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.
Ancestral predisposition
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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,1 D. Jean Lodge,5 Matthew E. Smith,6 Thomas W. Kuyper,7 Ana E. Franco-Molano,8 Timothy J. Baroni,9 and Duur K. Aanen1,10

1Laboratory of Genetics, Wageningen University & Research, Droevendaalsesteeg 1, 6708PB Wageningen, the Netherlands
2Microbial Ecology Department, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, the Netherlands
3Laboratorio de Micología y Fitopatología-LAMFU, Universidad de Los Andes, Carrera 1 # 10A-12, Bogotá, Colombia
4Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa
5Department of Plant Pathology, 2105 Miller Plant Sciences Building, University of Georgia, Athens, GA 30606, USA
6Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
7Soil Biology Group, Wageningen University & Research, Box 47, 6700AA Wageningen, the Netherlands
8Laboratorio 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
9Department of Biological Sciences, State University of New York, College at Cortland, PO Box 2000, Cortland, NY 13045, USA
10Lead contact
*Correspondence: lennartvdeppe@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 termitomyctoid 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.1 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.3,4 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
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 Termitomyces and Arthromyces is sister to the genus 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 assexual 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).
**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 (84%) 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-mycophellomeloid group is the clade consisting of the genera *Astrophora*, *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. For fungus-growing ants, it is proposed that either insect-facilitated dispersal or mycophagy was the initial step. 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, 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. 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 *Astrophora* or a (short) pseudorhiza in some specimens of *Tricholomella*. 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. Previous research showed that *Termitomyces* has a reduced complement for the breakdown of these substances, 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 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.
spores (conidia), either on the mycelium or in rare cases on the mushroom. However, several species in the termitomycetoid clade produce conidia (Figure 1). In species of *Termitomyces* only the mycelium produces conidia (Figure 3A–3D), which are ingested by the termites and mixed in the gut to inoculate fresh fungus combs. 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. 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. 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 and has evolved independently several times in Basidiomycota. The nutrient substrate can be a tree root in the case of *Phaeocollybia* and *Xerula radicata*, a wood mouse or mole latrine in the case of *Hebeloma radicosum*, 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. Examples are the multinucleate cells of the fungi cultivated by leaf-cutting ants. The fungi forming arbuscular mycorrhizae with plants, the polyploid endosymbiotic plastids and mitochondria of eukaryotic cells, and even our own domesticated crops, most of which are polyploids. *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. This striking similarity suggests that increased ploidy of symbionts may be selected as
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. 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 termitomyceotid 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. 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. 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. Some species of termites are also known to consume mushrooms and fungus-growing termites have been observed to consume mushrooms other than Termitomyces. The observation that termites consume and are attracted to rotting wood led to the hypothesis that mycophagy was the initial step toward domestication. 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 termitomyceotid clade suggest that the ancestor was not a generalist wood degrader but already possessed a suite of traits that predisposed it toward domestication. This 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 termitomyceotid clade exhibit various combinations of these five traits.

All non-Termitid termite families rely on cellulolytic protist gut symbionts for the digestion of wood. However, these gut symbionts have been lost in the Termitidae. The loss of gut symbionts was correlated with a diversification of feeding habits, including fungus farming, bacteria farming, and soil feeding. Recent evidence suggests that the subfamily Sphaerotermitinae, the only extant member of which constructs combs similar to fungus-growing termites, had 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. 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. After the loss of the flagellated protists, the ancestral comb may have served as an “external rumen” in fungus-growing termites, 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, 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 usable calories in the comb substrate, which directly benefitted...
the domestication process was local dispersal by insects. Lends support to the hypothesis that one of the first steps in myces produces conidia and is associated with insect feces and the nodules of myces is that the appendiculate conidiophores formed by Arthromyces and include the following:

**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.

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., 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

REFERENCES

1. Bignell, D.E. (2000). Introduction to symbiosis. In Termites: Evolution, Sociality, Symbioses, Ecology, T. Abe, D.E. Bignell, and M. Higashi, eds. (Springer Netherlands), pp. 189–208.

2. Chouvenc, T., Sobotnik, J., Engel, M.S., and Bourguignon, T. (2021). Termite evolution: mutualistic associations, key innovations, and the rise of Termitidae. Cell. Mol. Life Sci. 78, 2749–2769.

3. Nobre, T., and Aanen, D.K. (2012). Fungiculture or termite husbandry? The ruminant insect. Insects 3, 307–323.

4. Aanen, D.K., Eggleton, P., Rouland-Lefèvre, C., Guldborg-Frosliev, T., Rosendahl, S., and Boomsmma, J.J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. Proc. Natl. Acad. Sci. USA 99, 14887–14892.

5. Hofstetter, V., Redhead, S.A., Kauff, F., Moncalvo, J.M., Matheny, P.B., Vilgalys, R., and Moncalvo, J.M. (2002). Phylogenetic analyses of the Lyophyllaceae (Basidiomycota, Agaricales) based on a multigene phylogeny. Cryptogram. Mycol. 35, 399–425.

6. Hofstetter, V., Clemenson, H., Vilgalys, R., and Moncalvo, J.M. (2002). Phylogenetic analyses of the Lyophyllaceae (Agaricales, Basidiomycota) based on nuclear and mitochondrial rRNA sequences. Mycol. Res. 106, 1043–1059.

7. Matheny, P.B., Curtis, J.M., Hofstetter, V., Aime, M.C., Moncalvo, J.M., Ge, Z.W.,Slot, J.C., Ammirati, J.F., Baroni, T.J., Bouger, N.L., et al. (2006). Major clades of Agaricales: a multilocus phylogenetic overview. Mycologia 98, 982–995.

8. Bellanger, J.M., Moreau, P.A., Corniol, G., Bidaud, A., Chalange, R., Dudova, Z., and Richard, F. (2015). Plunging hands into the mushroom matrix: a phylogenetic framework for Lyophylloaceae (Agaricales, Basidiomycota). Genetica 143, 169–194.

9. Kubatko, L.S., and Degnan, J.H. (2007). Inconsistency of phylogenetic estimates from concatenated data under coalescence. Syst. Biol. 56, 17–24.

10. Ané, C., Larget, B., Baum, D.A., Smith, S.D., and Rokas, A. (2007). Bayesian estimation of concordance among gene trees. Mol. Biol. Evol. 24, 412–426.

11. Minh, B.Q., Hahn, M.W., and Lanfear, R. (2020). New methods to calculate concordance factors for phylogenetic datasets. Mol. Biol. Evol. 37, 2727–2733.

12. Harrington, T.C. (1987). New combinations in ophiostoma of ceratocystis species with leptographium anamorphs. Mycotaxon 28, 39–43.

13. Harrington, T.C. (2005). Ecology and evolution of mycophagous bark beetles and their fungal partners. In Insect-Fungal Associations: Ecology and Evolution, M.B.F.E. Vega, ed. (Oxford University Press), pp. 257–291.

14. Mueller, U.G., Gerardo, N.M., Aanen, D.K., Six, D.L., and Schultz, T.R. (2005). The evolution of agriculture in insects. Annu. Rev. Ecol. Evol. Syst. 36, 563–595.

15. De Duve, C. (2000). Singularity: Landmarks on the Pathways of Life (Cambridge University Press).

16. Burnett, A., and Ratcliff, W.C. (2020). The origin of phototrophy reveals the importance of priority effects for evolutionary innovation. Preprints. https://www.preprints.org/manuscript/202011.0700v2.

17. Buller, A.H.R. (1924). Agarics Which Are Parasitic on Other Agarics (Longmans Green and Co.).

18. Kalamees, K. (1992). Tricholomella, a new genus, with the distribution data of Tricholomella-constricrum, Comb-Nov in East Europe and Asia. Persoonia 14, 445–447.

19. Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henriass, B., Martinez, A.T., Otiliar, R., Spatafora, J.W., Yadav, J.S., et al. (2012). The Paleozoic origin of enzymic lignin decomposition reconstructed from 31 fungal genomes. Science 336, 1715–1719.

20. Poulsen, M., Hu, H., Li, C., Chen, Z., Xu, L., Otani, S., Nygaard, S., Nobre, T., Klaubauf, S., Schindler, P.M., et al. (2014). Complementary symbiont contributions to plant decomposition in a fungus-farming termite. Proc. Natl. Acad. Sci. USA 117, 14500–14505.

21. Revell, L.J. (2009). Size-correction and principal components for interspecific comparative studies. Evolution 63, 3258–3268.

22. Kües, U., Badalyan, S.M., Giefker, A., and Dörnte, B. (2016). Asexual sporulation in Agaricomycetes. In The Mycota, D. Growth, and J.W. Sexuality, eds. (Springer), pp. 269–328.

23. Botha, W.J., and Eicker, A. (1991). Cultural studies on the genus Termitomyces in South Africa. I. Macro-and microscopic characters of basidiome context cultures. Mycol. Res. 95, 435–443.

24. Hinze, B., Craiskeim, K., and Leuthold, R.H. (2002). Polysynt in food processing and social organisation in the nest of Macrotermes bellicosus (Isoptera, Termitidae). Insectes Soc. 49, 31–37.

25. Baroni, T.J., Franco-Molano, A.E., Lodge, D.J., Lindner, D.L., Horak, E., and Hofstetter, V. (2007). Arthromycyes and Blastosporrelae, two new genera of con-idia-producing lyophyllloid agarics (Agaricales, Basidiomycota) from the neotropics. Mycol. Res. 111, 572–580.

26. Horak, E. (1968). Synopsis Generum Agaricalium (Die Gattungstypen der Agaricales) (Druckerei Buchier).

27. Buller, A.H.R. (1934). Researches on Fungii VI (Longmans, Green & Company).

28. Campbell, A.H. (1938). Contribution to the biology of Collybia radicata (Reh.) Berk. Trans. Br. Mycol. Soc. 22, 151–159.

29. Redhead, S.A., and Malloch, D.W. (1986). The genus Phaeocollybia (Agaricales) in eastern Canada and its biological status. Can. J. Bot. 64, 1249–1254.

30. Sagara, N., Hongo, T., Murakami, Y., Hashimoto, T., Nagamasu, H., Fukiharu, T., and Asakawa, Y. (2006). Establishment of the case of Hebeloma radicosum growth on the latrine of the wood mouse. Mycol. Res. 109, 2727–2733.
Termitomyces fungi associated with fungus-growing termites is limited. BMC Evol. Biol. 14, 121.

Kool, P.W., Aanen, D.K., Schiett, M., and Boomsma, J.J. (2015). Evolutionarily advanced ant farms rear polyloid fungal crops. J. Evol. Biol. 28, 1911–1924.

Kuhn, G., Hijri, M., and Sanders, I.R. (2001). Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414, 745–748.

Bendich, A.J. (1987). Why do chloroplasts and mitochondria contain so many copies of their genome? BioEssays 6, 279–282.

Bretagnolle, F., and Thompson, J.D. (1995). Gametes with the somatic mating system and multinucleate cells. Mycol. Res.

Viana, A.B., Corêtes, M.O., Cornelissen, T.G., and Neves, F.D. (2018).

Zeder, M.A. (2015). Core questions in domestication research. Proc. Natl. Acad. Sci. USA 112, 3191–3198.

De Fine Licht, H.H., Andersen, A., and Aanen, D.K. (2005).

Bretagnolle, F., and Thompson, J.D. (1995). Gametes with the somatic mating system and multinucleate cells. Mycol. Res.

Wall, D.A., Lafage, J.P., Gilbertson, R.L., and Blackwell, M. (1987).

Inward, D.J., Vogler, A.P., and Eggleton, P. (2007). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. Mol. Phylogenet. Evol. 44, 953–967.

Darlington, J.P.E.C. (1994). Nutrition and evolution in fungus-growing termites. Fungal Ecol. 41, 92–100.

Catra, L.R., and Catra, S.W.T. (1979). Termit-Fungus Mutualism (Wiley), pp. 117–163.

Roulond-Lefèvre, C. (2000). Symbiosis with fungi. In: Termites: Evolution, Sociality, Symbioses, Ecology, T. Abe, D.E. Bigelli, and M. Higashi, eds. (Springer), pp. 289–306.

Brugerolle, G., and Radek, R. (2006). Symbiotic protozoa of termites. In: Intestinal Microorganisms of Termites and Other Invertebrates, H. König, and A. Varma, eds. (Springer), pp. 243–269.

Darlington, J.P.E.C. (1994). Nutrition and evolution in fungus-growing termites. In: Nourishment and Evolution in Insect Societies, J.H. Hunt, and C.A. Nalepa, eds. (Oxford), pp. 105–130.

Eggleton, P. (2006). The termite gut habitat: its evolution and co-evolution. In: Inintestinal Microorganisms of Termites and Other Invertebrates, H. König, and A. Varma, eds. (Springer), pp. 373–404.

Inward, D.J., Vogler, A.P., and Eggleton, P. (2007). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. Mol. Phylogenet. Evol. 44, 953–967.

Gamier-Sillam, E., Toutain, F., Villemin, G., and Renoux, J. (1989). Études préliminaires des meules originales du termite Xylophage Sphaerothermes pherothorax (Sjostedt). Insectes Soc. 36, 293–312.

Bucek, A., Sobotník, J., He, S., Shi, M., McMahon, D.P., Holmes, E.C., Shi, M., McMahon, D.P., Holmes, E.C., Sobotník, J., He, S., Shi, M., McMahon, D.P., Holmes, E.C.

Sobótka, J., He, S., Shi, M., McMahon, D.P., Holmes, E.C., Sobotník, J., He, S., Shi, M., McMahon, D.P., Holmes, E.C.

Diamond, J.M., and Orudnio, D. (1999). Guns, Germs, and Steel (Books on Tape).

Purugganan, M.D., and Fuller, D.Q. (2009). The nature of selection during plant domestication. Nature 457, 843–848.

Diamond, J. (2002). Evolution, consequences and future of plant and animal domestication. Nature 418, 700–707.

Leuthold, R.H., Badertscher, S., and Imboden, H. (1989). The inoculation of newly formed fungus comb with Termitomyces in Macrotomites colonies (Isoptera, Macrotermitinae). Insectes Soc. 36, 328–338.

Clarke, L.J., Soubrier, M., Weyrich, L.S., and Cooper, A. (2014). Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. Mol. Ecol. Resour. 14, 1160–1170.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Koval, O., Black, M., Hoew, V., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Epp, L.S., Boessenkool, S., Bellemain, E.P., Haile, J., Esposito, A., Riaz, T., Erseus, C., Gasuvar, V.I., Edwards, M.E., Johnsen, A., et al. (2012). New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. Mol. Ecol. 21, 1821–1833.

Zeale, M.R., Butlin, R.K., Barker, G.L., Lees, D.C., and Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Mol. Ecol. Resour. 11, 236–244.

Vogler, A.P., and DeSalle, R. (1994). Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle Cicindela dorsalis. Mol. Biol. Evol. 11, 393–405.

Seppey, M., Manni, M., and Zdobnov, E.M. (2019). BUSCO: assessing genome assembly and annotation completeness. Gene Prediction (Springer), pp. 227–245.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Pjihbelsky, A., Pjshkin, A., Siroton, A., Siroton, Y., et al. (2013). Assembling Genomes and Mini-Metagenomes from Highly Chimeric Reads. (Springer Berlin Heidelberg), pp. 158–170.

Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., van Haesel, A., and Lanfear, R. (2020). IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37, 1530–1534.

Stanke, M., and Morgenstern, B. (2005). AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. Nucleic Acids Res. 33, W465–7.

Palmer, J., and Stajich, J. (2019). nextgenusfs/funannote: funannote v1.5.3 (Version 1.5.3). Zenodo. https://doi.org/10.5281/zenodo.2604804.

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P.K., Xu, Y., and Yin, Y. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 46 (W1), W95–W101.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.

De Bie, T., Cristianini, N., Demuth, J.P., and Hahn, M.W. (2006). CAFE: a computational tool for the study of gene family evolution. Bioinformatics 22, 1269–1271.

Heim, R. (1977). Termites et Champignons; les Champignons Termitophiles d’Afrique Noire et d’Asie Meridionale (Editions Boub)
75. Walther, G., Garnica, S., and Weiss, M. (2005). The systematic relevance of conidiogenesis modes in the gilled Agaricales. Mycol. Res. 109, 525–544.
76. Thompson, G.E. (1936). Nyctalis parasitica and N. asterophora in culture. Mycologia 28, 222–227.
77. Baroni, T.J., Legon, N.W., Vilgalys, R., and Lodge, D.J. (1999). Calocybe cyanea - a rare and beautiful agaric is discovered in Puerto Rico. Mycologist 13, 7.
78. Singer, R. (1947). New genera of fungi. Mycologia 39, 77–89.
79. Moncalvo, J.M., Rehner, S.A., and Vilgalys, R. (1993). Systematics of lycoperdon section difformia based on evidence from culture studies and ribosomal DNA-sequences. Mycologia 85, 788–794.
80. Redhead, S.A. (1981). Parasitism of bryophytes by agarics. Can. J. Bot. 59, 63–67.
81. Nieuwenhuis, M., van de Peppel, L.J.J., Bakker, F.T., Zvaan, B.J., and Aanen, D.K. (2019). Enrichment of G4DNA and a large inverted repeat coincide in the mitochondrial genomes of termitomyces. Genome Biol. Evol. 11, 1857–1869.
82. Jusino, M.A., Banik, M.T., Palmer, J.M., Wray, A.K., Xiao, L., Pelton, E., Barber, J.R., Kawahara, A.Y., Gratton, C., Peery, M.Z., and Lindner, D.L. (2019). An improved method for utilizing high-throughput amplicon sequencing to determine the diets of insectivorous animals. Mol. Ecol. Resour. 19, 176–190.
83. Mirarab, S., Reaz, R., Bayzid, M.S., Zimmermann, T., Swenson, M.S., and Warnow, T. (2014). ASTRAL: genome-scale coalescent-based species tree estimation. Bioinformatics 30, i541–i548.
84. Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35, 526–528.
**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) | | |
| COI primers | | |
| COI primers (coleopteran specific) | | |
| COI primers (metabarcoding) | | |
| ITS1 primers (insect specific) | | |
| **Software and algorithms** | | |
| BUSCO | | https://busco.ezlab.org |

(Continued on next page)
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_017657315.1
- GCA_017657335.1
- GCA_018221615.1
- GCA_018221635.1
- GCA_018221655.1
- GCA_018221735.1
- GCA_018221755.1
- 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_018856625.1
- 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 ‘termitomyctoid’ clade. 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 lyophyllloid herbarium specimens were obtained from the Royal Botanical Gardens KEW herbarium and the Cort herbarium. Fresh Lyophyllloid 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.
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 *Tricholoma constricta* did not have a pseudorhiza; however, this trait is variable within this species, and therefore, we scored it as ‘present’. In the case of *Termityctes* 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*. However, because some authors describe it as weakly rooting, 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 and we therefore scored it as ‘present’.

The presence of clamp connections has been reported in the following genera or species: *Asterophora*, *Blastosporella*, *Calocybe cyanea* and *Hypsizygus*, *Lyophyllum*, *Myochromella*, *Tricholoma*, *S. paluster*. The absence of clamp connections is reported in the following genera: *Arthromyces* and *Termityctes*.

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. 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’), 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 and Jerry/Pat. We also attempted amplification of the internal transcribed spacer 1 (ITS1) marker using the Vogler primer pair and the 16S marker using the Coleoptera specific primers Coleop_16Sc and Coleop_16Sd. 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 diet in arthropod contents in fecal samples of insectivorous animals. We used two different DNA barcodes: a 157bp target region of the CO1 using the primer pair ZBJ-ArtF1c/ZBJ-ArtR2c and a 156bp target region of 16S using the primer pair Ins16S_1shortF/Ins16S_1shortR. 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). 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. 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 -predict using a pretrained Augustus dataset for *Arthromyces cryptogamus* T132 as reference. We also ran funannotate -iprscan, funannotate -remote with antiSmash, and funannotate -annotate all with default settings.

**Marker selection and phylogenetic analysis**

We collected conserved orthologs using BUSCO with the basidiomycete reference gene set odb9 provided on the BUSCO website. We then removed any sequence sets for which we found fewer than 25 matches. Finally, we aligned the remaining sequence sets using MAFFT v.7.4756 with the following parameters:--auto --maxiterate 1000 --adjustdirection.

To remove poorly aligned regions, we used Gblocks v.0.91b 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 -sp -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). 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. 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. 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 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.

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