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Termitomyces cryptogamus sp. nov. associated with *Macrotermes natalensis* in Africa

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Abstract—A new species of *Termitomyces* symbiotic with the termite *Macrotermes natalensis* is described from Africa. As there are no records of field collected basidiocarps within this lineage, traditional basidiocarp-based morphological taxonomy is not practical. While basidiocarps may be obtained rarely from incubation of fungal comb fragments, their practical use for taxonomical purposes is limited. Therefore, the species is described based on an ITS nucleotide sequence, with comparisons to an asexual culture. Based on samples with similar ITS sequences, this species is likely associated with multiple termite hosts across a large part of Africa.

Key words—*Agaricales*, *Lyophyllaceae*, phylogeny

Introduction

In Africa and Asia, a subfamily of the *Termitidae,* the *Macrotermitinae,* live in obligate symbiosis with members of the basidiomycete genus *Termitomyces* (*Lyophyllaceae*). The fungus resides inside the termite nest in so-called "fungus gardens," with mushroom production being periodically triggered by rain or the potential health of the nest (Koné & al. 2011). The mushrooms vary in size and are generally medium to large, although some species, such as *T. microcarpus* (Berk. & Broome) R. Heim, produce hundreds of small

mushrooms. Traditionally, taxonomy in this genus has been based on basidiocarp morphology, with molecular evidence only recently added (Frøslev 2003, Mossebo & al. 2017).

During research of termites and their associated fungi in Africa, one lineage was found that associates with *Macrotermes bellicosus* and *M. subhyalinus* when found in northern Africa, but only with *M. natalensis* when found in South Africa (Nobre & al. 2011). Surprisingly, sampling over ten years has not resulted in the discovery of mature basidiocarps of this species from any of its termite hosts, frustrating efforts for naming this species. Rarely, while excavating termite mounds, mushroom primordia are recovered (de Fine Licht & al. 2005; Vreeburg & al. 2020) that can occasionally be incubated under laboratory conditions in the absence of termite workers. Presumably due to this laboratory incubation the mushrooms are misshapen, as their appearance is not consistent with other known *Termitomyces* species. This may be similar to development that occurs in the production of "Enoki" mushroom from *Flammulina velutipes* which causes long thin stipes, smaller caps, and pale coloration (Kües & Navarro-González 2015). Despite the need to provide a name for this well-studied fungus, the lack of basidiocarps has prevented comparisons with published literature descriptions (Botha & Eicker 1991b), leading to an argument for using readily obtained asexual cultures as a stable type against which to compare (Makonde & al. 2013).

As extensive comparisons of ITS sequences from herbarium material from South Africa recovered no matches with our *M. natalensis* symbiont (van de Peppel & al., unpublished data), we provide a description based on sequence identity and asexual morphology. While there is a previous description of asexual characteristics from South African *Termitomyces,* only mounds producing basidiocarps were used, with *M. natalensis* mounds apparently not sampled (Botha & Eicker 1991b). As basidiocarp records are lacking, asexual cultures are a logical solution to this taxonomic issue. Three previous publications addressed differences between species in asexual cultures, and while interspecific differences were found, asexual characteristics alone were not considered sufficient to delineate species (Botha & Eicker 1991a,b; Tibuhwa 2012).

Recently an ecological study of *Macrotermes* symbionts in Kenya uncovered results consistent with previous sampling of this lineage (Vesala & al. 2017). Vesala & al. recovered the same fungal species as the one collected from South Africa (Nobre & al. 2011, de Fine Licht & al. 2005; Vreeburg & al.

2020) from mounds of *M. bellicosus, M. herus, M. jeanneli, M. michaelseni,* and *M. subhyalinus.* Vesala & al. (2020), who selected related ITS sequences from GenBank including symbionts of *M. natalensis* and *M. bellicosus*, found that these formed a monophyletic clade. These sequences form two well-supported groups with 97% and 98.5% support, with the exception of a single sequence (GenBank AF357024) isolated from an unidentified *Macrotermes* species. Based on these results we describe the species based on a fungal isolate from South Africa symbiotic with *M. natalensis* for which the fungal genome has been published (Poulsen & al. 2014).

Materials & methods

Samples and Isolates

A heterokaryotic culture, Mn103, was obtained by opening a termite mound and carefully removing nodules containing asexual spores from the fungal combs without soil contamination. These nodules were placed on agar plates without antibiotics to establish hyphal cultures. A homokaryotic culture, P5, was obtained by protoplasting the Mn103 heterokaryotic culture. Additional details regarding sampling, isolation, and subsequent protoplasting of this culture are found in Poulsen & al. (2014) and Nobre & al. (2014). A dried holotype specimen of the Mn103 heterokaryotic culture and a living ex-holotype culture of the P5 homokaryotic culture are conserved at the Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands (CBS).

DNA extraction, PCR amplification, sequencing

Genomic DNA was extracted from the protoplasted P5 homokaryotic culture using the cetyltrimethylammonium bromide (CTAB) protocol using mycelium and spores scraped from a petri dish. The nuclear ribosomal region containing the ITS1 + 5.8S + ITS2 region (ITS) was amplified using a standard PCR reaction using Promega GoTaq polymerase and the fungal specific primer ITS1F and the general reverse primer ITS4 (White & al. 1990, Gardes & Bruns 1993). The 28S region (LSU) was amplified using primers LR0R and LR5 (Vilgalys & Hester 1990).

As no basidiocarps have been reported from *M. natalensis* termite mounds, we also surveyed herbarium samples from the South African National Collection of Fungi (PREM) and Schweickerdt Herbarium (PRUM) (van de Peppel & al. unpublished data).

Sequence alignment & phylogenetic reconstruction

We used a previously published set of ITS sequences (Vesala & al. 2017), six GenBank sequences from *Termitomyces* symbiotic with *M. natalensis,* and a sequence generated from the specimen we designate here as the type for the new species. Sequences were aligned using the web software MAFFT v. 7 with default settings (Katoh & Standley 2013). Maximum likelihood trees were reconstructed using IQ-TREE v. 2.0.6 with default settings (Trifinopoulos & al. 2016).

Culture & microscopy

Cultures were maintained on MYA (20g Malt Extract, 2g Yeast Extract, 1 L H_2O), and incubated at 25 °C. Microscopical examinations were conducted using a Zeiss Axio Imager A1 with 63X objective lens under DIC optics.

Taxonomy

Termitomyces cryptogamus van de Peppel, **sp. nov.** Fig. 1

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Differs from *Termitomyces schimperi* by its clearly separated LSU sequence; there are no useful diagnostic differences in the morphology of the asexual morphs of these species.

Type—South Africa, Pretoria, Rietfontein 321-Jr, 25.7292°S 28.2347°E, May 2011, DK Aanen, heterokaryotic isolate (Mn103) obtained from asexual nodules on a fungal comb from a mound of *Macrotermes natalensis* (**Holotype**, CBS H-24752 [metabolically inactive dried culture]; living ex-type culture CBS 147190].

Etymology: *cryptogamus*, referring to the hidden marriages of a genetically well-mixed species without recorded basidiocarps in vivo.

Sexual state—not observed in vivo.

Asexual state—Growth of colonies on MYA medium reaching 5–6 cm diam. in 3 weeks at 25 °C (somewhat faster at 30 °C). Growth consisting of white hyphae mostly submerged in agar, with 1–2 mm diam. clusters of asexual spores produced on the agar surface. Conidia highly variable in size (10–100 mm long) and shape, with 2–5 nuclei per spore. Heterokaryotic colonies consistently producing heterokaryotic conidia.

Comments—In the absence of in vivo basidiocarps, *T. cryptogamus* cannot be distinguished readily from closely related *Termitomyces* species based on asexual characters. However, ITS and LSU sequence analyses clearly separate a well-supported clade that includes the holotype of *T. cryptogamus* and several other strains obtained from fungal combs of *Macrotermes natalensis* in South Africa, as well as from those of *M. bellicosus, M. herus, M. jeanneli, M. michaelseni,* and *M. subhyalinus* in Cameroon, Kenya, Senegal, and Ivory Coast.

Phylogenetic results

NCBI GenBank accession numbers were obtained for the nucleotide sequences generated from the protoplasted homokaryotic culture P5: ITS (MW251838), LSU (MW567773), whole genome (GenBank id: GCA_001263195).

The ITS sequence of our isolate P5 places *Termitomyces cryptogamus* inside Group 1 of Vesala & al. (2017). This group includes fungal individuals symbiotic

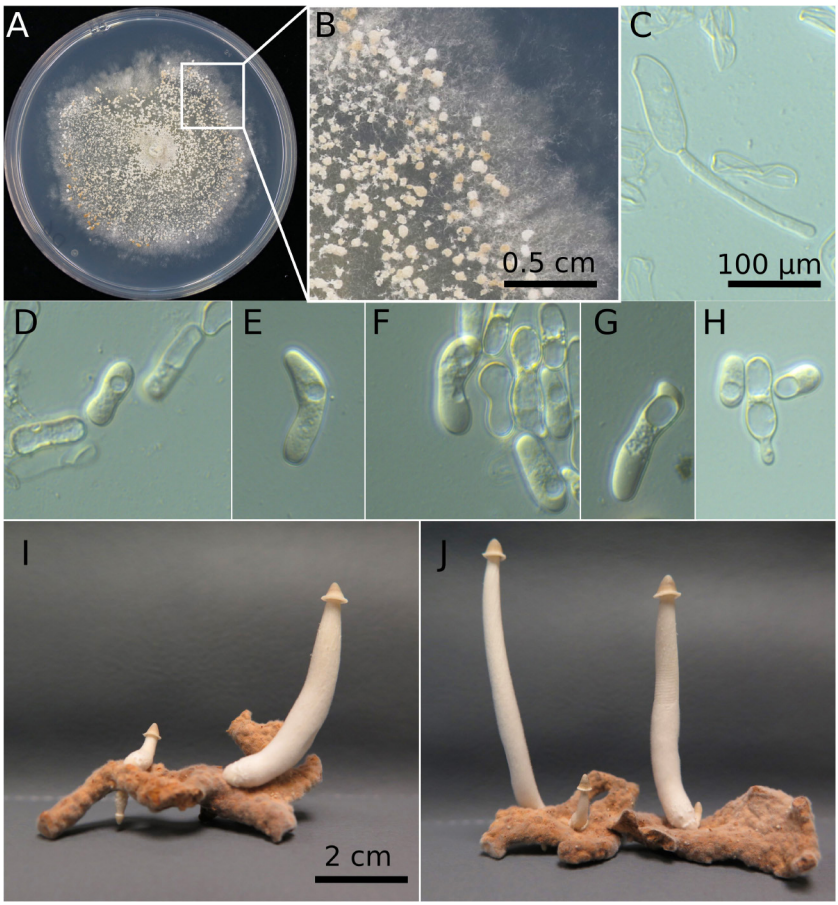

Fig. 1. *Termitomyces cryptogamus*: morphology. A. Growth on Malt Yeast Agar showing abundant nodule formation of asexual conidia; B. Close up view of A; C–H. Variable morphology of conidia; I, J. Morphology of mushrooms on fragments of excavated fungus garden comb produced after incubation for 5–10 days; note the small caps (<1 cm), which produce viable basidiospores.

with *Macrotermes natalensis, M. bellicosus, M. subhyalinus, M. michaelseni, M. herus* and *M. jeanneli* (Fig. 2). Except for *M. natalensis*, these termite species are found with sister species also closely related to *Termitomyces cryptogamus*.

To compare with other common *Macrotermes* mound symbionts, we also extracted DNA from samples of *Termitomyces schimperi* (Pat.) R. Heim. However, repeated PCR amplifications of the ITS region were not successful.

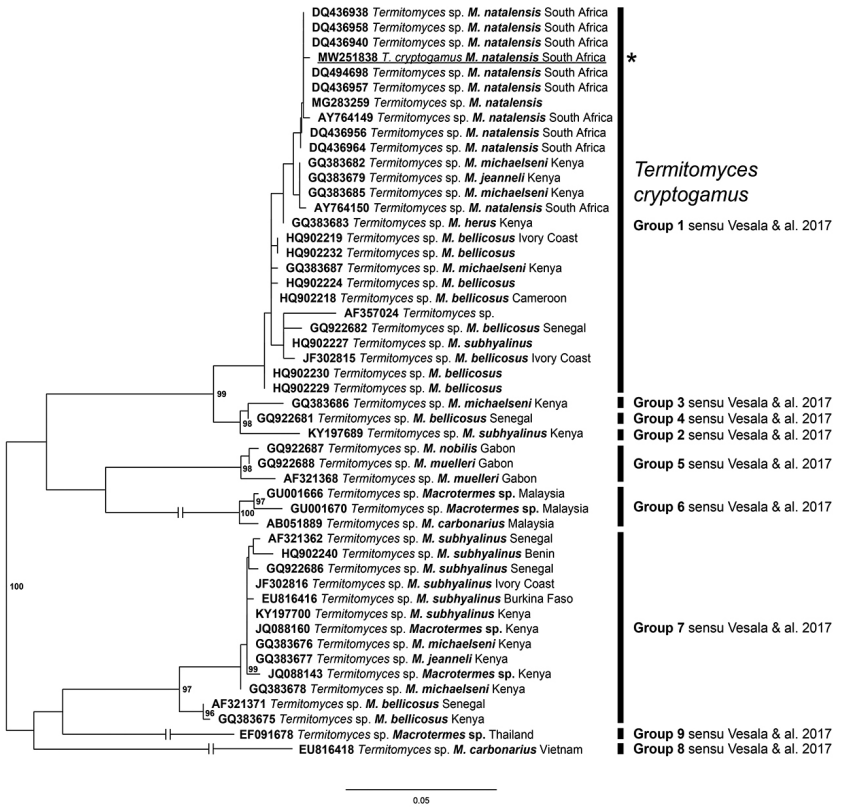

Fig. 2. Maximum likelihood tree based on ITS1-5.8S-ITS2 sequences of *Termitomyces cryptogamus* and allied *Macrotermes* symbionts with their host termite species (in bold) and their collection location. Outgroups were removed to increase readability of the tree. Numbers at the nodes indicate ultrafast bootstrap values, only significant node values >95% are displayed. Species delimitation groups with 97% ITS identity (sensu Vesala & al. 2017) are displayed on the right side of the tree. A sequence of the *T. cryptogamus* ex-type P5 is underlined and indicated by an asterisk.

We were able to amplify LSU sequences from both *T. cryptogamus* and herbarium samples of *T. schimperi* (PREM41964), which were then analysed together with other GenBank LSU sequences of *T. schimperi* and unidentified *M. natalensis* symbionts (Fig. 3). The LSU sequence of our P5 isolate was identical to that from a fungal symbiont of a *M. natalensis* mound. LSU sequences from the commonly recovered *Macrotermes* symbiont, *T. schimperi*, were not monophyletic.

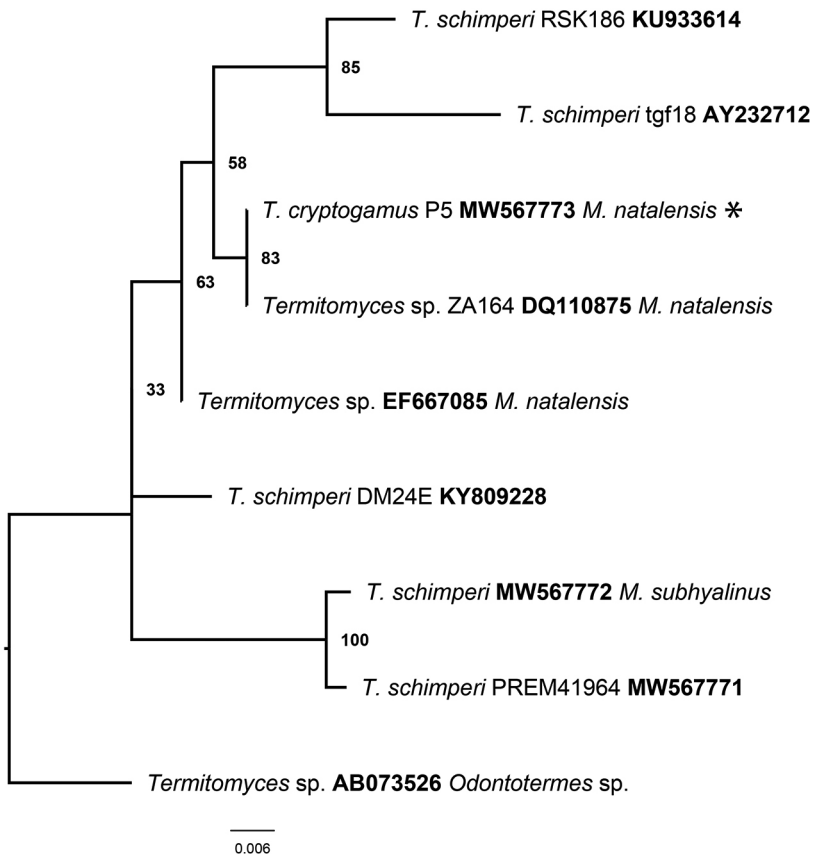

Fig. 3. Phylogenetic tree based on LSU sequences for *Termitomyces cryptogamus*. and publicly available LSU sequences of *T. schimperi*, a common *Macrotermes* symbiont. Sequence of AB073526 used as an outgroup to root the tree. Node values indicate bootstrap support.

Microscopical investigations revealed highly variable morphology of conidia harvested from laboratory grown cultures (Fig. 1c–h), which limits morphological comparison with asexual cultures of other *Termitomyces* species.

Discussion

There is accumulating evidence, both direct and indirect, that sexual reproduction does occur in *T. cryptogamus*. First, sexual reproduction between strains associated with *M. natalensis* was inferred as occurring

sufficiently frequently (at least 100 sexual events per generation) to explain the observed signature of free recombination (de Fine Licht & al. 2006). Second, mating tests between homokaryons retrieved from heterokaryons demonstrated that the *M. natalensis*-associated strains represent a single biological species (Nobre & al. 2014). Finally, strains associated with *M. natalensis* were observed to produce mushrooms and viable basidiospores in vitro (de Fine Licht & al. 2005; Vreeburg & al. 2020). These findings make it all the more surprising that mushrooms have not been found in nature. One hypothesis is that sexual reproduction of this species occurs belowground synchronously with alate dispersal.

Nevertheless, recovery of in-vitro basidiocarps only after prolonged laboratory incubation prevents their use as a morphological type specimen, as presumably incubation conditions greatly influence the resulting morphology. Additionally, the infrequency and unpredictability of these mushroom primordia in nests precludes their use for identification. Further, the asexual spores produced are highly polymorphic, and thus cannot be used for reliable identification with this group. As such, although comparisons of asexual cultures remain valuable, molecular markers provide the only reliable way to identify samples (Lücking & al. 2020). Based on the work of Makonde & al. 2013, it is likely that other *Termitomyces* species exist where the only practical identification markers will be molecular.

Using the 3% ITS similarity threshold, we find one lineage (group 1, sensu Vesala & al. 2017) that is distributed across Africa, with hosts differing geographically, but always within *Macrotermes*. The different termite symbionts combined with geographic isolation may indicate barriers to gene flow. Further study should show whether or not geographically separated populations of *T. cryptogamus,* including populations associated with *Macrotermes* species other than *M. natalensis,* all form a single biological species.

Although comparisons of ITS sequences from *T. cryptogamus* and *T. schimperi* are not currently available, we feel these sequences represent at least two species for two reasons. Firstly, the fact that we are unable to amplify the ITS regions successfully using the ITS1F and ITS4 primers, while we can amplify the LSU sequence indicates that there are likely mutations in the primer binding site not found in *T. cryptogamus* (for which ITS sequences are readily amplified). This suggests that *T. schimperi* likely has fixed substitutions not shared with *T. cryptogamus*. Secondly, the relationships between the LSU sequences generated from *T. schimperi* and *T. cryptogamus*

(Fig. 3) show significant genetic difference, although the backbone nodes of the phylogeny do not receive statistical support of bootstrap values greater than 70. Additionally, the recovery of two clades of *T. schimperi* indicates that *T. schimperi* is potentially paraphyletic and deserves further study.

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Literature cited

Botha WJ, Eicker A. 1991a. Cultural studies on the genus *Termitomyces* in South Africa. I. Macro- and microscopic characters of basidiome context cultures. Mycological Research 95(4): 435–443. https://doi.org/10.1016/S0953-7562(09)80843-5

Botha WJ, Eicker A. 1991b. Cultural studies on the genus *Termitomyces* in South Africa. II. Macro- and micromorphology of comb sporodochia. Mycological Research 95(4): 444–451. https://doi.org/10.1016/S0953-7562(09)80844-7

de Fine Licht HH, Andersen A, Aanen DK. 2005. *Termitomyces* sp. associated with the termite *Macrotermes natalensis* has a heterothallic mating system and multinucleate cells. Mycological Research 109(3): 314–318. https://doi.org/10.1017/S0953756204001844

de Fine Licht HH, Boomsma JJ, Aanen DK. 2006. Presumptive horizontal symbiont transmission in the fungus-growing termite *Macrotermes natalensis*. Molecular Ecology 15(11): 3131–3138. https://doi.org/10.1111/j.1365-294X.2006.03008.x

Frøslev TG, Aanen DK, Laessøe T, Rosendahl S. 2003. Phylogenetic relationships of *Termitomyces* and related taxa. Mycological Research 107(11): 1277–1286. https://doi.org/10.1017/S0953756203008670

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2): 113–118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Koné NA, Dosso K, Konaté S, Kouadio JY, Linsenmair KE. 2011. Environmental and biological determinants of *Termitomyces* species seasonal fructification in central and southern Côte d'Ivoire. Insectes Sociaux 58(3): 371–382. https://doi.org/10.1007/s00040-011-0154-1

Kües U, Navarro-González M. 2015. How do agaricomycetes shape their fruiting bodies? 1. Morphological aspects of development. Fungal Biology Reviews 29(2): 63-97. https://doi.org/10.1016/j.fbr.2015.05.001

Lücking R, Aime MC, Robbertse B, Miller AN, Ariyawansa HA, Aoki T, Cardinali G & al. 2020. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA fungus 11(14): [32 p.]. https://doi.org/10.1186/s43008-020-00033-z

Makonde HM, Boga HI, Osiemo Z, Mwirichia R, Stielow JB, Göker M, Klenk HP. 2013. Diversity of *Termitomyces* associated with fungus-farming termites assessed by cultural and culture-independent methods. PLoS ONE 8(2): e56464. https://doi.org/10.1371/journal.pone.0056464

Mossebo DC, Essouman EPF, Machouart M C, Gueidan C. 2017. Phylogenetic relationships, taxonomic revision and new taxa of *Termitomyces* (*Lyophyllaceae*, *Basidiomycota*) inferred from combined nLSU-and mtSSU-rDNA sequences. Phytotaxa 321(1): 71–102. https://doi.org/10.11646/phytotaxa.321.1.3

Nobre T, Fernandes C, Boomsma JJ, Korb J, Aanen DK. 2011. Farming termites determine the genetic population structure of *Termitomyces* fungal symbionts. Molecular Ecology 20(9): 2023–2033. https://doi.org/10.1111/j.1365-294X.2011.05064.x

Nobre T, Koopmanschap B, Baars JJP, Sonnenberg ASM, Aanen DK. 2014 The scope for nuclear selection within *Termitomyces* fungi associated with fungus-growing termites is limited. BMC Evolutionary Biology 14(121): [12 p.]. https://doi.org/10.1186/1471-2148-14-121

Poulsen M, Hu HF, Li C, Chen ZS, Xu LH, Otani A, Nygaard S, Nobre T et al. 2014 Complementary symbiont contributions to plant decomposition in a fungus-farming termite. Proceedings of the National Academy of Sciences of the United States of America 111(40): 14500–14505. https://doi.org/10.1073/pnas.1319718111

Tibuhwa DD. 2012. *Termitomyces* species from Tanzania, their cultural properties and unequalled basidiospores. Journal of Biology and Life Science 3(1): 140–159. https://doi.org/10.5296/jbls.v3i1.1723

Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Research 44(W1): W232–W235. https://doi.org/10.1093/nar/gkw256

Vesala R, Niskanen T, Liimatainen K, Boga H, Pellikka P, Rikkinen J. 2017. Diversity of fungus-growing termites (*Macrotermes*) and their fungal symbionts (*Termitomyces*) in the semiarid Tsavo Ecosystem, Kenya. Biotropica 49(3): 402-412. https://doi.org/10.1111/btp.12422

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172(8): 4238–4246. https://doi.org/10.1128/JB.172.8.4238-4246.1990

Vreeburg SME, de Ruijter NCA, Zwaan BJ, da Costa RR, Poulsen M, Aanen DK. 2020. Asexual and sexual reproduction are two separate developmental pathways in a *Termitomyces* species. Biology Letters 16(20200394): [5 p.]. https://doi.org/10.1098/rsbl.2020.0394

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis & al. (eds). PCR protocols: a guide to methods and applications. Academic Press, San Diego CA. https://doi.org/10.1016/B978-0-12-372180-8.50042-1