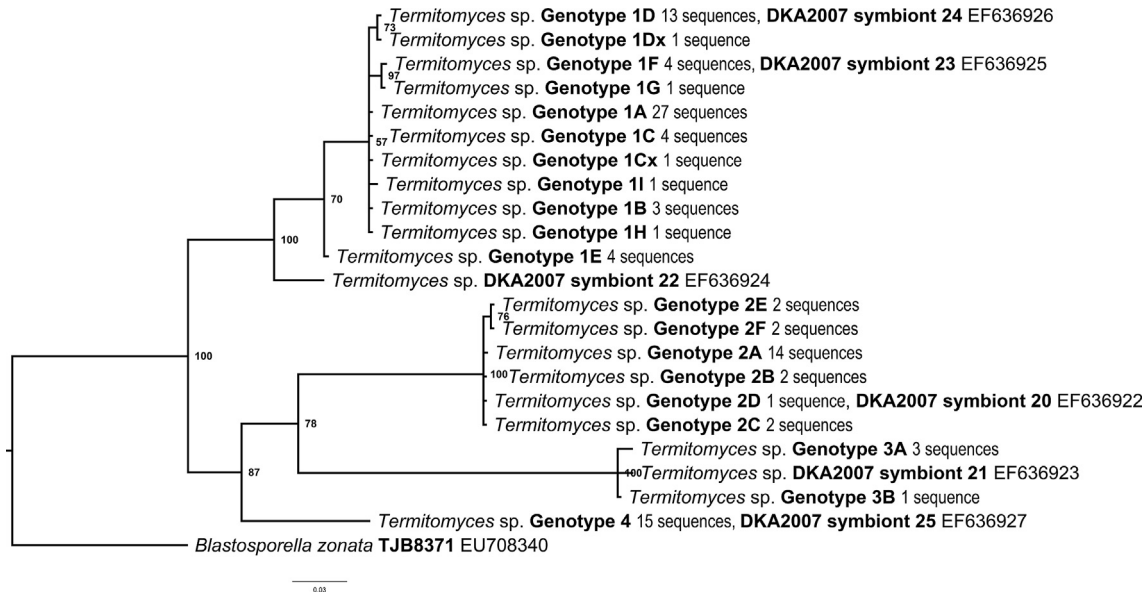

Fig. 4. Majority rule consensus tree of unique Termitomyces ITS sequences. The number of identical sequences found in this study for each genotype is indicated at the tip label. Node labels indicate posterior probabilities.

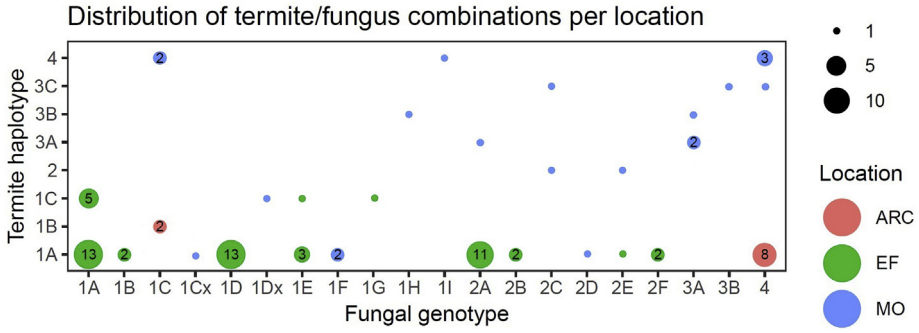

Fig. 5. Distribution of termite and fungal haplotypes for each location. The sampling locations are indicated by colour (ARC = red, EF = green and MO = blue). The size of the dot indicates the number of samples with the same types that were found.

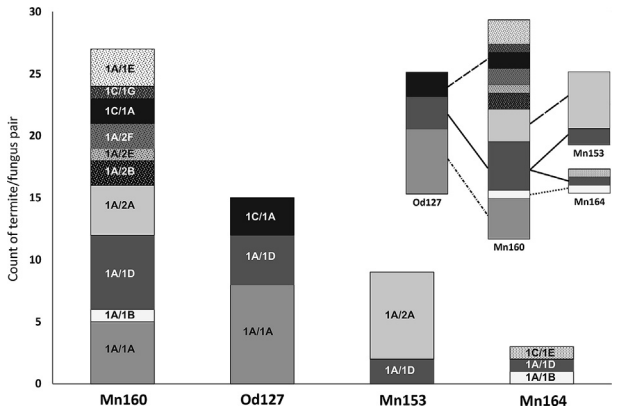

Fig. 6. Distribution of colonies over the different mounds at location EF. Each different shade and pattern indicates a unique type combination. Potential shared colonies between mounds are indicated in the top right panel. Colony 1A/1D is shared between all four mounds, colony 1A/1A, 1A/1B, 1A/2A and 1C/1A at location EF are shared between two mounds. At the two remaining locations, MO and ARC, each mound only has unique colonies which are not shared with the other mounds and are not shown in this picture.

encountered two during the entire study. We also found a high number of different colonies at each mound with up to ten different colonies at mound Mn160 (Fig. 6). This high cryptic diversity indicates that *Microtermes* spp. may play an important role in the functioning of the ecosystem (Hausberger et al., 2011).

We did not find shared occurrence of identical haplotype/genotype combinations at the different collection sites, which indicates that our method gives sufficient resolution to distinguish different colonies between different populations. We found five cases in which colonies were shared between different mounds at one collection site. These could be different colonies founded by sister queens or by female progeny from subsequent generations. If we assume these shared occurrences belong to a single colony it means that colonies may stretch as far as several hundreds of meters (Fig. 2). This also implies that a major part of the colony may be found in the open field, which also explains why we only found two royal chambers during this study.

For a more realistic estimate of colony size the sampling radius should be drastically increased and the open space in between mounds of other Macrotermitinae should be systematically excavated. Whether the density of *Microtermes* fungus gardens is higher in the large mounds of other species, and if so, what attracts *Microtermes* to those, is currently not known.

Although our data offer enough resolution to study patterns of fungal diversity in relation to host diversity and how this is distributed in space, we cannot rule out that some identical combinations belong to different colonies. Neither can we rule out that a single termite colony contains genetically different fungi, since we assumed that a termite colony has a single fungal genotype, which has been found in all other studied species of fungus-growing termites, so far. Microsatellite data are needed to confirm that termites belong to the same colony and additional polymorphic markers are needed to confirm that the symbionts are the same clone.

4.3. Host-specificity

The five studied species of the genus *Microtermes* all have vertical symbiont transmission via the female reproductives (Johnson et al., 1981; Johnson, 1981). Vertical transmission is a derived trait in the fungus-growing termites, and horizontal transmission via the sexual spores produced by fruit bodies of the fungus is the ancestral state (Aanen et al., 2002). It is expected that sexual reproduction in the symbionts of *Microtermes* termites does not occur frequently, if it occurs at all. This is based on the absence of observations of fruit bodies for *Microtermes* symbionts (Johnson et al., 1981; Darlington, 1994). However, it is possible that sexual reproduction could occur in these symbionts when they are cultivated by species of other genera of fungus-growing termites (*Ancistrotermes*, *Allodontotermes, Acanthotermes* and *Synacanthotermes*), since these share genetically identical or closely related symbionts with *Microtermes* (Aanen et al., 2002; Nobre et al., 2011b). *Termitomyces medius*, which is genetically very similar to *Microtermes*-associated symbionts in central Africa, does produce mushrooms when associated with species of *Ancistrotermes* (Aanen et al., 2002). It is not known whether *Microtermes* actively suppresses fruiting of their symbiont or if other factors prevent fruiting of the fungus.

Over time, strict vertical transmission is expected to lead to a tight host-symbiont relationship and reduced genetic variation in the symbiont (Frank, 1997; Law and Dieckmann, 1998; Herre et al., 1999). Our findings are not consistent with these theoretical expectations, since we found that a single termite haplotype (1A) could be associated with up to 12 different fungal genotypes from three of the four main clades. Fungi were not specific either to a single termite host. For example, fungal genotype 4 was associated with three different termite haplotypes from three of the four main clades. These findings show that host-switching must occur relatively often. A similar result has been found in fungus-growing ants which also have vertical transmission of their symbiont but occasionally horizontal transmission as well (Green et al., 2002). Since *Microtermes* has vertical transmission of its symbiont and the symbionts do not fruit, the most likely way to obtain a different symbiont is by obtaining it from a neighbouring colony. Some of the *Microtermes* symbionts are shared with members from the genera *Ancistrotermes*, *Acanthotermes*, *Allodontotermes* and *Synacanthotermes* (Nobre et al., 2011b; Aanen et al., 2002). Fruit bodies associated with the nests of *Ancistrotermes* and *Acanthotermes* have frequently been found (Johnson et al., 1981; Koné et al. 2011, Koné et al., 2018). Theoretically a different symbiont could be obtained from basidiospores when fruiting occurs in these other genera. This does not seem very likely since there is little overlap in geographical range where species from these genera and *Microtermes* co-occur (Uys, 2002). It is also not known over what distances basidiospores of *Termitomyces* can disperse. Studies on fungus garden establishment in *Microtermes* are limited and previous studies in a laboratory setting have not mentioned rates of successful fungus comb inoculation (Johnson, 1981; Johnson et al., 1981;). It is expected that in rare cases establishment of a fungus garden in species with vertical symbiont transmission is not always successful, in which case the queen or the young workers may need to reobtain the symbiont from other colonies. We, therefore, expect that switching between fungal cultivars will most likely occur between generations because when the fungus is established within the colony the chance of it being outcompeted by an invading fungus is presumably low. In fungus-growing ants it has recently been observed that queens may lose their symbionts and are able to obtain a new symbiont from neighbouring fungus gardens (Howe et al., 2019). Similarly, fungus-growing ants in the genus *Cyphomyrmex* are able to reobtain their symbiont if they accidentally lose it by stealing it from other colonies (Adams et al., 2000). Fungal crop theft has also been observed in fungus-growing ambrosia beetles (Hulcr and Cognato, 2010). The high density of *Microtermes* colonies that we observed allows easy access to fungus gardens from other colonies in case a fungal symbiont is lost. However, we did not find any indication of symbiont exchange between different termite genera. Although all *Microtermes* sampled in this study were inhabiting mounds of either *M. natalensis* or *Odontotermes* spp. we never found *Microtermes* termites associated with the fungal symbiont of these genera.

Microtermes has not been studied in great detail yet, probably because of its cryptic lifestyle. We aimed to resolve some of the basic questions about the biology of the genus *Microtermes* especially regarding colony size and host specificity. *Microtermes* certainly deserves more attention since it is one of the two cases in which vertical symbiont transmission has evolved in the Macrotermitinae and could serve as a model system for understanding the evolution of vertical transmission of symbionts. Vertical transmission is the main mode of symbiont transmission for *Microtermes* but we demonstrate that frequent horizontal exchange of genetically diverse symbionts occurs. Whether this horizontal exchange is the consequence of primary or secondary loss of the symbiont or whether termites actively search for a more suitable symbiont remains to be answered.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.100917.

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