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Investigations

Species and Speciation in the Termite-Cultivated Fungus *Termitomyces*.

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Abstract

Termitomyces is a genus of basidiomycete fungi cultivated by termites of the subfamily Macrotermitinae. This symbiosis originated in central Africa, and subsequently, the fungus-growing termites have colonized almost the entire African continent including Madagascar as well as significant parts of Asia. Around 40 species of *Termitomyces* have been described based on morphology of the sexual fruit bodies, which are associated with some 330 species of fungus-growing termites distributed over 11 genera. However, the total number of fungal species may be higher as not all species regularly produce mushrooms, and morphological variation does not seem to be a reliable criterion for species delimitation in this group. In this study we estimated the total number of species based on ITS-barcode criteria and assessed host specificity and geographic differentiation to infer patterns of speciation. We estimated the total number of phylogenetic species using two methods of DNA sequence-based species delimitation; Automatic Barcode Gap Discovery (ABGD) and the Generalized Mixed Yule Coalescent (GMYC) model on a large dataset of over 1,500 ITS sequences from laboratory cultures, fungarium specimens and the public database NCBI Genbank. This resulted in an estimated 87 species hypotheses using ABGD and 94 species hypotheses using the GMYC model. A phylogenetic reconstruction was performed on representative sequences of the 87 species hypotheses identified by ABGD (the most conservative estimate) constrained by a well-supported phylogeny based on whole-genome data to address host specificity and geographical differentiation. Five main clades were recovered which generally were associated with species of one or two host genera, except for samples collected from the genera *Microtermes* and *Ancistrotermes*, which formed two separate non-sister clades. We did not find any evidence for long-term host fidelity as would be expected for species with strict uniparental vertical symbiont transmission. We found strict geographic separation between African and Asian species of *Termitomyces* and infer a minimum of seven inter-continental migrations. We show that epigeous fruiting of the *T. microcarpus* group has a single evolutionary origin in Africa and that fruiting in species of this group likely is induced by the fungus rather than by the host-termite species. In contrast, fruiting in the symbionts of some species of *Microtermes* and *Macrotermes* may be suppressed by the host-termite species, since mushrooms of certain fungal species are found when those species are associated with some termite-host genera, but never when associated with other host genera. We discuss some examples of incongruence between morphological and phylogenetic species concepts and give suggestions to improve the taxonomy of the genus *Termitomyces*.

Introduction

Basidiomycete fungi of the genus *Termitomyces* (family: Lyophyllaceae) are cultivated by species of a subfamily of termites, the Macrotermitinae. The symbiosis has a single origin around 30 million years ago in the rain forests of central Africa (Aanen et al., 2002; Aanen & Eggleton, 2005; Nobre, Kone, et al., 2011; Roberts et al., 2016). Since all extant species of *Termitomyces* form a clade, researchers have hypothesized that only a single successful domestication

event has occurred and no reversals to a free-living state have been reported (Aanen et al., 2002; van de Peppel et al., 2021). From central Africa fungus-growing termites expanded their range through sub-Saharan Africa, including a migration of the genus *Microtermes* to Madagascar (Nobre, Eggleton, et al., 2010). Four of the eleven described genera of fungus-growing termites have been established in parts of Asia (Nobre, Rouland-Lefèvre, et al., 2010).

Fungus-growing termites cultivate *Termitomyces* fungi on a structure called the fungus comb which is constructed from faecal pellets consisting of consumed plant material and asexual spores (these pellets are referred to as primary faeces). On the fungus comb *Termitomyces* produces small asexual structures called nodules. The nodules are consumed by the termites and several different functions have been attributed to them. One of these functions is efficient within-nest propagation of the fungus; upon consumption the conidia present in the nodules are mixed with the plant material in the gut, and after a short gut passage, these primary faeces are used to construct the fungus comb (Leuthold et al., 1989). The nodules are also thought to play a role in nutrition by providing the termites with a reliable protein source and the essential amino acid tryptophan (Chiu et al., 2019; Nobre & Aanen, 2012). The fungus may also provide the termites with additional digestive enzymes (Martin & Martin, 1978).

Mushrooms are observed for most fungus-growing termite species, which rely on horizontal acquisition of the basidiospores for inoculation of the fungus in newly established colonies (de Fine Licht et al., 2006; Johnson et al., 1981; Korb & Aanen, 2003). In only two independent cases vertical transmission of the symbiont has evolved: via the male reproductive of the species *Macrotermes bellicosus* and via the female reproductive of all studied species of the genus *Microtermes* asexual fungal spores are carried in the gut (Johnson, 1981; Johnson et al., 1981; Korb & Aanen, 2003; Nobre, Fernandes, et al., 2011). The production of mushrooms usually occurs during the rainy season which coincides with the production of the reproductives (alates) (Koné et al., 2011, 2018). There is a moderate degree of host-specificity between fungus-growing termites and *Termitomyces*, mainly on a generic level (Aanen et al., 2002, 2007; Osiemo et al., 2010).

The genus *Termitomyces* was erected by Roger Heim in 1942, before which fungus-growing termite symbionts were placed in several unrelated fungal genera (Heim, 1942). Currently, *Termitomyces* is a large genus with 107 names listed in Index Fungorum (November 2023, http://www.indexfungorum.org/). These names include all names that have been validly published and therefore also contain synonyms and different forms of a single species, which makes it unclear how many biological species are represented. Large differences in macromorphological characteristics exist within the genus, particularly in size. For example, the genus contains both *T. titanicus*, which is considered the largest mushroom in the world with a cap diameter of up to one metre, but also *T. microcarpus* which has a cap diameter of less than two centimetres (Pegler & Piearce, 1980; Piearce, 1987). For most species of *Termitomyces* mushroom formation starts underground; primordia are formed on the fungus comb in the termite nest and the immature mushroom is pushed to the soil surface by a root-like structure called a pseudorhiza. Some species also have a cap with a hard pointed umbo called a perforatorium which is thought to facilitate penetration through the soil (Heim, 1977). A notable exception to this way of fruiting is the species *T. microcarpus* which fruits aboveground on comb material ejected by the termites. Probably as a response to this particular way of fruiting it does not produce a pseudorhiza and also does not have a perforatorium.

Taxonomy of *Termitomyces* mostly uses the morphological species concept based on macro-morphological characters of the mushroom such as size, shape, colour of the pileus, shape of the perforatorium and even the colour of the pseudorhiza. This has led to a biased representation of the total diversity of the genus as some species are thought to rarely produce mushrooms or even may have lost the ability to fruit (Bingham, 2002; Darlington, 1994; Wood & Thomas, 1989). Due to current taxonomical practices many of these rarely-fruiting species remain undescribed, as researchers only possess laboratory cultures made by plating fungal nodules with no representative mushroom (Makonde et al., 2013). Only recently has *T. cryptogamus* been described based on morphological features of a laboratory culture in combination with molecular data (van de Peppel et al., 2022). Although this species has been studied extensively it remained unnamed for almost two decades (Aanen et al., 2009; de Fine Licht et al., 2005, 2006; Poulsen et al., 2014). Research has also shown major genetic differentiation between African and Asian *Termitomyces* taxa (Frøslev et al., 2003). This geographical differentiation has often not been considered in the naming of species of *Termitomyces*, as some names are shared between African and Asian specimens, while genetically they may be diverse (Frøslev et al., 2003).

There are different species concepts that can be used to delimitate species in fungi. The biological species concept can be tested in basidiomycete fungi by testing the ability of two homokaryotic strains from different individuals to form a dikaryon (Boidin, 1986). Traditionally, the morphological species concept is used to delimit fungal species. However, in *Termitomyces*, macromorphological characters of the mushroom can be used to distinguish distantly related species, but not closely related species for which additional genetic data are needed (Tibuhwa et al., 2010). Because of the problems with morphological characters for species delimitation, the difficulty of applying the biological species concept (but see de Fine Licht et al., 2005, 2006), the rarity or absence of mushrooms in some species and the potential cryptic diversity, we used DNA sequence data to apply a phylogenetic species concept to estimate the number of species in the genus *Termitomyces*. For fungi, the nuclear ribosomal RNA internal transcribed spacer (ITS) region has been accepted as a universal barcode sequence and is frequently used to distinguish different species (Schoch et al., 2012). The ITS region consists of two spacer regions, ITS1 and ITS2, and are separated by the highly conserved 5.8S. The ITS is highly variable between and relatively con-

served within species and because it occurs in multiple copies per cell, it is easy to amplify. The ITS marker has also been used in previous studies on the phylogeny and diversity of *Termitomyces* (Makonde et al., 2013; Nobre, Kone, et al., 2011; Osiemo et al., 2010; Rouland-Lefevre et al., 2002; van de Peppel & Aanen, 2020; Vesala et al., 2017). Here, we studied genetic diversity in the genus *Termitomyces* and estimated the number of phylogenetic species by using two different methods of DNA sequence-based species delimitation; Automatic Barcode Gap Discovery (ABGD) and the Generalized mixed Yule coalescent (GMYC) method on a large dataset of ITS sequences generated specifically for this study as well as sequences from Genbank. We combined our species hypotheses with metadata on geographical location and termite host genus to arrive at an improved phylogenetic species concept for *Termitomyces*. Using this improved phylogenetic species concept and further resolved intrageneric relationships we evaluated the importance of five factors which could potentially influence diversification and speciation in the genus *Termitomyces*: host-specificity, geographic separation, symbiont transmission mode, host control over fruiting and fruiting mode (hypogeous versus epigeous). Our species hypotheses should offer a framework for future taxonomists and may facilitate future taxonomical practices in *Termitomyces* as it offers diagnostic features other than mushroom-based morphological differences.

Methods

Taxon sampling

Our aim was to cover most of the genetic diversity present in *Termitomyces*. Since *Termitomyces* is a large genus, we combined a dataset with sequences that we generated ourselves with sequences that are publicly available in the nucleotide database of NCBI Genbank. The sequences that were generated for this study came from two major sources, the first being our in-house collection which consists of a culture collection of *Termitomyces* strains collected in South Africa and ethanol-preserved nodules and mushrooms collected from various places in central and western Africa. The second source were voucher specimens from fungarium collections from four different collections: Royal Botanic Gardens KEW, United Kingdom (K), Meise Botanic Garden, Belgium (BR) and two herbaria in South Africa: the South African National Collection of Fungi (PREM) and the Schweickerdt Herbarium (PRUM).

To extend our own dataset we retrieved all available *Termitomyces* ITS sequences from NCBI Genbank (June 2020). We used the following search filters: “Termitomyces”, “Lyophyllaceae” and “internal transcribed spacer”. We also added five ITS sequences which we extracted using BLAST from the following assemblies: *Termitomyces eurhizus* MG13 (GCA_003316525.1), *Termitomyces heimii* MG15 (GCA_003313675.1), *Termitomyces* sp. MG145 (GCA_003313055.1), *Termitomyces* sp. MG148 (GCA_003313785.1) and *Termitomyces* sp. JCM 13351 (GCA_001972325.1). Metadata such as species name, collection location, termite host species and isolation source were also extracted and put into a spreadsheet. To filter out any non-*Termitomyces* sequences we did an initial sequence alignment and phylogenetic tree reconstruction (data not shown). We investigated sequences on long branches using NCBI Blast. The top hits for these sequences were either ascomycetes or distantly related non-*Termitomyces* basidiomycetes and were therefore discarded from the dataset. In a few cases unreliable sequences were removed after manual inspection as some sequences had multiple mutations in the conserved 5.8S region between ITS1 and ITS2, which were most likely sequencing errors.

A sequence of *Blastosporella zonata* and *Arthromyces matolae* were added as outgroup species (van de Peppel et al., 2021). An overview of all the sequences and their corresponding Genbank accession numbers used in this study can be found in Table S1.

Species names and taxonomy

Although resolving ongoing taxonomical challenges was not the main focus of our study, we attempted to put names on some of our species hypotheses. Species identifications of sequences submitted to Genbank are often unreliable, as they are often not part of a publication or they cannot be linked to publicly available voucher specimens. Therefore, we sampled voucher specimens that have been identified to the species level and a total of 20 type specimens from the four different collections that we mentioned in the previous paragraph. In addition to these type specimens we added sequences from the online UNITE database (https://unite.ut.ee/). In this curated database species hypotheses are generated using sequence similarity thresholds of the ITS region (Abarenkov et al., 2010). Most of these species hypotheses are reliable identifications as sequences are linked to a fungarium or voucher specimen. To limit ambiguity among species names, we will only use species names that can be directly linked to a type specimen, voucher specimen or UNITE species hypothesis.

DNA isolation and PCR

For DNA extraction, a small piece of tissue of about 0.1-0.5g of either a laboratory culture, mushroom or dried herbarium specimen was used. DNA was extracted following cetyltrimethylammonium bromide (CTAB) protocol used in a previous study (Nieuwenhuis et al., 2019). As DNA from old herbarium material is largely degraded we aimed to increase the DNA yield by extending the precipitation step in our protocol to two weeks for the fungarium samples (Staats et al., 2011).

An initial PCR was done to test whether the ITS marker could be amplified in its entirety using either the fungus-specific ITS1f (Gardes & Bruns, 1993) or *Termitomyces-*specific ITS1fT forward primer (Aanen et al., 2007) and the universal reverse ITS4 primer (White et al., 1990). In case the ITS marker could not be amplified, ITS1 and ITS2 were amplified separately by using the ITS1fT and ITS2 primers for ITS1 and the ITS3 and ITS4 primers for ITS2 (White et al., 1990). In case amplification was still unsuccessful

another attempt was conducted using ITS1fT as forward primer and a newly designed *Termitomyces*-specific reverse primer; ITS2T (AGATCCGTTGCTGAAAGTTG) for ITS1. For ITS2 the newly designed *Termitomyces*-specific forward primer ITS3T (AGTGTCATTTAAATTCTCAACC) and the general reverse primer ITS4 were used (White et al., 1990). The program used for all PCR reactions was as follows: initial denaturation at 94°C for five minutes, followed by 35 cycles consisting of one minute of denaturation at 94°C, one minute of annealing at 53°C and elongation for one minute at 72°C, followed by a final extension step for 10 minutes at 72°C. Sanger sequencing of PCR products was performed by Eurofins (Ebersberg, Germany). The forward and reverse read were assembled in CLC genomics workbench version 8. In a couple of cases we were not able to amplify ITS, despite having sufficient DNA of good quality. In the case of *Termitomyces* sp. DKA64 we were able to extract an ITS sequence from the genome assembly (GCA_017657295.1) using BLAST.

DNA sequence alignment

Combining our set of 161 newly generated ITS sequences and the filtered set of 1,416 ITS sequences from Genbank resulted in a dataset consisting of a grand total of 1,577 sequences. All sequences were aligned using the web server of MAFFT (7.475) using an iterative refinement strategy (FFT-NS-i) (Katoh & Standley, 2013). The alignment was manually inspected and the ends were trimmed. This alignment was used for the two methods of DNA sequence-based species delimitation analyses.

Species delimitation

Two different DNA sequence-based methods of species delimitation were used; Automatic Barcode Gap Discovery (ABGD) and the Generalized Mixed Yule Coalescent (GMYC) model (Fujisawa & Barraclough, 2013; Puillandre et al., 2012). The GMYC method is a likelihood method used to delineate species by aiming to detect switches between Yule speciation to coalescent process in a phylogenetic tree (Fujisawa & Barraclough, 2013). ABGD uses genetic distance values and does not require a phylogenetic tree as input. It aims to automatically detect the location of a barcoding gap between intraspecific and interspecific divergence based on a distance matrix (Puillandre et al., 2012).

We ran Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), using default parameter settings; a Jukes-Cantor (JC69) substitution model, Pmin = 0.001, Pmax = 0.1, steps = 10, X (relative gap width) = 1.5, number of bins = 20). GMYC requires an ultrametric phylogenetic tree as input. To determine the nucleotide substitution model, we used ModelFinder (Kalyaanamoorthy et al., 2017). We ran BEAST v1.10.4 (Suchard et al., 2018) on the CIPRES Science Gateway (Miller et al., 2010) using a TN93+G nucleotide substitution model, strict molecular clock model, coalescent constant population size and other default priors. The analysis was run for 10^7 generations with trees sampled every 10^4 generations. We used TreeAnnotator v1.10.4 (Drummond & Rambaut, 2007) to summarize the maximum clade credibility tree after discarding 25% of the trees as burn-in. The resulting tree was used for the single threshold GMYC analysis (Fujisawa & Barraclough, 2013) which was executed using the Splits package (Ezard et al., 2009) in R.

Phylogenetic reconstruction

Because the resulting phylogenetic tree from the alignment with 1,577 sequences would be difficult to study due to its size, we ran a second phylogenetic reconstruction using only one sequence of each species hypothesis. The output of ABGD shows a list of sequence accession numbers that make up each species hypothesis, therefore we could select a single representative sequence for each species hypothesis, this was usually the longest sequence. These sequences were aligned using the web server of MAFFT (7.475) using an iterative refinement strategy (FFT-NS-i) and other default settings (Katoh & Standley, 2013).

After trimming the alignment, we conducted phylogenetic reconstruction using RAxML version 8.2.12 (Stamatakis, 2014) with 100 bootstrap replicates using a GTR+gamma substitution model and species 69 as the outgroup. Previous phylogenetic studies using one or two genetic markers recovered the main clades within *Termitomyces*, however, they could not resolve the relationships between those clades (Aanen et al., 2002, 2007; Frøslev et al., 2003; Nobre, Kone, et al., 2011). A recent phylogenetic study on the Lyophyllaceae, which included 25 representative species of *Termitomyces*, used 1,131 orthologous nuclear genes and resolved the relationships between the main clades (van de Peppel et al., 2021). To conserve the correct order of the main clades, we used the tree from van de Peppel et al., 2021 as a constraint tree.

Results

Species delimitation

Our two methods of species delimitation yielded different numbers of estimated species. The genetic distance-based method ABGD identified 89 species hypotheses; 87 species of *Termitomyces* and two outgroup species. The phylogenetic tree-based method GMYC estimated 96 species hypotheses (confidence interval 90-106); 94 species of *Termitomyces* and two outgroup species. An overview of all sequences and their corresponding species hypothesis assigned by both methods can be found in Table S1. For subsequent examination and analysis we chose for the grouping by ABGD as it was the most conservative species estimate. An overview of the 89 species hypotheses, including assigned species name, termite host species and geographical location can be found in Table S2.

Type specimens

We obtained 20 type specimens from herbarium collections, and for 11 of these we obtained either a full or partial ITS sequence, this included two specimens older than 140 years (Table 1).

Table 1. Overview of type specimens collected and sequenced with their corresponding herbarium or Genbank identification number, type status, part of ITS sequenced, species hypothesis number, continent of origin and collection year.

Name	Voucher ID	Reference	Type status	Sequence	Species	Continent	Year collected
*T. acriumbonatus**	LAH35345	Usman & Khalid, 2020	Holotype	ITS1+2	34	Asia	2020
T. biyi	K109558	This study	Holotype	-	-	Africa	1963
T. bulborhizus	K109284	This study	Isotype	ITS1+2	3	Asia	2002
T. cartilagineus	K188704	This study	Isotype	-	-	Asia	1845
T. clypeatus	BR5020032886033	This study	Holotype	-	-	Africa	1923
T. congolensis (*le-testui*)	BR5020032900173	This study	Holotype	-	-	Africa	1923
T. congolensis var. *uelensis*	BR5020032898159	This study	Holotype	ITS1+2	51	Africa	1926
*T. cryptogamus**	CBS H-24752	van de Peppel et al., 2022	Holotype	ITS1+2	18	Africa	2011
T. eurrhizus	K188704	This study	Isotype	-	-	Asia	1844
*T. fragilis**	HKAS:88912	Ye et al., 2019	Holotype	ITS1+2	31	Asia	2012
T. globulus	BR5020032896131	This study	Holotype	ITS1+2	77	Africa	1941
T. griseiumbo	K143970	This study	Holotype	ITS1+2	77	Africa	1999
T. heimii	K94755	This study	Isotype	ITS1+2	55	Asia	1977
T. microcarpus	K237642	This study	Holotype	-	-	Asia	1868
T. rabuorii	K109548	This study	Holotype	-	-	Africa	1962
T. reticulatus	PREM47247	This study	Holotype	ITS1+2	59	Africa	1982
T. robustus	BR5020032919366	This study	Syntype	ITS1+2	54	Africa	1925
T. sagittiformis	K177059b	This study	Syntype	ITS1+2	72	Africa	1881
*T. sheikhupurensis**	LAH35710	Izhar et al., 2020	Holotype	ITS1+2	37	Asia	2017
T. striatus	K237645	This study	Holotype	-	-	Africa	1935
T. titanicus	K142416	This study	Isotype	ITS1+2	52	Africa	1978
T. tylerianus	K109536	This study	Holotype	ITS1+2	43	Africa	1966
T. umkowaani	K237646	This study	Holotype	ITS1	72	Africa	1888

Species with an asterisks were not sampled during this study but sequence data was obtained from Genbank.

Phylogenetic analysis

Using a preliminary phylogenetic analysis of the 87 species, we selected 24 taxa that covered the diversity of *Termitomyces* in our sample for whole-genome sequencing, these results were published in a different study (van de Peppel et al., 2021). The phylogenetic reconstruction shown in Figure 1 used this phylogeny as a constraint. We recovered five main groups (Fig. 1). Group 1 contains the symbionts of three genera: *Acanthotermes*, *Pseudacanthotermes* and *Ancistrotermes*. Group 2 contains predominantly the symbionts of *Microtermes* but also those of *Ancistrotermes,* and *Allodontermes*. Group 3 contains the symbionts of *Macrotermes*, with only one exception, which is based on only a single sample. Group 4 is a small group with just two species hypotheses and is associated predominantly with *Microtermes* and *Ancistrotermes* but also *Synacanthotermes*. Group 5 is the largest group including more than half of the total number of species hypotheses and containing predominantly symbionts of the genus *Odontotermes* but also of its small sister genus *Protermes*. Within this group we can distinguish four subgroups.

We find several examples of incongruence between morphospecies and our phylogenetic species hypotheses. The three most notable examples are *T. clypeatus*, for which we could match specimens to three different species hypotheses (species 6, 43, 49) in three major groups (group 1, 3 and 5.3), *T. striatus* which we could match to four species hypotheses (species 12, 15, 49, 72) in four major groups (group 1, 2, 4, 5.3) and *T. microcarpus* which matched to four species hypotheses (species 23, 24, 30, 60), all belonging to group 5.4. There was also incongruence within morphospecies as species 72 containes the types of both *T. sagittiformis* (K177059b) and *T. umkowaani* (K237646) as well as a specimen identified as *T. striatus* (PREM55766). The opposite also occurred for species 38, which contains

six specimens of *T. umkowaani* (PREM42978, 56841, 56842, 56843, 57217, 57327) as well as a specimen of *T. sagittiformis* (PREM56742). Another example of this is species 77 which contains the type specimens of both *T. globulus* (BR5020032896131) and *T. griseiumbo* (K143970). The species *T. clypeatus* and *T. striatus* can be found scattered in the phylogenetic tree, but species 49 contains a voucher specimen of *T. clypeatus* (K29920) as well as a UNITE species hypothesis of *T. striatus*.

Discussion

Host specificity

Previous phylogenetic studies on the genus *Termitomyces* recovered either four or five main symbiont clades and showed moderate host specificity between termite hosts and their symbionts, mainly at the level of these clades (Aanen et al., 2002, 2007; Nobre, Kone, et al., 2011; van de Peppel & Aanen, 2020). The relationships between these five clades were not resolved by previous studies but a recent phylogenomic study resolved these deeper relationships (van de Peppel et al., 2021; Fig. 2). Interestingly this study confirmed the paraphyly of the symbionts of *Ancistrotermes* (and *Microtermes*) termites.

Symbiont sharing appears to be rare between the major genera *Macrotermes*, *Microtermes* and *Odontotermes* on the African continent. In two cases, an *Odontotermes* symbiont is shared with *Microtermes* termites (species 75 and 86) and in two cases it is the other way around (species 27 and 78). Although a rare case of symbiont exchange between these genera seems possible, one should be cautious as these observations based on a single collection could be potential misidentifications. The morphology or architecture of the mound on which a fruiting body is collected is not a reliable identifier for the host genus, as colonies of different genera often co-inhabit a single mound. Co-habitation of *Microtermes* in the mounds of *Ma. natalensis* and various *Odontotermes* spp. occurs frequently (Uys, 2002; van de Peppel & Aanen, 2020). There are several reports of smaller species of termites which construct their fungus gardens at the base of the large above-ground mounds of *Macrotermes* species, such as *An. latinotus*, *Odontotermes sp.*, *P. militaris*, *Allodontotermes* sp. and *Microtermes sp*. (Malaisse, 1978; Mujinya et al., 2014). A *Termitomyces* mushroom collected on a termite mound should always be traced back via the pseudorhiza to the host fungus garden where a termite should be sampled for a reliable identification of the termite host (preferably both morphologically and molecularly). The relationship can be confirmed if the fungal sequence from the gut of the worker termites matches the fruiting body sequence (Aanen et al., 2002). Reliable identifications are thus crucial for solid claims on host-specificity.

On the Asian continent there are more reports of *Termitomyces* species shared among different genera. These species include: *T. bulborhizus, T. clypeatus*, *T. eurhizus* and *T. microcarpus* (Pegler & Vanhaecke, 1994; Wei et al., 2009). We also find three Asian taxa associated with the three major termite genera: species 23 (*T. microcarpus*), species 6 (*T. clypeatus*) and species 44 (*T. eurhizus*).

We do not have an explanation for this difference in host-specificity between the continents, but independent migrations by host and symbiont may play a role. For example, if a termite species migrates from Africa to Asia but its symbionts do not, its survival may depend on making a new association with an aspecific symbiont from a different termite species or genus.

Geographic separation

Our analysis generally shows clear genetic divergence between African and Asian taxa as all of the putative species occur either on Africa or on Asia, except for species 12, consistent with a recent intercontinental migration. The divergence between African and Asian taxa is consistent with previous research (Frøslev et al., 2003). Even though the symbiosis originated in Africa and only four of the major fungus-growing termite genera occur in Asia, we see no large differences in diversity of *Termitomyces* symbionts between the continents. The Asian species hypotheses make up just over half of the total diversity, 48 out of the 87 species hypotheses (including the one mixed species hypothesis).

Our phylogenetic analysis shows at least seven independent intercontinental migrations in the genus *Termitomyces*, presumably from Africa to Asia, but back-migrations are also possible as our tree is not completely resolved. The migratory events occur in all five major groups, except for group 1, which is only represented by African species. We identified at least two additional migratory events to the five intercontinental migrations in *Termitomyces* already documented (Aanen et al., 2002) and four out-of-Africa migrations of the termites (Aanen & Eggleton, 2005). The lack of mixed populations within species suggests that migratory events are not very common.

Symbiont transmission mode

Uniparental vertical symbiont transmission evolved twice independently in the fungus-growing termites, via the female reproductives in the genus *Microtermes* and via the male reproductives in the species *Ma. bellicosus* (Johnson, 1981; Korb & Aanen, 2003; Nobre, Fernandes, et al., 2011). Although uniparental vertical symbiont transmission could increase host-specificity, previous studies did not find evidence for strong host-specificity in both the genus *Microtermes* and the species *Ma. bellicosus* (Nobre, Fernandes, et al., 2011; van de Peppel & Aanen, 2020). Our analysis is congruent with these previous findings as we found two species (species 16 and 18) from two different sub clades to be associated with *Ma. bellicosus*. Both these species are also shared with several other species of *Macrotermes*. The symbionts of the genus *Microtermes* are also found in two main groups (group 2 and 4) and are also shared with the termite genera *Ancistrotermes, Synacanthotermes* and in rare cases *Odontotermes*. That these particular symbionts are shared between different hosts, including those without uniparental vertical transmission, shows that there is little

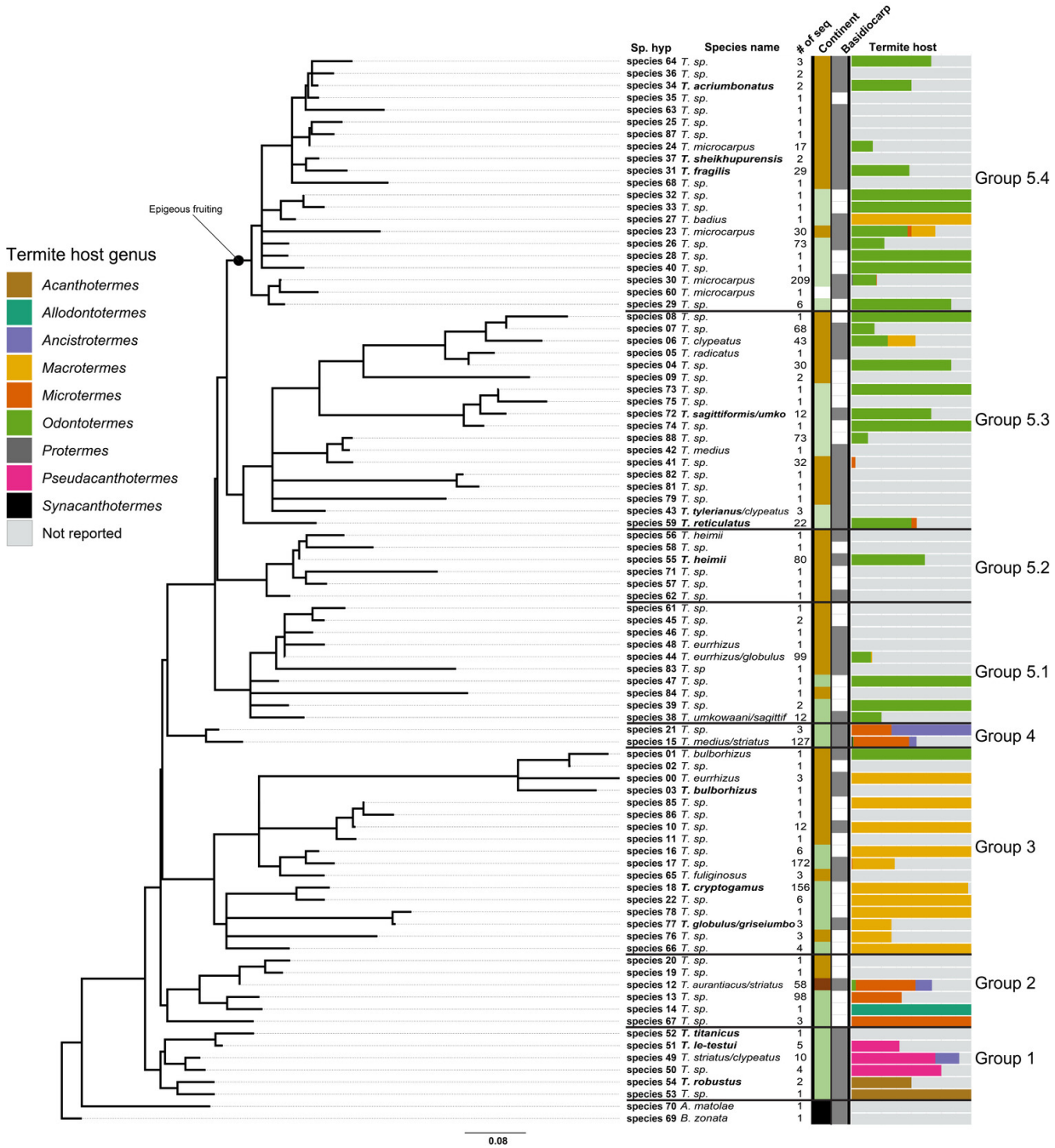

Figure 1. Maximum likelihood tree of representative ITS sequences for each species hypothesis, branches with bootstrap values lower than 60 were collapsed into polytomies.

The numbers in first column (Sp. Hyp) are the numbers of the species hypotheses generated by ABGD. The species names in the second column are based on a match of the respective species hypothesis to a sequence generated from a voucher specimen, UNITE species hypothesis, or a type specimen (in bold). The third column (# of seq) shows the number of sequences that ABGD assigned to the respective species hypothesis. The continent on which the species hypothesis occurs is indicated in the fourth column; Africa (light green), Asia (light brown), mixed (dark brown), not reported (white) or South America (black, outgroups). Whether a species hypothesis could be linked to a sequence obtained from a mushroom is indicated in the fifth column; mushroom present (dark grey) or absent/not reported (white). The termite host genus associated to each species hypothesis is indicated in the bar plots on the far right. The bars were created by taking the number of sequences for which the host termite was identified and dividing that by the total number of sequences.

opportunity for co-speciation. Therefore, it seems unlikely that symbiont transmission mode has played a major factor in speciation in the genus *Termitomyces*.

Host control over fruiting

We found that 37 of the 87 species hypotheses could not be associated with a sequence from a mushroom. About half of the African species (20 out of 38 species hypotheses) and approximately a third of the Asian species (17 out of 47 species hypotheses) could not be linked to a sequence obtained from a mushroom. Most species of *Termitomyces* produce mushrooms seasonally, usually correlated with the rainy seasons, and with alate production by the termites (Koné et al., 2011, 2018). Some species, especially the symbionts of the termite genus *Microtermes* and some African species of *Macrotermes*, seem to produce mushrooms in-

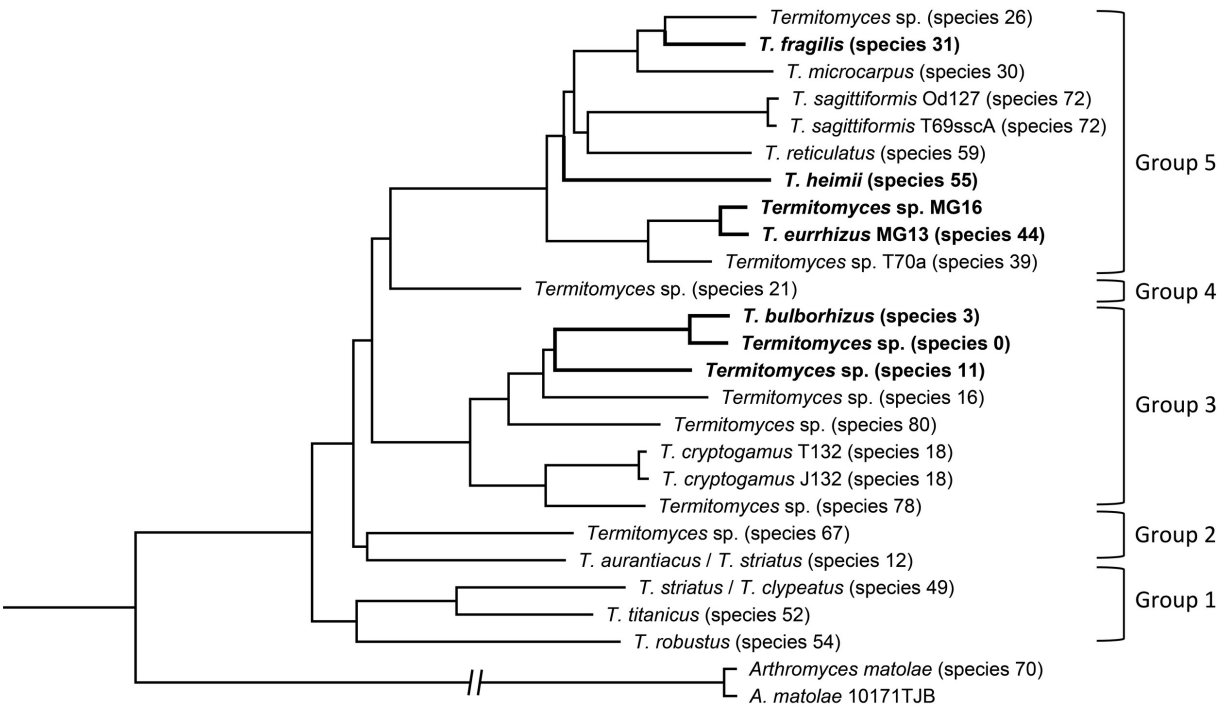

Figure 2. *Termitomyces* phylogeny adapted from van de Peppel et al. (2021) showcasing the five major groups within *Termitomyces*.

Names in bold indicate Asian taxa. Matches to species hypotheses from this study in parentheses. All branches are significantly supported by either 100 or 99 bootstrap replicates.

frequently or not at all (Darlington, 1994; Johnson, 1981; Korb & Aanen, 2003; Nobre, Fernandes, et al., 2011; van de Peppel & Aanen, 2020; Wood & Thomas, 1989). At least four species of *Microtermes* occur in South Africa associated with at least four species of *Termitomyces* (species 12, 13, 15, 67) (Aanen et al., 2007; van de Peppel & Aanen, 2020), yet none of the mushroom-forming species of *Termitomyces* known to occur in South Africa are associated with *Microtermes* (Van der Westhuizen & Eicker, 1990). This is also supported by our study on the herbarium collections of the PREM and PRUM herbaria in South Africa, none of the 92 specimens that we investigated, with collection dates ranging from 1912 to 2008, had *Microtermes* as the reported host or had a DNA match to one of the four *Microtermes* symbiont species. Nevertheless, for the absence of clonality among symbiont populations, indicates that sexual reproduction and thus mushroom formation should occur regularly (van de Peppel & Aanen, 2020). Of the four South African *Microtermes* symbiont species, species 12 and 15 are shared with other termite genera with horizontal symbiont transmission such as *Ancistrotermes* and *Synacanthotermes*. All mushrooms associated with species hypothesis 15, *T. medius*, were associated with termites of the genus *Ancistrotermes*, and never with *Microtermes*, which indicates the fungus has the capacity to form mushrooms, but could be suppressed by the termite host. The absence of clonality in *Microtermes* symbiont populations could be explained by occasional horizontal exchange with *Ancistrotermes* symbiont populations or invasions by novel symbiont genotypes. A similar pattern has been found for populations of the symbionts of a different species with vertical symbiont transmission, *Ma. bellicosus*, which are predominantly clonal when associated with *Ma. bellicosus*, but recombining when associated with other species of *Macrotermes* (Nobre, Fernandes, et al., 2011). A potential case of suppressed fruiting in a *Macrotermes* species with horizontal transmission is *M. natalensis* and its symbiont: *T. cryptogamus*. No natural mushrooms have been reported for this species, but under laboratory conditions in the absence of the termite host it can form mushrooms which produce viable spores (de Fine Licht et al., 2005; Vreeburg et al., 2020). It seems that differences in the frequency of mushrooms formation between groups can be explained by suppression of mushroom formation by some termite species but not by others (Korb & Aanen, 2003). Further experimental studies are needed to confirm this hypothesis.

The evolution of epigeous fruiting

With a cap diameter of less than two centimetres, *T. microcarpus* is the smallest species of *Termitomyces*. It grows in large patches of up to hundreds of mushrooms. It differs from other species of *Termitomyces* in that it fruits on comb material that termites eject from the nest (Fig. 3). Unlike all other species of *Termitomyces* it does not produce a pseudorhiza, although a tiny pseudorhiza has been reported (Horak, 1968). The atypical fruiting behaviour and the lack of a pseudorhiza made Heim (1977) think that this way of fruiting closely resembled the ancestral state and he therefore placed *T. microcarpus* in its own subgenus: *Prae-Termitomyces*. Molecular studies show that the opposite is true, as *T. microcarpus* is nested within *Termitomyces* so that this kind of mushroom formation is derived (Aanen et al., 2002; Frøslev et al., 2003).

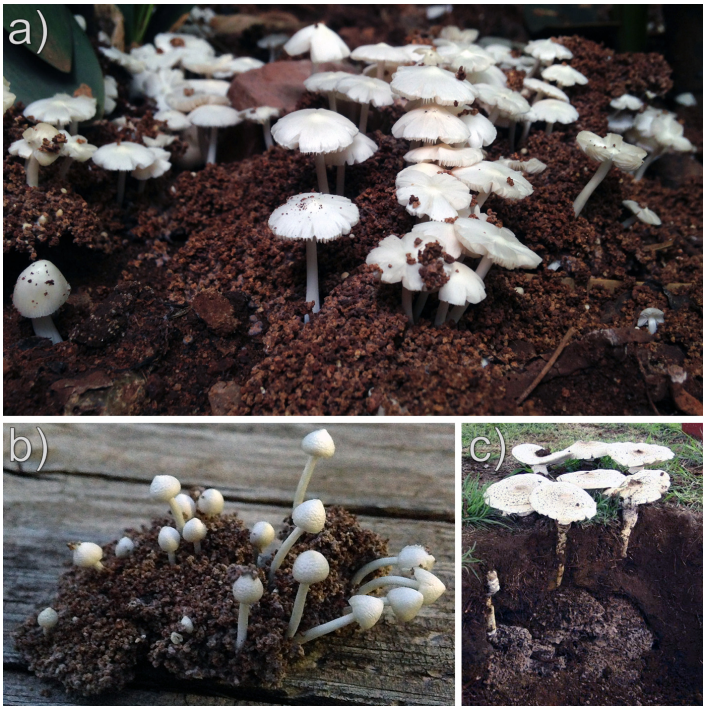

Figure 3. Photos depicting the differences between the two fruiting modes in *Termitomyces*.

a) Epigeous fruiting of African *T. microcarpus* (species 30) showing mushrooms produced on ejected comb material. b) No connection between comb and mushroom is present. c) An example of the more common fruiting mode; hypogeous fruiting, showing the attachment of *T. reticulatus* (species 59) to a fungus comb of *Odontotermes* sp.

It might seem likely that *T. microcarpus* has a limited host range because of this unique fruiting behaviour, if it would exploit a specific termite behavioural activity. However, this does not seem to be the case, as in Africa this species is associated with *O. badius*, *O. transvaalensis* and *O. vulgaris* (Bottomley & Fuller, 1921; Sands, 1969; Van der Westhuizen & Eicker, 1990) but also with two species of the genus *Protermes*; *P. minutus* and *P. prorepens* (Aanen et al., 2002). In Asia it is associated with *O. malaccensis*, *O. redemanni* but also species in the genus *Ancistrotermes* (Pegler & Vanhaecke, 1994), *Hypotermes xenotermitis* (Aanen et al., 2002) and *Ma. barneyi* (Wei et al., 2009). Here, we found additional host species, in Asia: *Ma. gilvus*, *Ma. annandalei*, *Microtermes* sp., *O. longignathus,* and *O. formosanus*, and in Africa: *Mi. subhyalinus*. Importantly, most of these species are also known to associate with different *Termitomyces* symbionts. For example, *O. badius* is known to associate with *T. umkowaani* and *T. reticulatus* and *O. transvaalensis* with *T. reticulatus* (Van der Westhuizen & Eicker, 1990). Surprisingly, this broad host range implies that this typical behaviour of ejecting comb material by the termites is induced by the fungus, as termites perform this behaviour only when associated with *T. microcarpus*. This is in sharp contrast with what we described in the previous paragraph in the termite genus *Microtermes*, where the termite seems to have some level of control over fruiting of the symbiont.

Our phylogenetic reconstruction shows that all samples identified as *T. microcarpus* are placed within one clade (group 5.4) indicating that epigeous fruiting evolved once. Since the Asian taxa form a sub-clade within the main clade, epigeous fruiting most likely evolved on the African continent. Group 5.4 consists of 21 species hypotheses and includes several species names, showing that the name *T. microcarpus* is paraphyletic and does not represent a single species but rather a group of species exhibiting a similar phenotype. This causes some taxonomical issues, as the type specimen has been described from a collection from Sri Lanka (Berkeley & Broome, 1871). Despite repeated attempts, we did not manage to obtain a sequence from the type specimen of *T. microcarpus* and therefore we cannot accurately assign the name to a species hypothesis. This means that the African *T. microcarpus* collections (species 30) represents a different phylogenetic species and should therefore be renamed, which may not be warranted as it is a very common species in Africa (Pegler, 1977; Van der Westhuizen & Eicker, 1990). This is also reflected by the 209 sequences which make up species 30 in our analysis. Other species with either epigeous fruiting or indications for epigeous fruiting (lack of a pseudorhiza) include: *T. badius*, *T. indicus*, *T. narobiensis*, and *T. orientalis* (Natarajan, 1975; Otieno, 1964, 1968), although some authors conclude that these species all are synonyms for *T. microcarpus* (Osiemo et al., 2010; Pegler, 1977; Pegler & Vanhaecke, 1994; Wei et al., 2009). Our single specimen identified as *T. badius* (species 27) is genetically distinct from specimens identified as *T. microcarpus*, this may indicate that *T. badius* could be a different species. We were unable to obtain specimens for *T. indicus*, *T. narobiensis* and *T. orientalis*, so their relationship to *T. microcarpus* remains unclear.

A 'semi-hypogeous' form of *T. microcarpus* has been reported of which the stipe can be up to eight centimetres belowground and multiple fruiting bodies can be connected (Heim, 1942, 1977). Heim also notes that there is no connection between the mushrooms and the fungus comb. We

were able to match an unidentified fruiting body (VDWALT1577) to this description in species hypothesis 26 (R. van der Walt, pers. comm.). It is therefore likely that this semi-hypogeous form represents a different species, although it belongs to clade 5.4. Other species from group 5.4 which produce a pseudorhiza and therefore may represent semi-hypogeous fruiters are *T. acriumbonatus*, *T. fragilis*, *T. radicatus* and *T. sheikhupurensis* (Izhar et al., 2020; Natarajan, 1977; Usman & Khalid, 2020; Ye et al., 2019). Growth on ejected comb material is reported for *T. sheikhupurensis* (Izhar et al., 2020). For *T. fragilis* a possible connection to a fungus garden has been reported, although the illustrations do not show a fungus comb (Ye et al., 2019).

We found no clear evidence for a reversal from fruiting on ejected comb material to fruiting directly from the fungus comb in group 5.4. Only in some species this ejected comb material may reach the soil surface and produce the typical habit lacking a pseudorhiza. In other cases, the comb material may be expelled from the nest but remain buried in the soil and therefore a (short) pseudorhiza is produced. The mechanism by which the fungus causes the termites to eject comb material from their nest is an interesting topic for future study.

Taxonomical challenges

Morphology-based taxonomy has greatly hampered the identification and description of new species of *Termitomyces*, as mushrooms are seasonal and not frequent in many species. The symbionts of several species of *Microtermes* (Darlington, 1994; van de Peppel & Aanen, 2020; Wood & Thomas, 1989) as well as several species of *Macrotermes* (de Fine Licht et al., 2006; Koné et al., 2011) even have no reports of mushrooms. The rarity of mushrooms in a substantial number of species begs for a different approach to the taxonomy of *Termitomyces*. Although differences in morphology occur in laboratory cultures, these differences are not sufficient and reliable enough to distinguish between species (Botha & Eicker, 1991a, 1991b; Tibuhwa et al., 2010). An approach to taxonomy using well-characterized and publicly accessible laboratory cultures in combination with molecular data has been suggested (Makonde et al., 2013). Thus far, only a single species, *T. cryptogamus*, has been described using this approach (van de Peppel et al., 2022).

In this study we used ITS for species delimitation as this marker shows plenty of genetic variation, even within species. However, in some cases we were unable to amplify ITS, this was particularly the case for specimens of *T. schimperi* (van de Peppel et al., 2022) and other *Macrotermes* symbionts such as species 80. In case we had difficulties amplifying ITS, we were able to amplify part of the ribosomal large subunit (28S). This marker also suffices for species identification.

This study shows that in general there is clear genetic differentiation between African and Asian taxa of *Termitomyces*, indicating substantial geographical isolation between continents. Although certain species names, such as *T. clypeatus*, *T. eurhizus* and *T. microcarpus* are used for the same morphospecies, African and Asian specimens of these morphospecies are clearly genetically divergent. This may cause problems in scientific communication as specimens from different continents identified under the same name generally represent different phylogenetic species and possibly also separate biological species. This is clearly the case for *T. microcarpus,* where the African and Asian are not part of the same species hypothesis and cluster in separate sub-clades within clade 5.4. Also different species hypotheses within continents bear the name *T. microcarpus*, indicating additional cryptic diversity. Two species with a very similar morphology, *T. umkowaani* and *T. eurhizus*, have been synonymized by some authors (Pegler & Vanhaecke, 1994; Van der Westhuizen & Eicker, 1990). This similar or identical morphology can be explained by shared ancestry as we find that *T. umkowaani* (species 38) and *T. eurhizus* (species 0, 44, 48) are part of two distinct sub-clades in group 5.1. In other cases, morphological similarity is not due to shared ancestry, but due to non-homologous processes, for example convergent evolution. This is the case for *T. striatus* and *T. clypeatus*, as specimens of these species are found scattered in the phylogenetic tree.

Previous examples indicate that a species concept based on morphological diagnostic features of the mushroom is not congruent with our phylogenetic species concept. A morphological species concept does not seem useful in the genus *Termitomyces* as it is very inconvenient to describe common species with rare fructification events and because there appears to be a high degree of cryptic species diversity. We therefore suggest delineating species primarily based on DNA-sequence similarity, preferably in combination with additional features such as termite host genus, geographic origin, and fruiting mode. Morphological features should be described to assist preliminary identification in the field but should never be used without DNA evidence for the final identification. Additionally, to understand the significance of DNA divergence for reproductive isolation, the application of a biological species concept will also be highly valuable.

Concluding remarks

In this study, we delineated species using two DNA sequence-based methods; automated barcode gap discovery (ABGD) and the Generalized Mixed Yule Coalescent (GMYC) model on a large dataset of ITS sequences. Using these methods, we identified between 87 and 94 phylogenetic species of *Termitomyces*. We identified several factors that may have contributed to the current diversity such as host-specificity, geographic separation, host control over fruiting and fruiting mode. We conclude that uniparental vertical symbiont transmission probably has only a minor contribution to diversification in *Termitomyces*. There are probably several other factors which may have caused diversification but which were not part of this study, such as substrate preference of changes in host diet (da Costa et al., 2019). This study helped to improve our understanding of several processes which may have shaped current diversity in *Termitomyces* but further study is needed to confirm some of the hypotheses that we formulated.

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Data availability

Newly generated ITS sequences were uploaded to Genbank (accession numbers: OP837112-OP837278). Additional sequence information such as specimen voucher number or strain number as well as a list of all sequences used in this study can be found in Table S1.

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