

Phylogenetic Relationships of the Mutualistic Fungi Associated with *Macrotermes subhyalinus* in Oman

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ABSTRACT

The symbiotic association between fungus-gardening termites *Macrotermes* and its fungal symbiont has a moderate degree of specificity—although the symbiotic fungi (*Termitomyces*) form a monophyletic clade, there is not a one-to-one association between termite species and their fungus-garden associates. Here, we aim to determine the origin and phylogenetic relationships of *Termitomyces* in Oman. We used sequences of the internal transcribed spacer region (ITS) and the nuclear large subunit ribosomal RNA (LSU rRNA, 25S) gene and analyzed these with sequences of *Termitomyces* from other geographic areas. We find no evidence for more than a single colonization of Oman by *Termitomyces*. Unexpectedly, we find *Termitomyces* in Oman is most closely related to the symbiont of *M. subhyalinus* in West Africa rather than to those of geographically closer populations in East Africa.

ARTICLE HISTORY

Received 21 October 2020
Revised 7 September 2023
Accepted 11 September 2023

KEYWORDS

Biogeography; fungal comb; ITS; 25S rDNA; Oman

1. Introduction

Fungus-gardening termites (Termitidae: Macrotermitinae) have obligate symbioses with the fungus *Termitomyces* (Basidiomycete: Agaricales: Lyophyllaceae). Fungus gardens inside the termite nest are provided with buffered temperature and humidity conditions, and the fungus provides food for its host termites. Termitophilic fungi are cultivated on plant-based substrates, called “fungus combs” (Figure 1), made of termite fecal matter from pre-digested plant parts (e.g. leaf litter, wood and grass) contained in special chambers inside the termite nest [1,2]. The fungus produces nodules, vegetative spherical growths about 0.1–2 mm in diameter (Figure 1), on the comb surface that serve as a food source for termite workers [3].


Unlike the fungi cultivated by attine ants, in which some species of fungus have close free-living close relatives [4–7], no *Termitomyces* are known to have a free-living state and they form a monophyletic group in phylogenetic analyses [8]. Fungus-gardening termites (Macrotermitinae) also form a monophyletic group [9–11] and all species cultivate *Termitomyces* as a food source [12]. Together, these observations imply a single origin of the termite-fungus mutualism: the relationship between termites and their cultivated fungus is symmetrical in that both partners have a single origin with no reversal

to a non-symbiotic state, and both partners are obligatorily dependent on this relationship [1,8].

The evolutionary relationships between fungus-gardening termites and their mutualistic fungi have yet to be fully resolved, partly due to their high complexity. For example, even in cases of vertical transmission where fungi are thought to be inherited from parental colonies, the symbiotic fungi used by a particular termite lineage do not necessarily form a monophyletic group [8,13,14]. For example, *Macrotermes bellicosus* has associations with four different lineages of *Termitomyces* that do not form a clade [8,15]. The pattern is consistent with horizontal transfer of the fungus between species [15]. In contrast, high specificity is found in some other lineages despite the potential for horizontal transfer when spores are collected from the surrounding environment for inoculating the comb. For example, *M. natalensis* is associated with a single symbiont lineage of *Termitomyces* [16,17]. Overall, fungus-gardening termites show a range of specificity with respect to their *Termitomyces* symbionts, and apparent transmission mode does not unambiguously predict host-symbiont specificity.

To date, the *Termitomyces* association with *M. subhyalinus* in Oman have not been included in studies of the fungus-termite associations. *Macrotermes*

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 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/12298093.2023.2258623>.

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Figure 1. Fungus comb in fungus garden of *Macrotermes subhyalinus* in Oman, yellow arrow showing fungus nodules. Photo by Hilal Al-Shamakhi.

subhyalinus occurs in the Dhofar region of southern Oman, a local center of endemism [18], where it forms mounds of up to 2 m in diameter. Here, we sample populations of *Termitomyces* from across the distribution of *M. subhyalinus* in Oman to assess its diversity and to determine its phylogenetic relationship with *Termitomyces* from other regions.

2. Materials and methods

2.1. Specimen sampling

Fungal combs were collected from 76 colonies of *M. subhyalinus* from across Dhofar, southern Oman (Figure 2; Supplementary Material Table S1) during June and July 2015. Nodules were separated from fungal combs and surface washed in 70% ethanol, then preserved in 99% ethanol until extraction of genomic DNA.

2.2. DNA extraction

A single nodule (2–3 mm) was selected from each of several combs in each colony. Genomic DNA was extracted by grinding the samples from one nest manually using a pestle in 600 μ l 2 M CTAB buffer and 10 μ l Proteinase K. The mixture was then incubated on a hot plate for two hours at 55 °C before heating at 95 °C for 10 min. After cooling to room temperature, 300 μ l of chloroform was added and the mixture was gently rocked for 15 min, followed by micro-centrifuging at 15800 RCF for 5 min. The supernatant was removed to a new tube and 600 μ l of 100% isopropanol was added, vortexed and centrifuged at 15800 RCF for 10 min. After discarding the supernatant, the DNA pellet was washed with 500 μ l of 80% ethanol and centrifuged at 15800 RCF for 1 min. The supernatant was removed and the pellet was air dried before being re-suspended in

50 μ l sterile distilled water. To assess the integrity of the genomic DNA, 2 μ l of template DNA was run through a 1% agarose gel at constant voltage V400/A120 for 60 min and visualized under ultraviolet light.

2.3. Amplification and sequencing protocol

We amplified the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal RNA repeat regions and the D2-D3 expansion regions of the nuclear large subunit ribosomal RNA gene (LSU rRNA, 25S). About 670 bp of ITS was amplified with primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') [19] and PCR consisted of an initial denaturing step of 4 min at 94 °C, then 35 cycles of denaturing at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (90 s), followed by an additional extension at 72 °C (10 min). About 530 bp of the 25S rDNA D2-D3 region was amplified using the primers 25S4R (5'-ACAAGTGCTGAGTTC CTCAG-3', a specific primer) and ITS4R (5'-GCATATCAATAAGCGGAGGA-3' [19], and PCR used the same thermocycle program as that for ITS. PCR products were prepared for sequencing using Antarctic Phosphatase and Exonuclease I (New England BioLabs, Australia) or the MacroGen purification service, then sequenced at MacroGen Inc. (Republic of Korea) using BigDye chemistry (Applied Biosystems) and the Sanger sequencing method using the same primers as that used for the initial amplification. All PCR products were sequenced in both the forward and reverse directions.

2.4. Phylogenetic analysis

Sequences were edited in GENEIOUS R9 [20] and aligned using Multiple sequence Alignment based on Fast Fourier Transform (MAFFT v7.017) [21] with options L-INS-I (iterative refinement method incorporating local pairwise alignments), gap opening penalty = 1.5, 200PAM/K=2 scoring matrix, offset value = 0.123 for nucleotide sequences, then adjusted by eye in GENEIOUS so that indels were aligned.

For analysis of ITS, 75 sequences of fungus symbionts of fungus-gardening termite species of subfamily Macrotermitinae (representing 19 species and five genera) were included from Genbank, along with one related, non-gardened fungus species (*Blastoporella zonata*) as outgroup (Supplementary Material Table S1). For 25S, we used 63 sequences of the symbiotic fungus from Genbank. These sequences belong to 29 species and seven genera (Figure 3). The non-gardened

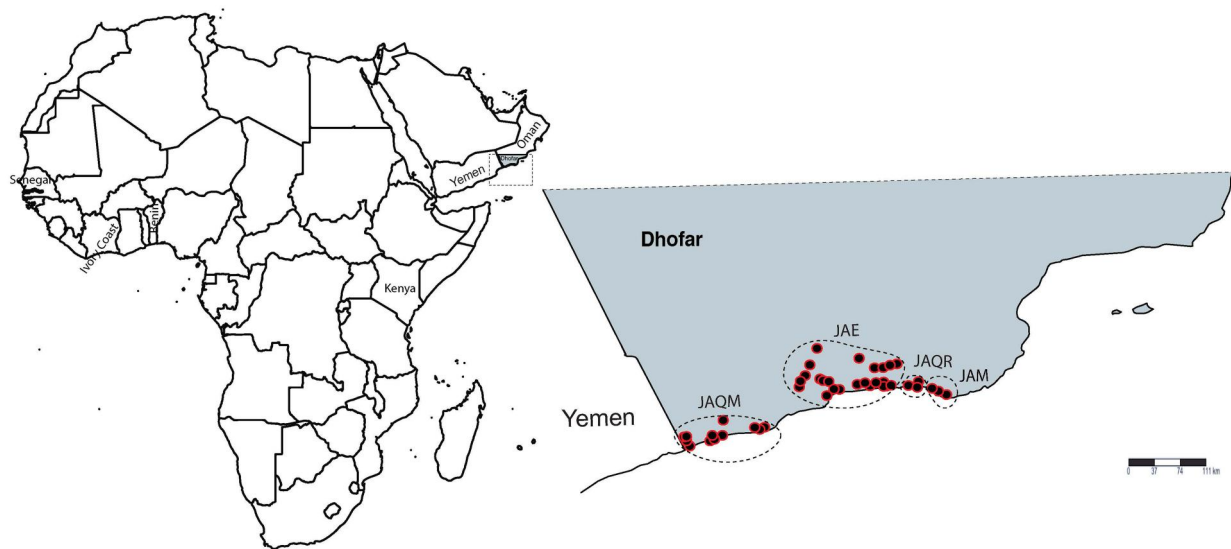


Figure 2. Map showing sampled localities across Dhofar region southern of Oman. (JAQM) Jabal qamar, (JAE) Jabal eitn, (JAQR) Jabal qara, and (JAM) Jabal murbat.

fungus species *Arthromyces claviformis* (EU708336) and *Arthromyces matolae* (EU708334) were included as outgroups. Although ITS and 25S sequences from Omani samples could have been concatenated because they represent a single functionally linked locus, this could not be done with Genbank data because the two DNA regions were sequenced from different studies using different specimens. This is also why the outgroups used for each DNA region were different.

Identical sequences from Omani samples were collapsed into a single representative prior to phylogenetic analysis to decrease computational intensity and over-representation of alleles in model estimation. Both ITS and 25S were tested for deviation from stationarity [22] using PAUP v4.0a146 [23]. Phylogenetic analyses were performed using two methods (maximum parsimony and Bayesian inference) because each has differing underlying assumptions, and congruence across methods increases confidence in results. These were also compared with a neighbor-joining dendrogram [24].

For neighbor-joining analyses, seeding was randomly initiated and the Hasegawa–Kishino–Yano (HKY) model [25], with a gamma shape parameter of 0.5 was used to calculate the genetic distances among alleles as identified as the best-fit evolutionary model for both ITS and 25S genes by JMODELTEST [26]. To evaluate support for nodes, fast stepwise addition bootstrapping using 1000 pseudoreplicates was used, and nodes with bootstrap values over 70% were regarded as supported (as per [27]).

Maximum parsimony searches were carried out using a heuristic search, 100 random addition replicates, tree bisection and reconnection (TBR) branch-swapping, all character positions weighted equally, gaps treated as missing data, and saving one

tree only at each step. The shortest trees saved were then used as starting trees in a subsequent heuristic search using TBR branch swapping and saving all most-parsimonious trees. This approach was adopted to maximize the number of replicates performed on a large data set and to overcome becoming "stuck" on only a few "tree islands". A strict consensus tree was generated from these MP trees. Nodes were evaluated using 1000 bootstrap pseudoreplicates using the full heuristic search option and the settings as before.

In order to efficiently select the most appropriate model of sequence evolution for Bayesian analyses we used JMODELTEST [26] and the Bayesian Information Criterion to select the best fitting model. This identified the Hasegawa–Kishino–Yano (HKY) [25] including a proportion of invariable sites (I), and gamma-distributed among-site rate variation (Γ). Bayesian inference with an outgroup was used in MrBayes v3.2.6 [28], sampling every 1000th generation of 10 million generations. Convergence of runs was assessed using Bayes Factors [29] ensuring that the harmonic mean of the two runs was less than 2, and checking ESS values using TRACER v1.6 [30]. Posterior probabilities ≥ 0.95 were considered as significant, as per [31].

BEAST v1.8.2 [32] was also used to estimate a phylogeny of *Termitomyces* alone because it does not require an outgroup. Two separate runs of 10 million generations were performed, sampling every 1000 generations. Runs were checked for stationarity as per MrBayes. Logcombiner was used to combine the results of the two runs, and TreeAnnotator was used to find the MCC (max clade credibility) tree. The analyses were run at least twice under both Yule and Birth-Death process model in tree priors

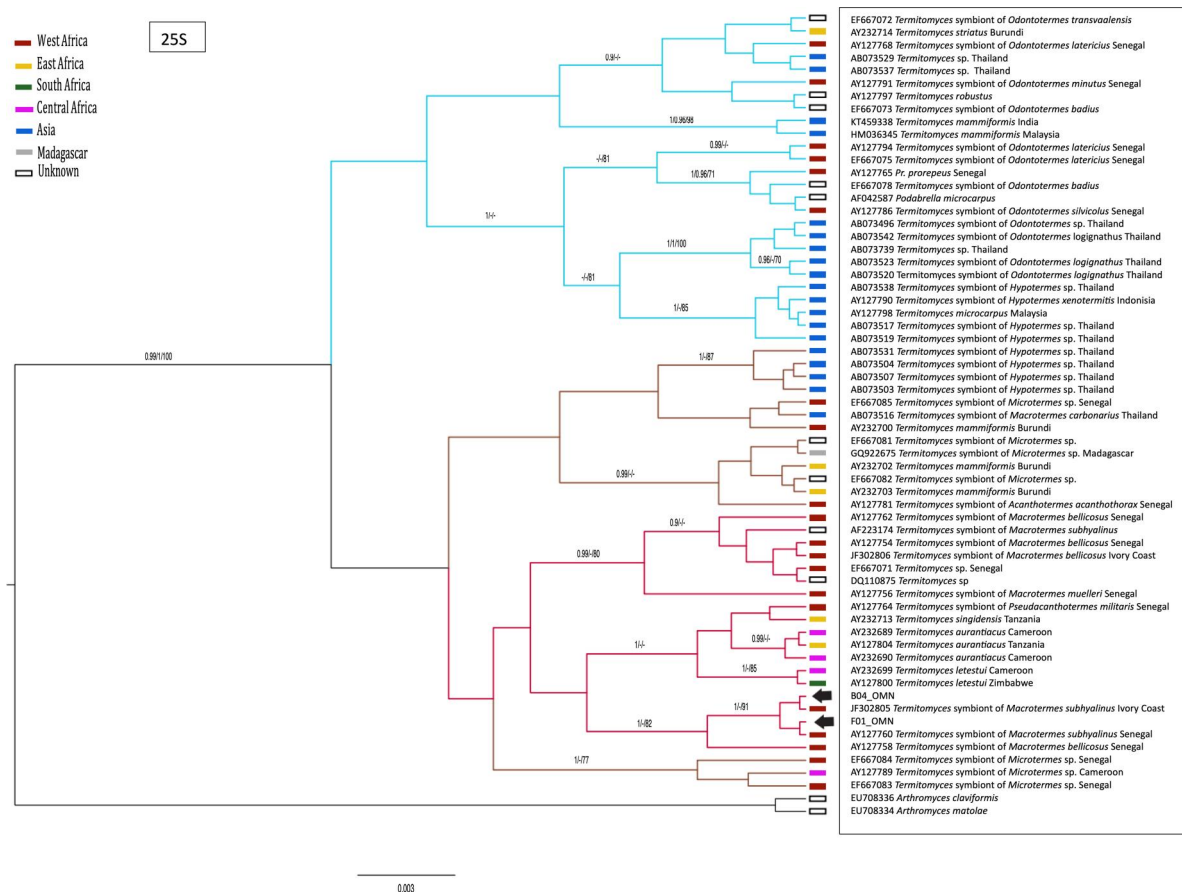


Figure 3. Phylogenetic relationships of the Omani populations of *Termitomyces* based on Bayesian analysis (BEAST) of the 25S gene. Support values are shown above nodes (MrBayes/BEAST/bootstraps support). Collection locality is indicated by colored bars next to tips. GenBank accession numbers correspond to those.

to ascertain the effect of these tree priors on the resulting estimates [33].

3. Results

There was no bias in base composition (non-stationarity) among alleles of each gene. Fungi from 61 of the 76 collection locations in Oman were successfully sequenced for ITS. All were identical and therefore only one representative sequence was used in further phylogenetic analyses. *Termitomyces* was generally grouped together based on their association with specific termite genera. However, there are some exceptions to this pattern. Some *Termitomyces* associated with termite genera like *Odontotermes*, *Microtermes*, *Pseudacanthotermes*, and *Cryptotrama* formed polyphyletic groups. In contrast, *Termitomyces* associated with the genus *Macrotermes* formed a monophyletic group. *Termitomyces* associated with different *Macrotermes* species did not make monophyletic groups, but they formed several sub-lineages. For example, *Termitomyces* associated with *Macrotermes bellicosus* appeared in two lineages. One of them included *Macrotermes bellicosus* from Senegal and Kenya and the other lineage included *Macrotermes bellicosus*

from Senegal, *Macrotermes michaelsoni* from Kenya, *Macrotermes herus* from Kenya, and *Macrotermes subhyalinus* from Benin. *Termitomyces* associated with *Macrotermes subhyalinus* appeared in two lineages. Most of them were included in a monophyletic lineage majorly consisted of *Macrotermes subhyalinus* from Senegal, Benin, Ivory Coast, and Kenya and *Macrotermes jeanneli* from Kenya, and *Macrotermes sp.* from Kenya. ITS sequences from *Termitomyces* samples collected in Oman were identical and showed 3 bp difference with the *Termitomyces* associated with *Macrotermes subhyalinus* from Benin. The Omani samples were nested within a monophyletic lineage consisting of *Termitomyces* associated with *Macrotermes subhyalinus* from West Africa. Separation of the lineage from the *Termitomyces* lineage associated with *Macrotermes subhyalinus*, *Macrotermes jeanneli*, and *Macrotermes sp.* from East Africa was weakly supported by bootstrap analysis. However, monophyly of them and sister relationship between the two lineages was maintained in maximum parsimony and Bayesian approaches.

The 25S gene was successfully amplified from 73 of the 76 fungal combs. There were multiple alleles present among Omani samples. Two alleles differed by a single nucleotide substitution, with 52 samples

Additional sampling of fungal combs across Africa may reveal new clades or many more examples of mixed clades, so it is not yet definitive that there are no Eastern African *Termitomyces* similar to those from Oman. An alternative explanation to insufficient sampling is that *Termitomyces* colonized Oman from Western Africa, either with the original colonization by *Macrotermes subhyalinus* or secondarily, replacing the original lineage of *Termitomyces*.

There is very little allelic diversity among the samples of *Termitomyces* from Oman, with the ITS region being identical across all samples sequenced. For both DNA regions (ITS and 25S), alleles sampled from Omani populations are nested among a diverse array of alleles sampled from Africa, indicating that *Termitomyces* in Oman is derived from African ancestry. That is, dispersal has occurred from Africa to Oman. The ITS allele and one allele of 25S from Oman were different from any sampled in Africa. If this differentiation is not due to insufficient sampling in Africa, it could indicate that Omani populations have been isolated for some time and evolved unique alleles. Nevertheless, the low diversity and nesting within clades of alleles from Africa indicate a single colonization event of Oman. More finely resolving genetic markers, such as SNPs derived from RADseq, are required to assess whether Omani populations show genetic structure consistent with long-term occupancy or whether they have only more recently arrived, potentially even within the human epoch.

5. Conclusion

Termitomyces symbionts of the fungus-gardening termites from Oman appear to share a more recent common ancestry with *Termitomyces* symbionts of *M. subhyalinus* from Western Africa than from Eastern Africa, but we cannot rule out alternative scenarios because of poor representation of other populations in Genbank. The phylogenetic pattern is consistent with dispersal to Oman from Africa. The lack of genetic diversity in Oman, and geographical contrasts in closest relative requires more investigation with more sampling from *Termitomyces* symbionts of *M. subhyalinus* is needed.

Author contributions

H. S. AlShamakhi was responsible for performing lab work and data analysis, interpreting the data, and drafting and writing the manuscript. L. G. Cook critically reviewed drafts and provided advice on analyses and interpretation of the data.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Royal Court Affairs – No Grant Number. Author H. S. Al Shamakhi has received research support from Royal Court Affairs.”

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Data availability statement

The datasets generated during and/or analyzed during the current study are available in the [Supplementary Materials](#).

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