Report

Current Biology

Ancestral predisposition toward a domesticated lifestyle in the termite-cultivated fungus *Termitomyces*

Highlights

- Insect-fecal associations predate the domestication of *Termitomyces* fungi
- A set of morphological traits predisposed lyophylloid fungi toward domestication
- Insect-associated lyophylloid fungi have reduced plant-degrading capabilities
- This symbiosis may have been facilitated by pre-adaptation of both partners

Authors

Lennart J.J. van de Peppel, Mathijs Nieuwenhuis, Benjamin Auxier, ..., Ana E. Franco-Molano, Timothy J. Baroni, Duur K. Aanen

Correspondence

lennartvdpeppel@gmail.com (L.J.J.v.d.P.), duur.aanen@wur.nl (D.K.A.)

In brief

How termites came to domesticate *Termitomyces* fungi is unknown. van de Peppel et al. identify a set of ecological, morphological, and genomic traits shared by domesticated *Termitomyces* and the insect-associated sister group *Arthromyces*. These may have served as the basis for domestication.

van de Peppel et al., 2021, Current Biology *31*, 4413–4421
October 11, 2021
https://doi.org/10.1016/j.cub.2021.07.070

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Report

Ancestral predisposition toward a domesticated lifestyle in the termite-cultivated fungus *Termitomyces*

Lennart J.J. van de Peppel,[1,10,*] Mathijs Nieuwenhuis,[1] Benjamin Auxier,[1] Alexey A. Grum-Grzhimaylo,[2] Martha E. Cárdenas,[3] Z. Wilhelm de Beer,[4] D. Jean Lodge,[5] Matthew E. Smith,[6] Thomas W. Kuyper,[7] Ana E. Franco-Molano,[8] Timothy J. Baroni,[9] and Duur K. Aanen[1,*]

[1]Laboratory of Genetics, Wageningen University & Research, Droevendaalsesteeg 1, 6708PB Wageningen, the Netherlands
[2]Microbial Ecology Department, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, the Netherlands
[3]Laboratorio de Micología y Fitopatología-LAMFU, Universidad de Los Andes, Carrera 1 # 10A-12, Bogotá, Colombia
[4]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa
[5]Department of Plant Pathology, 2105 Miller Plant Sciences Building, University of Georgia, Athens, GA 30606, USA
[6]Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
[7]Soil Biology Group, Wageningen University & Research, Box 47, 6700AA Wageningen, the Netherlands
[8]Laboratorio de Taxonomía y Ecología de Hongos (TEHO), Instituto de Biología, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia
[9]Department of Biological Sciences, State University of New York, College at Cortland, PO Box 2000, Cortland, NY 13045, USA
[10]Lead contact
*Correspondence: lennartvdpeppel@gmail.com (L.J.J.v.d.P.), duur.aanen@wur.nl (D.K.A.)
https://doi.org/10.1016/j.cub.2021.07.070

SUMMARY

The ancestor of termites relied on gut symbionts for degradation of plant material, an association that persists in all termite families.[1,2] However, the single-lineage Macrotermitinae has additionally acquired a fungal symbiont that complements digestion of food outside the termite gut.[3] Phylogenetic analysis has shown that fungi grown by these termites form a clade—the genus *Termitomyces*—but the events leading toward domestication remain unclear.[4] To address this, we reconstructed the lifestyle of the common ancestor of *Termitomyces* using a combination of ecological data with a phylogenomic analysis of 21 related non-domesticated species and 25 species of *Termitomyces*. We show that the closely related genera *Blastosporella* and *Arthromyces* also contain insect-associated species. Furthermore, the genus *Arthromyces* produces asexual spores on the mycelium, which may facilitate insect dispersal when growing on aggregated subterranean fecal pellets of a plant-feeding insect. The sister-group relationship between *Arthromyces* and *Termitomyces* implies that insect association and asexual sporulation, present in both genera, preceded the domestication of *Termitomyces* and did not follow domestication as has been proposed previously. Specialization of the common ancestor of these two genera on an insect-fecal substrate is further supported by similar carbohydrate-degrading profiles between *Arthromyces* and *Termitomyces*. We describe a set of traits that may have predisposed the ancestor of *Termitomyces* toward domestication, with each trait found scattered in related taxa outside of the termite-domesticated clade. This pattern indicates that the origin of the termite-fungus symbiosis may not have required large-scale changes of the fungal partner.

RESULTS AND DISCUSSION

Phylogenetic relationships of the termitomycetoid clade

The family Lyophyllaceae (Basidiomycota), to which *Termitomyces* belongs, harbors species with diverse ecologies, including saprotrophic, parasitic, and mutualistic lifestyles, with frequent transitions between them.[5] The genus *Termitomyces*, which engages in a mutualistic symbiosis with termites, presents an enigma on how such an intricate symbiosis could have evolved. Previous studies were unable to confidently identify the origin of this symbiosis due to a limited number of phylogenetic markers in the analyses or because closely related taxa were not included in the datasets.[5–8] To reveal the origin of the termite-*Termitomyces* symbiosis, using field collections and herbarium material we collected 39 samples of 11 genera spanning Lyophyllaceae. We obtained whole-genome sequences from these samples and added another seven publicly available assemblies. We reconstructed the phylogeny of these 46 taxa using 1,131 conserved nuclear genes (Figure 1A). The topologies generated by both coalescent-based ASTRAL analysis (Figure S1) and concatenation-based IQ-TREE analysis were in agreement. Therefore, we will focus on the IQ-TREE phylogeny for our discussion. As

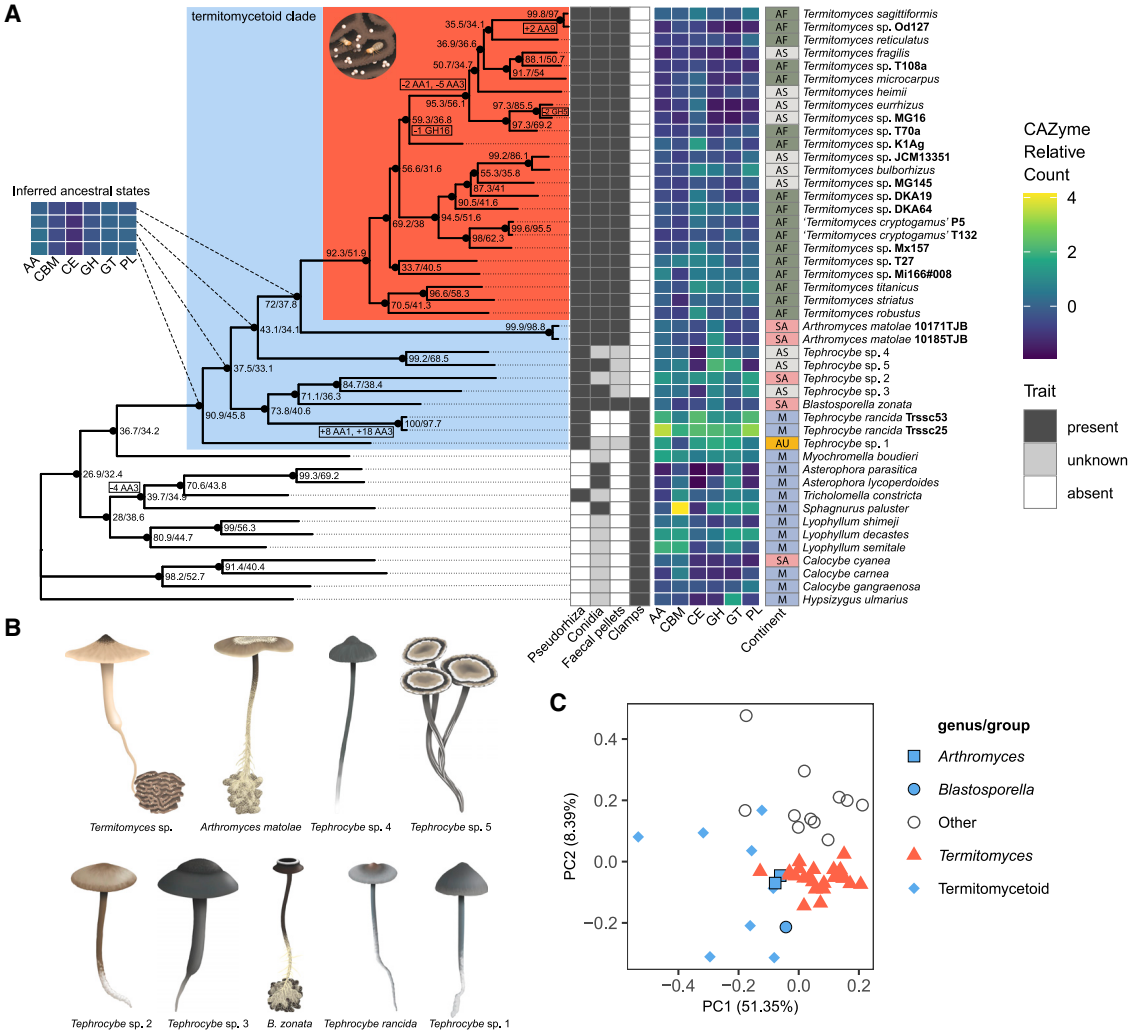

Figure 1. Phylogenomic and CAZyme analysis of the Lyophyllaceae showing that the insect-associated *Arthromyces* is sister to the genus *Termitomyces* and showing a reduction in CAZyme complement prior to the symbiosis

(A) Maximum-likelihood tree based on 1,131 core nuclear (BUSCO) genes. Numbers at the nodes represent gene concordance factor/site concordance factor, dots indicate 100% bootstrap support, and numbers in boxes indicate significant changes in CAZymes. *Hypsizygus ulmarius* was used as an outgroup. Termitomycetoid clade highlighted in blue; termite-associated species highlighted in red. The most parsimonious reconstruction of the origin of the pseudorhiza is in the most recent common ancestor of the sister group of *Tephrocybe* sp. 1 as indicated. Matrix of morphological and CAZyme data: the left section of the matrix contains the character states of the predisposition traits: a pseudorhiza, production of asexual spores (conidia), an insect-fecal association, and the presence of hyphal clamp connections. The right section of the matrix shows a scaled heatmap of the total number of CAZymes split into the six main biochemical groups (auxiliary activity [AA], carbohydrate-binding module [CBM], carbohydrate esterase [CE], glycoside hydrolase [GH], glycosyltransferase [GT], and polysaccharide lyase [PL]). Colors indicate the difference of a taxon's CAZyme content from the average of the group, in standard deviations. CAZyme data of selected ancestral nodes inferred by CAFE are indicated to the left of the phylogeny, using the same color scale as the main matrix. Significant changes in CAZymes not explained by the phylogeny are indicated in boxes next to the nodes. The column on the right indicates the continent on which each species occurs: Australia (AU), Africa (AF), Asia (AS), South America (SA), or multiple continents (M).

(B) Drawings of representative species in the termitomycetoid clade showing their overall morphology and the presence and/or absence of the predisposition traits.

(C) Principal component analysis of the same CAZyme data as in the CAZyme matrix except that the six main groups are split into 119 CAZyme subfamilies; numbers in parentheses indicate the percentage of variance explained by a principal component. Note that samples of *Termitomyces* (red triangles) and *Arthromyces* (blue squares) cluster together.

See also Figures S1 and S2.

high marker numbers inflate bootstrap support (BS),[9] we additionally calculated gene and site concordance factors (gCF and sCF; Figure 1A),[10,11] which respectively show the fraction of gene trees or informative alignment sites supporting each node. Although concordance factors cannot be used to statistically test whether a node is well supported by the data, they reflect the amount of ambiguity among loci for a given bifurcation.

Consistent with previous findings,[5,8] we recover a well-supported termitomycetoid clade, which consists of the genera

Arthromyces, *Blastosporella*, *Tephrocybe*, and *Termitomyces* (Figure 1). The sister group of the termitomycetoid clade is the genus *Myochromella*, although there is conflict among gene trees as indicated by the low gCF (36%). This indicates that the majority (64%) of gene trees do not support the quartet composition of this node, but it is the quartet supported by the largest share of gene trees. The sister group of the termitomycetoid-myochromelloid group is the clade consisting of the genera *Asterophora*, *Tricholomella*, *Sphagnurus*, and *Lyophyllum*. The position of the genus *Calocybe* remains unresolved as we did not recover significant BS for this split. Our phylogenomic analyses provide strong support for the hypothesis that the sister group of *Termitomyces* is the genus *Arthromyces* (100BS, 72.1% gCF, 37.7% sCF) (Figure 1). The sister group of the *Arthromyces-Termitomyces* clade is the clade consisting of two Asian species, *Tephrocybe* sp. 4 and *Tephrocybe* sp. 5, supported by 100% bootstrap, but showing some discordance among gene trees (43.1% gCF, 34% sCF). The sister group of the *Arthromyces-Termitomyces-Tephrocybe* sp. 4-*Tephrocybe* sp. 5 clade is the clade represented in our dataset by the European and North American *T. rancida*, *Blastosporella zonata* from Central and South America, *Tephrocybe* sp. 2 from South America, and sp. 3 from Asia (100BS, 37.6% gCF, 33.1% sCF). The increased discordance for these branches is not surprising as their branch lengths are relatively short, indicating a rapid diversification event. The deepest split in the termitomycetoid clade is represented by *Tephrocybe* sp. 1 from Australia, and this relationship is well supported (100BS, 90.7% gCF, 45.7% sCF).

Predispositions toward domestication in the ancestor of *Termitomyces*

The evolutionary steps in the three main groups of fungus-insect symbiosis remain unclear. For fungus-growing beetles, the accepted hypothesis is that domestication was contingent on dispersal by the beetle's partner, as the wild relatives of the cultivated fungi are also dispersed by insects.[12,13] For fungus-growing ants, it is proposed that either insect-facilitated dispersal or mycophagy was the initial step.[14] The fungal partners in these two groups have multiple origins, and in the case of beetles also multiple origins of farmers, suggesting that fungal domestication in these groups did not require many changes. In contrast, a single fungal lineage has been domesticated by a single group of termites,[4] which makes the mutualism between termites and fungi a "singularity." Such singularities can be interpreted as either difficult low-probability evolutionary events or, alternatively, due to evolutionary priority effects, where first-movers suppress subsequent independent origins.[15,16] The biological data we collected and our phylogenetic reconstruction allowed us to identify a set of five traits shared by *Termitomyces* and the non-termite-associated sister group *Arthromyces*: a carbohydrate-degrading profile with a reduced potential to degrade plant cell wall components, a rooting stipe (pseudorhiza), the formation of asexual spores (conidia), an insect-fecal association, and the loss of clamp connections (Figure 1). Strikingly, these traits are shared to varying degrees by other members of the termitomycetoid taxa, suggesting that termitomycetoid fungi have a predisposition to domestication. Furthermore, some of these traits are found outside the termitomycetoid clade, such as the conidia-producing *Asterophora* or a (short) pseudorhiza in some specimens of *Tricholomella*.[17,18] We hypothesize that the combination of these traits in the ancestor of *Termitomyces* allowed it to colonize the comb formed by the most recent common ancestor of fungus-growing termites.

Reduced capacity for carbohydrate breakdown predates domestication

Fungi use a broad set of secreted carbohydrate-active enzymes (CAZymes) to break down and metabolize carbohydrates outside of their hyphal bodies. The CAZyme profiles of a species correlate with their ecology.[19] Previous research showed that *Termitomyces* has a reduced complement for the breakdown of these substances,[20] but the timing of this reduction, whether predating the termite symbiosis or not, remained an open question. To test whether this reduction occurred pre- or post-domestication, we assessed the predicted CAZymes of the taxa in our dataset (Table S1). On average we identified 219 CAZymes per taxon (maximum, 375; minimum, 144). Species of *Termitomyces* have 197 CAZymes on average (maximum, 251; minimum, 144). The related non-termite-associated taxa in the genera *Arthromyces* and *Blastosporella* have a slightly higher CAZyme complement, with 233 and 232 CAZymes, respectively. There is no clear pattern of change related to *Termitomyces* in all six functional CAZy classes (Figure 1A). Principal component analysis of a finer separation of CAZymes revealed that generally *Termitomyces* species cluster together, with *Blastosporella* and *Arthromyces* nearby, despite the large genetic distance between *Termitomyces* and *Blastosporella* (Figure 1C). Correcting the principal component analysis for phylogeny[21] did not reveal any source of CAZyme variation from other members of the Lyophyllaceae (Figure S2E). Variation in CAZyme sets is unlikely to be related to sequencing quality, as we found no correlation between the number of reads used in the assemblies and the CAZyme, BUSCO, or entire predicted proteome content (Figures S2A–S2D).

Analysis of the evolutionary history of the CAZyme gene families revealed no significant changes that could be ascribed to the transition to the termite symbiosis, which indicates that the reduced CAZyme complement predates the symbiosis (Figure 1). Five gene families of the 119 total had evolutionary histories that were not explained by the phylogeny alone as tested using the CAFE analysis: AA1, AA3, AA9, GH16, and GH5. The changes in the evolutionary history of AA1 and AA3, both used in the oxidation of lignin, were based on increased gene family number in the two *Tephrocybe rancida* strains. The changes in the GH5 and AA9 families, both families involved in cellulose degradation, while significant, were restricted to individual species, with no internal nodes showing unexpected changes. The GH16 family, involved in breaking various β-1,3-glucan bonds, showed changes at the tips of the tree, as well as a reduction within *Termitomyces*.

Insect-fecal associations predate domestication

During field work, collections of *A. claviformis*, *A. matolae*, and *B. zonata* showed that these species were associated with aggregated clumps of insect-fecal pellets in all cases (Figure 2). Fecal pellets upon which *A. matolae* was growing were composed of woody plant material (Figure S3). We were unable to identify the insect source of the pellets.

Figure 2. Insect-fecal associations found in *Arthromyces matolae* and *Blastosporella zonata*

(A) Mushroom of *A. matolae* connected to a fecal pellet mass.
(B) Close-up of the fecal pellets.
(C) Pileus of *A. matolae* showing the dark arthroconidia.
(D) *B. zonata* mushrooms and pellet mass.
(E) Detail of the mushroom of *B. zonata* showing the attachment with the pseudorhiza to the pellet substrate.
(F) Beetle larva next to fresh fecal pellets.
(G) Close-up of the fecal pellet mass showing white rot in the pellets.
See also Figure S3.

To identify the source of the fecal pellets in our *B. zonata* samples, we collected nearby abundant beetle larvae whose fresh fecal pellets were macroscopically similar to those found with *B. zonata* samples (Figure 2D). We used DNA barcode sequences to identify these as larvae of a Scarab beetle (Scarabaeidae), although we could not identify them to the species level due to a lack of identical or close matches in NCBI GenBank (STAR Methods). Our novel finding of the insect-fecal pellet substrate of *Arthromyces* and *B. zonata* raises the question of whether other species in the termitomycetoid clade are also associated with insect feces. We were unable to re-collect any of the species of *Tephrocybe* except *T. rancida*, but despite extensive efforts, we found no indication that *T. rancida* grows on insect feces (STAR Methods; Figure S3). This apparent lack of an insect-fecal relationship is supported by the higher total number of CAZymes in the *T. rancida* genome compared to *B. zonata* (Table S1), including significant increases in AA1 and AA3 (Figure 1A). The phylogenetic position of the five unidentified species of *Tephrocybe* in the termitomycetoid clade, the pseudorhiza, and their similarity in CAZyme profiles warrant re-collection and detailed study of the substrate. It remains unclear if these taxa have unrecognized associations with insect feces.

Conidial production predates domestication

While all mushroom-forming fungi produce sexual spores (basidiospores) for reproduction, only some species produce asexual spores (conidia), either on the mycelium or in rare cases on the mushroom.[22] However, several species in the termitomycetoid clade produce conidia (Figure 1). In species of *Termitomyces* only the mycelium produces conidia[23] (Figures 3A–3D), which are ingested by the termites and mixed in the gut to inoculate fresh fungus combs.[24] These conidia are produced both inside termite mounds and when grown in laboratory culture. Previously, it has been reported that *Arthromyces* species produce conidial chains by fragmentation of terminal hyphae (arthroconidia) on the entire mushroom while *B. zonata* produces ornamented conidia in small spore heads by a budding process (so-called blastoconidia) on the mushroom cap.[25] Here, we report that *A. matolae* also produces dikaryotic conidia on the mycelium in laboratory culture (Figures 3E–3H and S4A). These conidia are encased in an elaborate structure composed of a hyphal net with large extended setae composed of single cells. We further found that *B. zonata* produces both dikaryotic blastoconidia and arthroconidia on the mycelium in culture (Figures S4B–S4D). Arthroconidia were also detected on the mushrooms of *Tephrocybe* sp. 3 and *Tephrocybe* sp. 5. We did not find conidia in the following taxa: *T. rancida*, *Tephrocybe* sp. 1, *Tephrocybe* sp. 2, and *Tephrocybe* sp. 4. The current lack of laboratory cultures for the various *Tephrocybe* spp. prevents the confident pinpointing of the origin, or origins and subsequent losses, of conidial production in the termitomycetoid clade. However, the most parsimonious reconstruction is that the common ancestor of *Arthromyces* and *Termitomyces* produced conidia.

A rooting stipe predates domestication

All species within the termitomycetoid clade are able to produce a rooting stipe (pseudorhiza). Within *Termitomyces*, *T. microcarpus* may only produce a tiny pseudorhiza in some cases, probably as a response to epigeous fruiting on expelled comb material.[26] The pseudorhiza is a specialized part of the stipe that pushes the immature mushroom of the fungus from the buried subterranean nutrient substrate to the soil surface[27] and has evolved independently several times in Basidiomycota. The nutrient substrate can be a tree root in the case of *Phaeocollybia* and *Xerula radicata*,[28,29] a wood mouse or mole latrine in the case of *Hebeloma radicosum*,[30,31] and insect feces in the case of *Arthromyces*, *B. zonata* (Figure 2), and *Termitomyces*, where insect feces form a specialized fungus comb. All termitomycetoid species are able to produce a pseudorhiza (Figure 1), which indicates a single transition toward growth on a buried nutrient substrate in this group.

Loss of clamp connections predates domestication

There is a striking pattern in a range of mutualisms with convergent increases in genome copy numbers per cell, either through polyploidy or multiple nuclei.[32] Examples are the multinucleate cells of the fungi cultivated by leaf-cutting ants,[33] the fungi forming arbuscular mycorrhizae with plants,[34] the polyploid endosymbiotic plastids and mitochondria of eukaryotic cells,[35] and even our own domesticated crops, most of which are polyploids.[36] *Termitomyces* also fits in this pattern with multinucleate cells containing up to 10 nuclei, lacking the specialized morphological structures, clamp connections, that maintain nuclei per cell in most other basidiomycete fungi.[32,37] This striking similarity suggests that increased ploidy of symbionts may be selected as

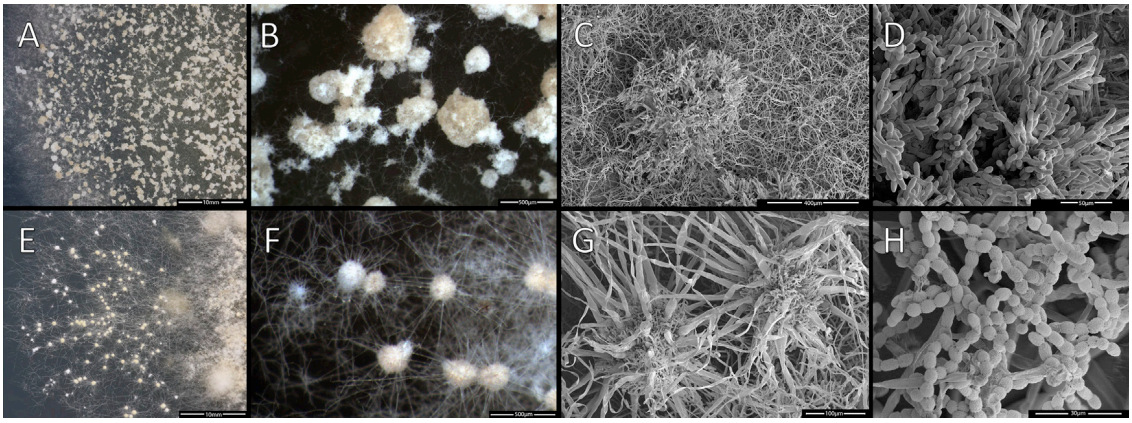

Figure 3. Macroscopic and SEM microscopic images of the conidiophores of "*Termitomyces cryptogamus*" and *Arthromyces matolae*

(A and B) Conidiophores in laboratory culture of "*T. cryptogamus*" (P5). Scale bars, 10 mm, 500 μm.

(C) Electron microscope image of a conidiophore showing the chains of conidia. Scale bar, 400 μm.

(D) Close-up of a conidiophore showing the branched chains of conidia of "*T. cryptogamus*." Scale bar, 50 μm.

(E and F) Laboratory culture of *A. matolae* (FLAS-F-62734) showing the conidiophores. Scale bars, 10 mm, 500 μm.

(G) Electron microscope image of *A. matolae* showing two conidiophores with large aerial setae. Scale bar, 100 μm.

(H) A close-up of an *A. matolae* conidiophore showing the chains of arthroconidia. Scale bar, 30 μm.

See also Figure S4.

a consequence of a symbiotic lifestyle. However, the most parsimonious reconstruction is that clamp connections were lost before domestication of *Termitomyces* since its sister group *Arthromyces* also lacks clamp connections.[25] The origin of multinucleate cells without clamp connections presumably is even older, predating the split between the *Termitomyces-Arthromyces* clade and the *Tephrocybe* sp. 4 and sp. 5 clade, but following the second split in the termitomycetoid clade, between *Tephrocybe* sp. 1, which has clamp connections, and its sister group, most members of which lack clamp connections.

Origin of termite-fungus symbiosis

Three pathways have been described to explain the evolution of a domestication interaction: commensal, prey-or-harvest, and direct.[38,39] The latter two primarily apply to human domesticators since they involve conscious selection of a potential domesticate. In the commensal pathway, a two-way partnership can arise if species A enters the habitat of species B where it can make use of a niche provided by species B.[39] Previously, the sister group of *Termitomyces* and its biology have remained unknown, preventing identification of this potential niche. The consumption of fungus-infested wood has been shown to increase survival in several different species of termites.[40] Some species of termites are also known to consume mushrooms,[41] and fungus-growing termites have been observed to consume mushrooms other than *Termitomyces*.[42] The observation that termites consume and are attracted to rotting wood led to the hypothesis that mycophagy was the initial step toward domestication.[14,43,44] This explanation may be too simplistic as it does not recognize a potential niche provided by the termites that could be utilized by the fungus. Our novel findings on the biology of species in the termitomycetoid clade suggest that the ancestor was not a generalist wood degrader but already possessed a suite of traits that predisposed it toward domestication. This is unique among the fungus-growing insects as ancestral predispositions have not been documented in fungus-growing ants and ambrosia beetles.

Our phylogenetic analysis provides strong support for a sister-group relationship between *Arthromyces* and *Termitomyces*. Both genera share the five traits discussed above, so the most parsimonious reconstruction is that the common ancestor of *Termitomyces* grew on insect feces and had the carbohydrate-degrading enzymatic profile to utilize that growth substrate, produced conidia, had multinucleate cells with no clamp connections, and had a rooting stipe. Our results therefore imply that an insect-fecal association predated the termite-fungus symbiosis. This insect-fecal association may even predate the common ancestor of *Arthromyces* and *Termitomyces* as several other taxa in the termitomycetoid clade exhibit various combinations of these five traits.

All non-Termitid termite families rely on cellulolytic protist gut symbionts for the digestion of wood.[45] However, these gut symbionts have been lost in the Termitidae.[46,47] The loss of gut symbionts was correlated with a diversification of feeding habits, including fungus farming, bacteria farming, and soil feeding.[2,48] Recent evidence suggests that the subfamily Sphaerotermitinae, the only extant member of which constructs combs similar to fungus-growing termites but that are instead colonized by bacteria, is the sister group of the fungus-growing termites.[49,50] The most parsimonious reconstruction for comb evolution is that comb-building was present in the common ancestor of the Sphaerotermitinae and the fungus-growing termites.[50] After the loss of the flagellated protists, the ancestral comb may have served as an "external rumen" in fungus-growing termites,[3] providing a suitable substrate for a fungus adapted to grow and reproduce on a similar fecal substrate. As wood-decay fungi are more efficient lignin degraders compared to lignocellulolytic bacteria,[51] the increased capacity to degrade lignin and cellulose combined with a reduced capacity to break down oligosaccharides of the ancestors of *Termitomyces* may have increased the amount of useable calories in the comb substrate, which directly benefitted

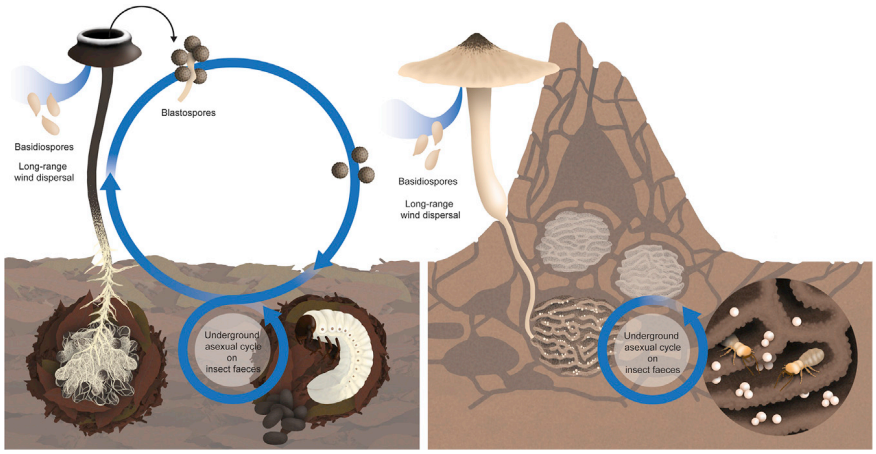

Figure 4. Proposed insect-fungal interaction model of insect-fecal-associated mushroom-forming fungi compared to the life cycle of the domesticated genus *Termitomyces*

In the aboveground part, asexual spores produced on the mushroom are used for local dispersal and rapid inoculation of the fecal pellets. In the belowground part, asexual spores may stick to an insect and inoculate fresh batches of fecal pellets. Additionally, the conidia could also facilitate faster colonization by reinoculation of the substrate via the insect host. Wind-dispersed basidiospores are used for long-range dispersal and sexual recombination.

the termites. Given the sister-group relationship between *Arthromyces* and *Termitomyces*, the most parsimonious reconstruction is that the appendiculate conidiophores formed by *Arthromyces* and the nodules of *Termitomyces* are homologous. The conidia may have served an important role in maintaining the fungus as the dominant species within the ancestral comb since the conidia could facilitate local dispersal and continuous substrate reinoculation (Figure 4).

The conidia produced on the mushroom in *Arthromyces* and several other species in the termitomycetoid clade may be involved in local dispersal since they are thick-walled and this likely increases their chances of survival during periods when substrate is unavailable. This strategy has been suggested for the mycoparasitic genus *Asterophora* as well because their hosts are only available seasonally and patchily.[17,52] It seems likely that they serve the purpose of survival structures such as chlamydospores or sclerotia that are found in a wide array of Basidiomycota.[22,53]

According to our hypothesis, the niche provided by the termites for the ancestor of *Termitomyces* was the pre-digested plant material in the ancestral comb. Besides mineral nutrients obtained from consuming the fungus, the termites could also benefit from the increase in available calories of the comb as a result of fungal degradation and the oligosaccharides left behind by the fungus. The fungus benefitted from a physically pre-treated substrate with an enlarged surface area combined with local dispersal and reinoculation by the termites.

Non-wind-dispersed asexual spores (produced by the fungus) that are locally dispersed by a different species (the termites) are analogous with human domestication and propagation of food plants. It has been proposed that the reduction of natural seed dispersal, which includes the loss of seed shattering, is a key trait selected for under cultivation.[54,55] Most seed crops have non-shattering seeds, which renders a plant species with limited dispersal abilities primarily dependent on humans for survival and propagation.[56] Similarly, *Termitomyces* depends on termites for substrate colonization and accordingly for survival and propagation.[57] The finding that the sister group of *Termitomyces* produces conidia and is associated with insect feces lends support to the hypothesis that one of the first steps in the domestication process was local dispersal by insects.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.07.070.

ACKNOWLEDGMENTS

We would like to thank the Colombian students from the University of Antioquia who assisted with collecting *B. zonata* during fieldwork. We thank Dirk Stubbe for providing a specimen of *Tephrocybe* sp. 5. We thank Marc Maas for creating Figure 4. We thank the Royal Botanical Gardens KEW herbarium for letting us use their facilities and providing specimens. We thank the field mycologists who helped us with taxon sampling: Carolien Reindertsen, Mirjam Veerkamp, Arthur Grupe, Jenny Rogers, Jacob Kalichman, and Gert Immerzeel. We thank Natascha Oosterwijk for making some of the microscopic images. We thank Marcel Giesbers from the Wageningen Electron Microscopy Centre for his help in making the electron microscopy images. D.K.A.,

L.J.J.v.d.P., M.N., A.A.G.-G., and B.A. were supported by the Netherlands Organization for Scientific Research (D.K.A., L.J.J.v.d.P., M.N., and A.A.G.-G. by VICI: NWO 86514007; D.K.A. and B.A. by ALWGR.2017.010). T.J.B. and D.J.L. acknowledge grants from the NSF (DEB-9525902 and DEB-0103621 – Belize, Puerto Rico, Dominican Republic), and T.J.B. acknowledges support from the National Geographic Society (Belize) and the New York Botanical Gardens (Thailand and Australia), supporting field research that allowed discovery of many of the novel non-*Termitomyces* taxa included in this study. The Colombian biological material used in this work was collected under the permission given to the University of Antioquia according to resolution 0524 of May 27, 2014, issued by the National Environmental Licensing Authority - ANLA.

AUTHOR CONTRIBUTIONS

Conceptualization, L.J.J.v.d.P. and D.K.A.; Methodology, L.J.J.v.d.P., D.K.A., M.N., and B.A.; Investigation, L.J.J.v.d.P., D.K.A., M.N., A.A.G.-G., T.J.B., M.E.S., M.E.C., A.E.F.-M., T.W.K., and Z.W.d.B.; Formal Analysis, L.J.J.v.d.P., M.N., and B.A.; Resources, T.J.B., M.E.S., M.E.C., A.E.F.-M., D.J.L., and T.W.K.; Data Curation, M.N.; Writing – Original Draft, L.J.J.v.d.P. All of the authors commented on the first draft and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 15, 2021
Revised: June 22, 2021
Accepted: July 27, 2021
Published: August 16, 2021

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fungal samples used for DNA isolation	Author's collection, Cort herbarium, Royal Botanic Gardens Kew herbarium, NCBI GenBank	CORT; K; Table S2
Chemicals, peptides, and recombinant proteins		
NucleoSpin Soil, Mini kit for DNA from soil	Macherey-Nagel	REF 740780.50
cetyl trimethylammonium bromide (CTAB)	Sigma-Aldrich	CAS 57-09-0
chloroform	Merck	CAS 67-66-3
Isopropanol (2-propanol)	Merck	CAS 67-63-0
ethanol	Merck	CAS 64-17-5
proteinase K	Merck	CAS 39450-01-6
malt extract	Merck	CAS 8002-48-0
yeast extract	Becton Dickinson Difco	Difco 212750
agar	DUCHEFA	CAS 9002-18-0
streptomycin	Merck	CAS 3810-74-0
Deposited data		
Raw reads	This study	SRA: SRX10313000-SRX10313007; SRX10337354-SRX10337371; SRX4910404-SRX4910415
Fungal genomes	This study	Table S2
Coleopteran larva CO1 sequences	This study	Genbank: MW698941; MW698942
R-scripts	Github	https://github.com/BenAuxier/Termite.Domestication
Experimental models: Organisms/strains		
Fungal samples	Author's collection, Cort herbarium, Royal Botanic Gardens Kew herbarium, NCBI GenBank	CORT; K; Table S2
Oligonucleotides		
16S primers (metabarcoding)	[58]	Ins16S_1shortF; Ins16S_1shortR
COI primers	[59]	C1-J-2183 ('Jerry'); TL2-N-3014 ('Pat')
COI primers	[60]	LCO1490; HCO2198
COI primers (coleopteran specific)	[61]	Coleop_16Sc; Coleop_16Sd
COI primers (metabarcoding)	[62]	ZBJ-ArtF1c; ZBJ-ArtR2c
ITS1 primers (insect specific)	[63]	N/A
Software and algorithms		
BUSCO	[64]	https://busco.ezlab.org

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SPAdes 3.5.0	[65]	http://cab.spbu.ru/software/spades/
MAFFT v7.475	[66]	https://mafft.cbrc.jp/alignment/software/
Geneious 10.0.9	[67]	https://www.geneious.com
IQ-TREE 2.1.2	[68]	http://www.iqtree.org
AUGUSTUS	[69]	http://bioinf.uni-greifswald.de/augustus/
funannotate v.1.7.4	[70]	https://doi.org/10.5281/zenodo.1134477
dbCAN2	[71]	http://bcb.unl.edu/dbCAN2/index.php
Gblocks v.0.91b	[72]	http://molevol.cmima.csic.es/castresana/Gblocks_server.html
CAFE version 5	[73]	https://github.com/hahnlab/CAFE5/releases
R studio version 1.3.959	RStudio	https://rstudio.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Lennart van de Peppel (lennartvdpeppel@gmail.com)

Materials availability

All samples used in this study, either preserved or living, are available upon request (see Table S2). A living culture of *Arthromyces matolae* (FLAS-F-62734) was submitted to the culture collection of the Westerdijk Fungal Biodiversity Institute and is publicly under the accession number CBS 147616.

Data and code availability

Newly generated raw reads of the fungal taxa are available in the NCBI Sequence Read Archive (SRA): SRX10313000-SRX10313007, SRX10337354-SRX10337371 and SRX4910404-SRX4910415. Assembled genomes are available at NCBI GenBank: GCA_017580835.1, GCA_017607575.1, GCA_017657195.1, GCA_017657225.1, GCA_017657235.1, GCA_017657275.1, GCA_017657295.1, GCA_017657315.1, GCA_017657335.1, GCA_017657355.1, GCA_017657375.1, GCA_018220975.1, GCA_018221615.1, GCA_018221635.1, GCA_018221655.1, GCA_018221735.1, GCA_018221785.1, GCA_018221805.1, GCA_018282005.1, GCA_018282025.1, GCA_018849495.1, GCA_018850235.1, GCA_018850255.1, GCA_018850275.1, GCA_018850815.1, GCA_018851285.1, GCA_018851305.1, GCA_018851325.1, GCA_018851835.1, GCA_018854895.1, GCA_018855395.1, GCA_018855915.1, GCA_018856295.1, GCA_018857265.1, GCA_018857285.1, GCA_018857305.1, GCA_018858115.1. Beetle larva sequences are available at NCBI GenBank: MW698941 and MW698942.

Scripts used for filtering contigs and removing bacterial contamination from the assemblies in the bioinformatic analyses as well as the scripts used in the CAZyme analysis are available at: https://github.com/BenAuxier/Termite.Domestication

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We sampled a total of 47 taxa, of which 25 were *Termitomyces*, across the entire family of the Lyophyllaceae and within the Lyophyllaceae we focused primarily on the 'termitomycetoid' clade.[5] For the three remaining clades we sampled one to four representative species. We collected specimens from various sources; the *Termitomyces* samples were obtained from our in-house culture collection, the Royal Botanical Gardens KEW herbarium and from mushrooms stored in ethanol collected in Ivory Coast. Other non-*Termitomyces* lyophylloid herbarium specimens were obtained from the Royal Botanical Gardens KEW herbarium and the Cort herbarium. Fresh Lyophylloid mushrooms were provided by various collectors from the Netherlands, DNA was isolated either directly from these mushrooms or from tissue cultures. Tissue cultures were made by cutting the stipe or pileus and moving a sterile piece of tissue from the inside of the stipe or pileus with sterile forceps into a Petri dish containing malt yeast extract agar (per liter demi water: 20 g malt extract, 2 g yeast extract, 15 g agar) and streptomycin (30mg/L) against bacterial contamination. A full overview of all samples can be found in Table S2.

METHOD DETAILS

Detection and collection of fecal pellets

Mushrooms of *Blastosporella zonata* and pellets were collected in Murillo - Tolima, Colombia. Between ten and 15 pellets were stored in 1.5ml Eppendorf tubes with pure ethanol for subsequent analysis. In some cases, pellets were found very close to the pellet mass on which *B. zonata* was growing. These pellets, which did not show fungal colonization, were collected separately.

On two occasions (collection Bzo6 and Bzo8) scarabid beetle larvae were found in close proximity of the pellet substrate. A total of six larvae were collected, three larvae were found within a 15cm radius of collection Bzo6 and one larva was found within the same radius of collection Bzo8. Two additional larvae were collected randomly in leaf litter.

The larvae were collected in 50ml tubes to collect fresh fecal pellets. Fresh pellets and beetle larvae were stored in pure ethanol. Fresh fruiting bodies were stored in 50ml tubes and tissue cultures were made on the same day. Conidial cultures were made by streaking conidia from the pileus of the fruiting body on a Petri dish containing the same medium. Cultures were stored at room temperature.

To detect potential fecal pellets and to study the function of the pseudorhiza of *Tephrocybe rancida*, we sampled mushrooms at one location on the property of the Nyenrode Business University in Breukelen, the Netherlands. We sampled in late October of 2015, 2017 and 2019. The rooting base was carefully excavated using a small gardening trowel. The pseudorhiza was traced into the soil but no clear connection to fecal pellets or any buried substrate could be found. Fruiting bodies were collected in 50ml tubes and were either dried or used to make tissue cultures. Single-spore isolates were made by attaching a pileus with petroleum jelly to the lid of a Petri dish and spores were captured on MYA medium with streptomycin. When basidiospores had germinated a single colony was transferred to a new MYA plate.

Scoring of the morphological predisposition traits

The four morphological traits were scored after inspection of the specimens that were collected. In most cases the traits could unambiguously be detected from our collections. However, in some cases we were not able to score all four traits as we only had a culture and no mushroom or the other way around. In these cases, scoring was done combining our personal observations and those from literature.

Our single collection of *Tricholomella constricta* did not have a pseudorhiza; however, this trait is variable within this species,[18] and therefore, we scored it as 'present'. In the case of *Termitomyces* we made the general assumption that all species have a pseudorhiza although there may be a single case in which there may not always be a pseudorhiza, which is in *T. microcarpus*.[74] However, because some authors describe it as weakly rooting,[26] it is probably still able to produce the pseudorhiza and we therefore scored it as 'present'. The ambiguity of this trait in *T. microcarpus* is most likely a response to epigeous fruiting on expelled comb material.

We were not able to detect conidia in our culture of *Sphagnurus paluster;* however, conidial production has been reported for this species[75] and we therefore scored it as 'present'.

The presence of clamp connections has been reported in the following genera or species: *Asterophora*,[76] *Blastosporella*,[25] *Calocybe cyanea*[77] and *Hypsizygus*,[78] *Lyophyllum*,[79] *Myochromella*,[5] *Tricholomella*,[18] *T. rancida*,[5] *S. paluster*.[80] The absence of clamp connections is reported in the following genera: *Arthromyces*[25] and *Termitomyces*.[32,74]

DNA isolation

For DNA isolation of the fungal samples a small piece (0.2-0.5g) of mycelium from a laboratory culture, the pileus of a dried herbarium specimen or the pileus of a specimen stored in ethanol was frozen in liquid nitrogen and disrupted in a 1.5ml Eppendorf tube with glass beads prior to DNA isolation. DNA isolation for all samples was performed using the cetyltrimethylammonium bromide (CTAB) as previously described.[81] DNA from beetle larvae was isolated from a leg part using the same protocol.

DNA from fecal pellets was isolated by using a Nucleospin Soil DNA extraction kit (Macherey-Nagel) following the manufacturer's instructions. For the fresh pellets that were directly obtained from living beetle larvae only a single pellet was used for DNA isolation, from the pellets that were part of the fungal substrate between five and 20 pellets were used depending on the size of the pellets and availability.

Beetle larvae identification

As the identification of beetles from the larval stage is very difficult and requires a field expert, we attempted molecular identification of the six larvae that we collected. This was done by obtaining a partial sequence of the mitochondrial cytochrome *c* oxidase (CO1) and using NCBI BLAST for identification. A partial sequence of the CO1 was amplified using the primer pair C1-J-2183 ('Jerry')/ TL2-N-3014 ('Pat'),[59] using the following PCR program: denaturation at 94°C for 60 s, then five cycles consisting of 30 s denaturation at 94°C, 40 s annealing at 47°C and elongation for 60 s at 72°C, followed by 30 cycles consisting of 30 s denaturation at 94°C, 40 s annealing at 52°C and elongation for 60 s at 72°C, followed by a final extension step for 10 min at 72°C. After Sanger sequencing of amplified products, we were able to distinguish two different genotypes, of which four larvae with identical sequences were of genotype 1 (MW698941) and two larvae with identical sequences of genotype 2 (MW698942). We could not make a reliable identification for either genotype because searches against the GenBank database did not return a close match (97% similarity). The closest match for genotype 1 was a 85.75% match to a *Cryptodus* sp. sequence (KF801857), while genotype 2 had a 84.17% match to a *Pimelopus dubius dubius* sequence (EF487738). Both of these species belong to the subfamily Dynastinae (Rhinoceros beetles) within the Scarabaeidae.

Fecal pellet identification

Identification of the depositor of the fecal pellets of *B. zonata* or *Arthromyces* could shed light on the interaction between the fungus and the insect partner. As we were only able to obtain fresh pellets in ethanol for *B. zonata* we focused on these pellets. We attempted amplification of the mitochondrial cytochrome *c* oxidase (CO1) on DNA extracted from pellets using PCR with primer pairs, LCO1490/HCO2198[60] and Jerry/Pat.[59] We also attempted amplification of the internal transcribed spacer 1 (ITS1) marker using the Vogler primer pair[63] and the 16S marker using the Coleoptera specific primers Coleop_16Sc and Coleop_16Sd.[61] PCR was performed using protocols and conditions described for each primer pair specified in the original publications. We could not confidently observe amplification of host DNA as we obtained multiple different PCR products per reaction.

Environmental DNA barcoding

Our standard PCR protocol was insufficient to identify the depositor of the pellets so we opted for an environmental DNA barcoding approach. A recent study used environmental DNA barcoding approaches to determine dietary arthropod contents in fecal samples of insectivorous animals.[82] We used two different DNA barcodes: a 157bp target region of the CO1 using the primer pair ZBJ-ArtF1c/ZBJ-ArtR2c[62] and a 156bp target region of 16S using the primer pair Ins16S_1shortF/Ins16S_1shortR.[58] PCR was performed using protocols and conditions described for each primer pair specified in the original publications. A total of 19 PCR reactions were done using the protocol below on 13 different DNA samples; six from a *B. zonata* pellet substrate, five from pellets not visibly colonized by *B. zonata* and two from fresh beetle pellets. Unique barcode adapters were used for each PCR reaction and all samples were pooled after PCR and sequenced using an Oxford nanopore MinION. To test whether the pellets were of beetle origin we used the sequences generated from the larvae that we collected as reference. We used Geneious 10.0.9 (https://www.geneious.com/) to match reads (between 1,000 and 27,000 reads per sample) against the reference but were unable to find any significant matches (data not shown).[67] Since we used two DNA samples from fecal pellets directly obtained from these larvae this suggests that our method may not be sensitive enough to pick up host DNA (from gut epithelial cells) from the fecal pellets.

Electron microscopy

Scanning electron microscopy on laboratory cultures of *B. zonata* (Bzo9), *A. matolae* (FLAS-F-62734) and '*T. cryptogamus*' (P5) was performed at the Wageningen Electron Microscopy Centre. To preserve the delicate conidiophores in *A. matolae* and the nodules in '*T. cryptogamus*' samples were frozen in liquid nitrogen prior to imaging (cryoSEM). The *A. matolae* culture that was used was grown for 14 days at 15°C on MYA agar. The '*T. cryptogamus*' culture was grown for 25 days at 25°C on MYA.

Library preparation and whole genome sequencing

Library preparation and whole-genome sequencing was performed by Novogene (Hong Kong) using the Illumina Hiseq 2500 platform. The paired-end reads that were generated were 150bp long and the insert size was 500bp.

Assembly and annotation

We assembled paired Illumina reads using SPAdes v.3.5.0 with default settings.[65] Short contigs smaller than 300bp or contigs with a coverage lower than 5x were removed from the assembly using a script. Presumed bacterial contigs were removed from the assembly using a script which matched contigs using BLAST against a reference library of 500 randomly selected bacterial genomes. Contigs with a BLAST hit with an expect value (E) of less than 1e-7 were removed from the assembly. We applied automatic annotations to each assembly using the funannotate pipeline (v.1.7.4). We ran funannotate -mask with default options for repeat masking, followed by funannotate -sort. We then ran funannotate -predict using a pretrained Augustus dataset for '*Termitomyces cryptogamus*' T132 as reference.[69] We also ran funannotate -iprscan, funannotate -remote with antiSmash, and funannotate -annotate all with default settings.[70]

Marker selection and phylogenetic analysis

We collected conserved orthologs using BUSCO with the basidiomycete reference gene set odb9 provided on the BUSCO website.[64] We then removed any sequence sets for which we found fewer than 25 matches. Finally, we aligned the remaining sequence sets using MAFFT v.7.475[66] with the following parameters:–auto -–maxiterate 1000 –-adjustdirection

To remove poorly aligned regions, we used Gblocks v.0.91b[72] with the following input: -o -b5 = h -t = DNA.

We then concatenated all trimmed alignments and ran a maximum likelihood phylogenetic analysis with IQ-TREE (version 2.1.2) with the following settings: -s -spp -o -bb 1000 -bsam GENESITE -m TESTMERGE–runs 100, with a partition for each BUSCO locus, and *Hypsizygus ulmarius* as outgroup. Using -bsam GENESITE reduces bootstrap inflation by resampling partitions first and then resampling sites within partitions. We ran 100 independent runs and all produced the same topology with minimal variation in likelihood estimates. To estimate phylogenetic conflicts between loci we compared species tree to individual locus trees estimated with IQ-TREE (version 2.1.2).[68] We reconstructed the locus trees with the following parameters: -s -S. The gene and site concordance factors were then computed with IQ-Tree using the command: -t –-gcf -s–scf 100. In addition, we used the locus trees generated by IQ-TREE to perform a coalescent-based species tree reconstruction using ASTRAL.[83] We performed ASTRAL with default settings using the command: java -jar astral.5.6.3.jar -i -o 2 > out.log.

CAZyme analysis

Predicted CAZymes were collected from the funannotate output for each assembly in our dataset. These predictions are made from the dbCAN2 database based on Hidden Markov Model predictions. To increase the confidence of the predictions, we then submitted the corresponding amino acid sequences to the dbCAN2 webserver, to obtain the predictions for this set of proteins using DIAMOND and Hotpep.[71] Only predicted CAZymes that were also identified using either the DIAMOND or Hotpep pipelines were used for the analysis.

CAFE analysis

To detect significant changes in CAZyme composition along our phylogenetic tree we ran CAFE[73] on our CAZyme dataset using default settings. The phylogenetic tree reconstructed using IQ-tree was made ultrametric using the package Ape in R and was used as input for the analysis.[84]

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical support values for the phylogenetic analyses were calculated using IQ-tree as described in the Method details.