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Termitomyces sp. associated with the termite *Macrotermes natalensis* has a heterothallic mating system and multinucleate cells

Henrik H. DE FINE LICHT, Anders ANDERSEN† and Duur K. AANEN*

Department of Population Biology, Biological Institute, University of Copenhagen, DK-2100 Copenhagen O, Denmark.
E-mail : dkaanen@bi.ku.dk

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Fungi of the genus *Termitomyces* live in an obligate symbiosis with termites of the subfamily *Macrotermitinae*. Many species of *Termitomyces* frequently form fruit bodies, which develop from the fungus comb within the nest. In this study, we determined the mating system of a species of *Termitomyces* associated with the South African termite *Macrotermes natalensis*. Termite nests were excavated and a *Termitomyces* sp. was isolated into pure culture from the asexual fruit bodies (nodules) growing in the fungus gardens. For one strain, single basidiospore cultures were obtained from basidiomes growing from the fungus comb after incubation without termites. Using nuclear staining, we show that both comb cultures and single spore cultures have multinucleate cells and that the majority of spores has a single nucleus. However, DNA sequencing of the ITS region in the nuclear RNA gene revealed that the comb mycelium had two different ITS types that segregated in the single spore cultures, which consequently had only a single ITS type. These results unambiguously prove that the strain of *Termitomyces* studied here has a heterothallic mating system, with the fungus garden of the termite mound being in the heterokaryotic phase. This is the first time the mating system of a *Termitomyces* species has been studied.

INTRODUCTION

Fungi of the genus *Termitomyces* (*Termitomyceteae*: *Tricholomataceae*: *Basidiomycota*) live in an obligate mutualistic symbiosis with termites of the subfamily *Macrotermitinae*. The termites cultivate the fungus on special structures within the nest called fungus combs. These fungus combs are continuously provided with externally derived plant material (e.g. wood, dry grass, leaf litter), while the older parts, consisting of partially degraded plant material and fungal mycelium and nodules (asexual fruit bodies covered with conidia) are consumed (Batra & Batra 1979, Rouland-Lefevre 2000, Aanen *et al*. 2002). All species in the genus *Termitomyces* are completely dependent on the termites since they have never been found free-living and they are rapidly overgrown by other fungi, when removed from the termite nest (Darlington 1994). In turn, the termites are apparently completely dependent on *Termitomyces* fungi, since they have never been found without *Termitomyces* and experiments have shown that they are unable to survive without the fungus (Sands 1956, Grassé 1959, Rouland-Lefèvre 2000).

Mushrooms (basidiomes) of *Termitomyces* species are commonly observed on termite mounds and are collected by humans as food (Darlington 1994) but the mating systems of *Termitomyces* species have not been studied (Korb & Aanen 2003). This is partly due to difficulties in obtaining single spore cultures *via* spore germination. Moreover, in contrast to most other higher basidiomycetes, *Termitomyces* species do not form clamp connections between adjacent cells (Heim 1977), which makes a heterokaryon not easily distinguishable from a homokaryon. Most basidiomycetes have a heterothallic mating system (Burnett 2003). With a heterothallic mating system, a single haploid basidiospore germinates to form a haploid mycelium, the homokaryon. This mycelium usually has a single nucleus per cell (i.e. a monokaryon), but sometimes multiple identical nuclei are present within a single cell (Kües 2000). Contact between two compatible homokaryons is followed by cell fusions and reciprocal nuclear exchange and migration through both mycelia, so that all cells in this heterokaryon have two nuclei (i.e. a dikaryon), one from each component monokaryon. The heterokaryon can give rise to sexual fruit

* Corresponding author.
† Current address: Unit of Gastrointestinal Infections, Department of Bacteriology, Mycology and Parasitology (ABMP) Statens Serum Institut, DK-2300 Copenhagen S, Denmark.

Table 1. *Termitomyces* sp. isolates from South Africa *Macrotermes* samples used in this study.

Fungus	Termite	Location	Date, Collector	DAPI	ITS sequence	GenBank accession no.
Comb cultures						
ZA4	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+		
ZA8	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+	Double	
ZA15	*M. natalensis*	Pietersburg	Jan. 2003, D.K.A.	+		
ZA33	*M. natalensis*	Pietersburg	Jan. 2003, D.K.A.	+		
Monospore cultures						
ZA8–6	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+	Type 2	AY764150
ZA8–7	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+	Type 2	AY764150
ZA8–16	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+	Type 2	AY764150
ZA8–18	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+		
ZA8–21	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.		Type 1	AY764149
ZA8–22	*M. natalensis*	Pretoria	Jan. 2003, D.K.A.	+	Type 1	AY764149
Spores						
ZA120	*M. natalensis*	Pretoria	Jan. 2004, H.H.d.F.L.	+		

bodies where the basidia develop in which the nuclei fuse and enter a short diploid stage before they undergo meiosis (Carlile, Watkinson & Gooday 2001, Burnett 2003). A minority of basidiomycetes have homothallic, secondarily homothallic or amphithallic (a mixture of secondarily homothallic and heterothallic; Petersen 1995) mating systems (Lamoure 1989). In (secondarily) homothallic mating systems no pairing between homokaryotic mycelia occurs and a single spore can complete the life-cycle.

Macrotermes natalensis is the most widespread termite species of southern Africa and a major pest to buildings and plantations in the region (Uys 2002). Like the other members of *Macrotermes*, this species often builds conspicuous termite mounds in the landscape.

In this study we examine single spore cultures and mycelium isolated from nodules of the fungus comb of *M. natalensis*, to determine whether its symbiotic *Termitomyces* species has a heterothallic or (secondarily) homothallic mating system. We use epifluorescence microscopy to determine the number of nuclei in the cells of the growing mycelium. Molecular DNA techniques are applied to determine whether somatic cells with multiple nuclei are hetero- or homokaryotic. To our knowledge this is the first time the mating system of a species of *Termitomyces* has been studied.

MATERIALS AND METHODS

Fungal isolates

Samples of *Termitomyces* sp. were collected by D.K.A. and H.H.d.F.L. in January 2003 and 2004 from termite mounds near Pretoria and Pietersburg, South Africa (Table 1). Comb material was excavated as carefully as possible and transferred to the laboratory in plastic bags. *Termitomyces* was isolated in pure culture by aseptically (in a fume cupboard, using sterile forceps) placing four nodules per colony on malt yeast extract agar Petri dish (MYA; 20 gl^{-1} malt extract, 2 gl^{-1} yeast extract, and 15 gl^{-1} agar). The plates were inspected daily and when a mycelium developed from a nodule without contamination, this mycelium was further subcultured on a new plate. Parts of the comb were placed in closed glass containers of approximately $7 \times 7 \times 10$ cm and incubated (without termites) in the dark at 25 °C. A high humidity was maintained by placing a wet tissue paper inside the glass container. In most cases stromata of the ascomycete genus *Xylaria* quickly developed, but in one case the nodules developed into sexual fruit bodies. Basidiospore prints of these mushrooms were directly made on MYA Petri dishes by connecting pilei to the lid with Vaseline for periods ranging from five minutes to several hours to obtain different basidiospore densities. Agar plates were incubated in the dark at 25 ° and inspected daily using a stereomicroscope. Only a very small proportion of the basidiospores germinated. Single basidiospore cultures (further referred to as to single spore cultures) were isolated on new MYA plates. Additionally, the single spore cultures were also set up with a sterile coverslip between two blocks of mycelia to facilitate growth on the coverslip for microscopic purposes. Similarly, spore prints from the basidiomes were made on sterile empty Petri dishes for later staining and microscopy of the spores. Dried fruit bodies have been deposited in the herbarium of the Botanical Museum, University of Copenhagen (accession no. C 66585).

Fluorescence microscopy

From the fungal media with a coverslip, the coverslip was removed and turned 180 ° so that the upper side was facing downwards. The coverslip with mycelium attached to the surface was subsequently placed on a droplet of DAPI solution (4,6-diamidino-2-phenylindole dihydrochloride; stock solution: 2 mg DAPI dissolved in 1 ml DMSO (dimethyl sulfoxid); work solution: 2 µl stock solution mixed with 1 ml 0.1 M Na-phosphate buffer (pH 7.0), yielding a final concentration of 2µg ml^{-1}). DAPI binds selectively to AT-rich

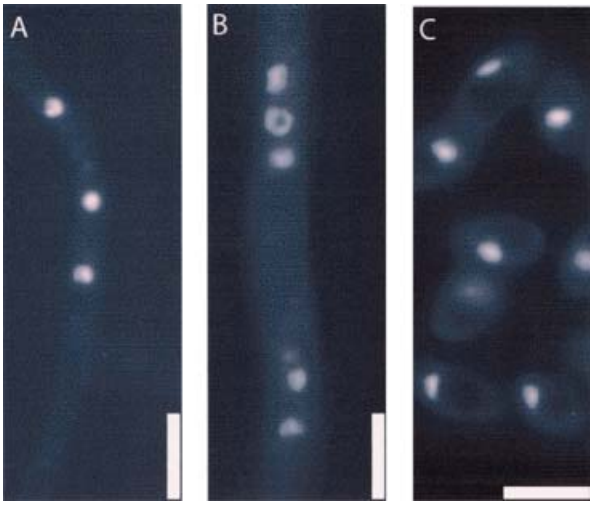

Fig. 1. Nuclear distribution in the fungus *Termitomyces* sp. from *Macrotermes natalensis* (A–B) and in basidiospores of a basidiome from the mound of the termite *Odontotermes* sp. (C). (A) Nuclear staining with DAPI shows three nuclei in a cell (septa outside photograph) of a monospore culture (ID#ZA8). (B) The segment of a hypha from a comb culture (ZA33) shows five nuclei (septa outside photograph). (C) The basidiospores showing one nucleus in each spore. Bar = 5 μm.

regions in double stranded DNA (Meixner & Bresinsky 1988, Butt *et al*. 1989) and nuclei thus become fluorescent and are visible using fluorescence microscopy. From the fungal media without a coverslip, a small amount of arial mycelium was scraped off and immediately placed in a droplet of DAPI solution. Slides were examined on a Leica Leitz laborlux S microscope equipped for epifluorescence with a Leica D filter setup (BP 355–425, RKP455 & LP460). Pictures were taken with a Coolsnap camera (RS photometrics) and Coolsnap vers. 1.2 for PC (Roper Scientific, Tucson, AZ) attached to the microscope.

Five single spore cultures, four comb cultures, and dried spores from a basidiome were used (Table 1).

DNA sequence analysis

DNA was purified using a DNeasy® Plant Mini Kit (cat. no. 69104, Qiagen, Hilden) from five single spore cultures and the original comb culture, all from the same fungus comb (Table 1). The highly polymorphic internal transcribed spacer (ITS) region between the 18 S and the 25 S nuclear RNA gene was amplified using the primer pair ITS1 (5′ tccgtaggtgaacctgcgg 3′) and ITS4 (5′ tcctccgcttattgatatgc 3′) (White *et al*. 1990). PCR reactions (using AmpliTaq gold; Applied Biosystems, Foster City, CA) consisted of an initial denaturing step of 10 min at 95 ° followed by 35 cycles (30 s at 95 °, 30 s at 57 ° and 30 s at 72 °) finished by a final elongation step of 5 min at 72 °. The samples were purified with Qiagen DNA purification kit and sequenced by MWG – Biotech, Germany. Sequences were aligned and analyzed with Sequencer 3.1.1 for Macintosh. DNA sequences have been deposited to GenBank (accession nos. AY764150, and AY764149).

RESULTS

Fluorescence microscopy

It was possible to distinguish the number of nuclei in the cells by comparing the fluorescence image showing the nuclei and the normal bright field image showing the cell walls and septa between adjacent cells. Both comb cultures and single spore cultures had multinucleate cells with varying numbers of nuclei in each cell. Nuclear numbers per cell ranged from one to six for the single spore cultures ($n = 36$; Fig. 1). The comb cultures showed the same pattern, but with up to 12 nuclei in a single cell ($n = 36$). Empty cells with no visible nucleus were present in both single spore and comb cultures. There was thus no clear difference between the single spore cultures and the comb cultures in the number of nuclei per cell, although we did not quantify the exact numbers of nuclei in the multinucleate cells. Fluorescence staining of the basidiospores revealed a single nucleus per spore in ∼99 % of the stained spores ($n = 83$). A single spore (∼1 %) contained two nuclei.

DNA Sequence analysis

Visual inspection of the sequence-chromatograms showed that the comb culture contained two different sequences, differing by an AT length mutation (Fig. 2). These two ITS types segregated into the five studied single spore offspring as these contained only a single ITS type, with two cultures having the one type and

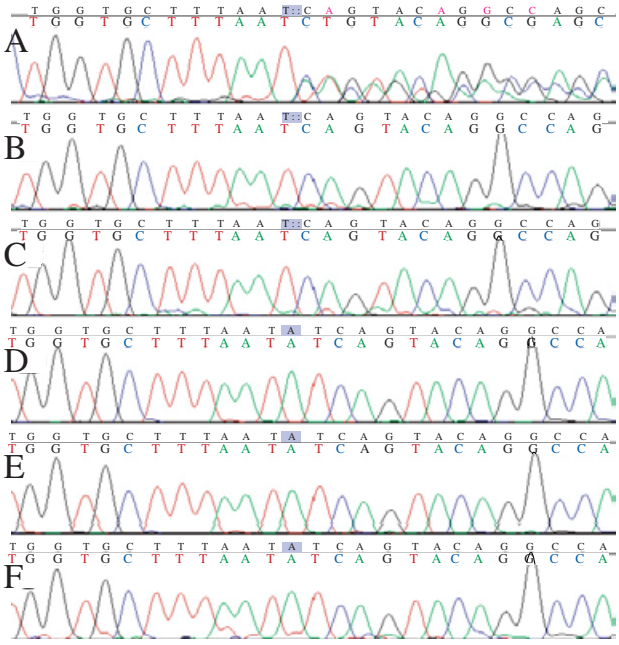

Fig. 2. A section of the 600 bp ITS region between the 18 S and the 25 S nuclear RNA gene. (A) DNA from the ITS sequence of the heterokaryotic comb culture. From the central C and onwards to the right the chromatogram has double peaks. (B–F) The ITS sequence of five single spore cultures showing a single sequence of nuclear genes with (B–C) having type 1 and (D–F) having type 2.

three cultures having the other. We therefore conclude that the comb culture is heterokaryotic, while the single spore cultures are homokaryotic. These findings are direct evidence for a heterothallic mating system of *Termitomyces sp*. with two vegetative phases, the homokaryon and the heterokaryon. Blast searches (http://www.ncbi.nlm.nih.gov/BLAST/) of the sequences gave the highest fit with an unidentified *Termitomyces* symbiont collected from *Macrotermes subhyalinus* (accession no. AF357024).

DISCUSSION

The results of this study unambiguously show that the *Termitomyces* strain studied here, associated with the termite *Macrotermes natalensis*, has a heterothallic mating system with a homokaryotic and a heterokaryotic phase. In contrast to the majority of heterothallic basidiomycetes, both the homokaryon and the heterokaryon of this *Termitomyces* have multinucleate cells. However, multinucleate cells are not uncommon in basidiomycetes (Lamoure 1989). Other examples are *Heterobasidion annosum* (Stenlid & Rayner 1991) and the genus *Stereum* (Ainsworth 1987, Burnett 2003, Calderoni, Sieber & Holdenrieder 2003). Because the single spore cultures and comb cultures could not be distinguished on morphological criteria, we had to use molecular methods. We made use of an intra-strain ITS polymorphism to distinguish between the primary, homokaryotic, and the secondary, heterokaryotic phases. That the ITS polymorphism segregated in the single spore offspring unambiguously showed that the ITS variation was between nuclei and not between the ribosomal repeats within a nucleus. If the mating system were (secondarily) homothallic both ITS sequences would be inherited in the progeny, and they would not segregate as we show. Such ITS variation between nuclei (in heterokaryons), but not within nuclei (in homokaryons), has been found before in basidiomycetes (e.g. Aanen, Kuyper & Hoekstra 2001). In contrast, evidence for between repeat variation within nuclei, but not between nuclei, has recently been found in arbuscular mycorrhizal fungi (Pawlowska & Taylor 2004).

Our study does not reveal any information on the regular transmission mode of the *Termitomyces* sp. symbiont of *M. natalensis*. The few studies on symbiont transmission modes for other species indicate that most termite species rely on horizontal acquisition of *Termitomyces* species, and probably this usually means the acquisition of fungal spores (Korb & Aanen 2003). For (secondarily) homothallic species a single spore would be sufficient to complete the fungal life cycle, but for a heterothallic species like the *Termitomyces* symbiont of *M. natalensis* in this study, two compatible spores ultimately need to colonize a nest. We found a low percentage of spores with two nuclei and we found the same pattern for the spores of a *Termitomyces* sp. symbiont associated with a termite of the genus *Odontotermes* (data not shown). The nuclear composition of these spores was not studied further here. If the extra nucleus is the result of an additional mitotic division, as has been found in several species (e.g. in *Hebeloma*, Aanen & Kuyper 1999), it has no consequences for the mating system. However, if the two nuclei have compatible mating types, this would mean that a small percentage of the spores do not require outcrossing to complete the sexual life cycle (Petersen 1995).

Vertical transmission for *M. natalensis* would be the clonal propagation of fungal symbionts from a termite colony to the offspring of this colony. In the related species *M. bellicosus*, it has been shown that such vertical transmission is the main transmission mode, whereby asexual fungal spores are carried in the crop of the male reproductives (Johnson *et al*. 1981). In dissected individuals of *M. natalensis* we never found any contents in the crop of male reproductives, and only in two female reproductives ($n = 30$, data not shown). This suggests that *M. natalensis* does not have a mechanism of vertical inheritance of fungal symbionts, but instead relies on horizontal acquisition.

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