

Current Biology

Two Basidiomycete Fungi in the Cortex of Wolf Lichens

Highlights

- Most wolf lichens contain three fungal species in their cortex
- A *Tremella* fungus thought to be restricted to rare galls is ubiquitous as a yeast
- When in hyphal form, *Tremella* enwraps algal cells
- Bright-field microscopy underestimates secondary fungal occurrence in lichens

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In Brief

Lichens have historically been treated as symbioses of a single fungus and an alga, but shotgun DNA sequencing is enabling a re-evaluation of this census. Tuovinen et al. report a third fungal species in the cortex of 95% of sampled wolf lichens. The findings suggest that lichens may not be defined by any one universal combination of organisms.



Two Basidiomycete Fungi in the Cortex of Wolf Lichens

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SUMMARY

Since the late 1800s, mycologists have been detecting fungi above and beyond the assumed single fungus in lichen thalli [1–6]. Over the last century, these fungi have been accorded roles ranging from commensalists to pathogens. Recently, Cyphobasidiales yeasts were shown to be ubiquitous in the cortex layer of many macrolichens [7], but for most species, little is known of their cellular distribution and constancy beyond visible fruiting structures. Here, we demonstrate the occurrence of an additional and distantly related basidiomycete, *Tremella*, in 95% of studied thalli in a global sample of one of the most intensively studied groups of lichens, the wolf lichens (genus *Letharia*). *Tremella* species are reported from a wide range of lichen genera [8], but until now, their biology was deduced from fruiting bodies (basidiomata) formed on lichen thalli. Based on this, they have been thought to be uncommon to rare, to occur exclusively in a hyphal form, and to be parasitic on the dominant fungal partner [9, 10]. We show that, in wolf lichens, *Tremella* occurs as yeast cells also in thalli that lack basidiomata and infer that this is its dominant stage in nature. We further show that the hyphal stage, when present in *Letharia*, is in close contact with algal cells, challenging the assumption that lichen-associated *Tremella* species are uniformly mycoparasites. Our results suggest that extent of occurrence and cellular interactions of known fungi within lichens have historically been underestimated and raise new questions about their function in specific lichen symbioses.

RESULTS

We recently found *Cyphobasidium* yeasts (Cystobasidiomycetes) to be present in the cortex of many lichen species,

including *Letharia* [7]. In the course of analyzing metatranscriptomes and metagenomes from four species of *Letharia* lichens (Figure 1A) from western Montana, USA, we identified a third fungus, belonging to the basidiomycete class Tremellomycetes. The only previously described tremellomycete fungus from *Letharia* lichens is *Tremella lethariae* Diederich, heretofore recognized solely from basidiomata (hereafter referred to as “galls,” in keeping with convention) that had been found on three *Letharia vulpina* thalli [9, 11]. The specimens used for metagenome sequencing lacked galls and were morphologically unremarkable. In order to assess whether the tremellomycete DNA detected in the metagenomes from *Letharia* was identical to that from gall-inhabiting *T. lethariae*, we screened 440 newly collected and 369 herbarium specimens of *Letharia* for galls from which to extract DNA. We found galls on a total of nine thalli (Figure 1B; Data S1, sheet 3). Microscopic investigation confirmed the presence of *T. lethariae* in these galls (Figure 1C), and from each of the eight galls subjected to DNA extraction, PCR, cloning, and sequencing, we obtained internal transcribed spacer (ITS) rDNA sequences identical to those extracted from the metagenomes.

The occurrence of *T. lethariae* in all four investigated metagenomes motivated us to test how frequently it can be found in *Letharia* thalli that lack galls. We used *Tremella*-specific PCR primers to screen for the presence of *Tremella* in 316 of the new samples from *Letharia vulpina* and all five other species in the genus [12] collected in North America and Europe. We amplified *Tremella* DNA from 95% (300 out of 316) of the *Letharia* specimens (Data S1, sheet 1). Of these, 273 yielded readable sequences and 27 gave ambiguous sequences or failed sequencing reactions. The readable sequences could be assigned to two different lineages, *T. lethariae* and another strain previously detected from the lichen genus *Bryoria* and referred to as *Tremella* sp. B [13]. *Tremella lethariae* was found in all six studied species of *Letharia* and is not known from any other lichens or barcoding studies to our knowledge. *Tremella* sp. B. was found in *L. “barbata,” L. columbiana, L. lupina, and L. vulpina* (Data S1, sheet 1).

The occurrence of *Tremella* species in *Letharia* thalli exhibited a geographical pattern (Figure 1D). *T. lethariae* was detected in all 137 specimens from the United States. In addition, 19 of these



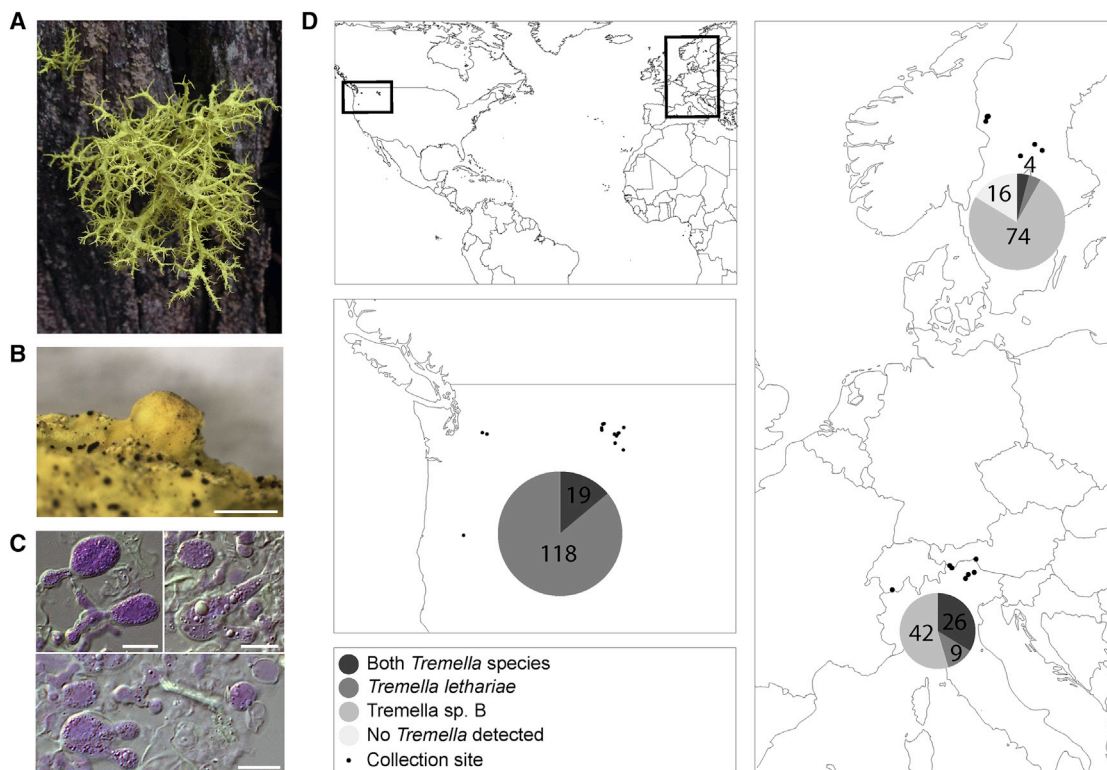


Figure 1. Letharia-Associated Tremella

(A) *Letharia vulpina*.

(B) *Tremella lethariae* gall on *Letharia vulpina*; scale bar 100 μm.

(C) Squash preparation of a *T. lethariae* gall pretreated with KOH and stained with phloxine, showing basidia in different developmental stages, basidiospores, and hyphae. Scale bars, 10 μm.

(D) Collection sites of *Letharia* specimens used for molecular studies and *Tremella* species detected in them.

specimens also harbored *Tremella* sp. B, and in Europe (Sweden, Italy, and Switzerland), *T. lethariae* was detected in 42 thalli (24% of the European specimens) and *Tremella* sp. B. in 146 thalli (83%). Both *Tremella* species were detected in the same thallus in 30 European specimens (Data S1, sheet 1). The sixteen specimens that resulted in negative PCR were all collected from Sweden.

Phylogenetic analysis of a three-locus alignment of *Tremella* sequences from *Letharia* metagenomes and a subset of previously published sequences of Tremellomycetes returned a topology (Figure S1) consistent with previous results from the group [13–15]. In this tree, *T. lethariae* forms a well-supported sister clade to *T. hypogymniae* Diederich & M.S. Christ. and is not closely related to *Tremella* sp. B. The clade with *T. lethariae* includes other *Tremella* species associated with lichens from the same ascomycete family to which *Letharia* belongs, *Parmeliaceae*, corresponding to the *Tremella* clade III from previous phylogenetic studies [14, 16].

We used fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy to simultaneously visualize the three fungi in *Letharia* thalli: *Letharia* (the ascomycete which bears the name of the lichen, as per the International Code of Nomenclature for algae, fungi, and plants); *Tremella*; and *Cyphobasidium*. We developed a new rRNA probe to ensure reciprocal exclusion of *Cyphobasidium* and *Tremella*, which are both basidiomycetes.

The *Tremella*-specific probe stained two types of cells: hyphae and yeasts. We observed hyphae of *T. lethariae* only in galls, but they were constant in all 25 galls imaged, from three geographical localities (Montana and British Columbia). Within each gall, the hyphae formed sexual fruiting structures (basidia) near the surface (Figures 2A–2F). Below the layer with basidia, hyphae extend into the gall interior, enwrapping and forming wall-to-wall contacts with algae (Figures 2A–2C and 2G–2J; Video S1). It is in this layer that we also observed what appear to be *Tremella* haustoria (Figure 2J). In contrast to the hyphal stage, the yeast stage was present in the gall-free cortex (Figure 3). This pattern was consistent in each imaged thallus ($n = 20$, from nine geographical localities in Montana, Alberta, and British Columbia). Yeast cells of *T. lethariae* were recorded budding and forming pseudohyphae in the gall-free cortex, as well as with possible conjugation tube initials in the algal layer inside galls (Video S1). From these experiments, we infer that the *Tremella* that we detected in non-gall-bearing thalli, including of *Tremella* sp. B (not visualized), is of the yeast form. Taken together, our imaging supports the conclusion that *T. lethariae* is dimorphic, with hyphal stages in galls and yeasts widespread in gall-free lichen cortex.

In both galls and gall-free cortex, *T. lethariae* always occurs intermixed with hyphae of *Letharia* and, especially in the cortex, with *Cyphobasidium* yeasts (Video S2). In addition to these fungi, the non-gall-bearing cortex of *Letharia* thalli also always includes

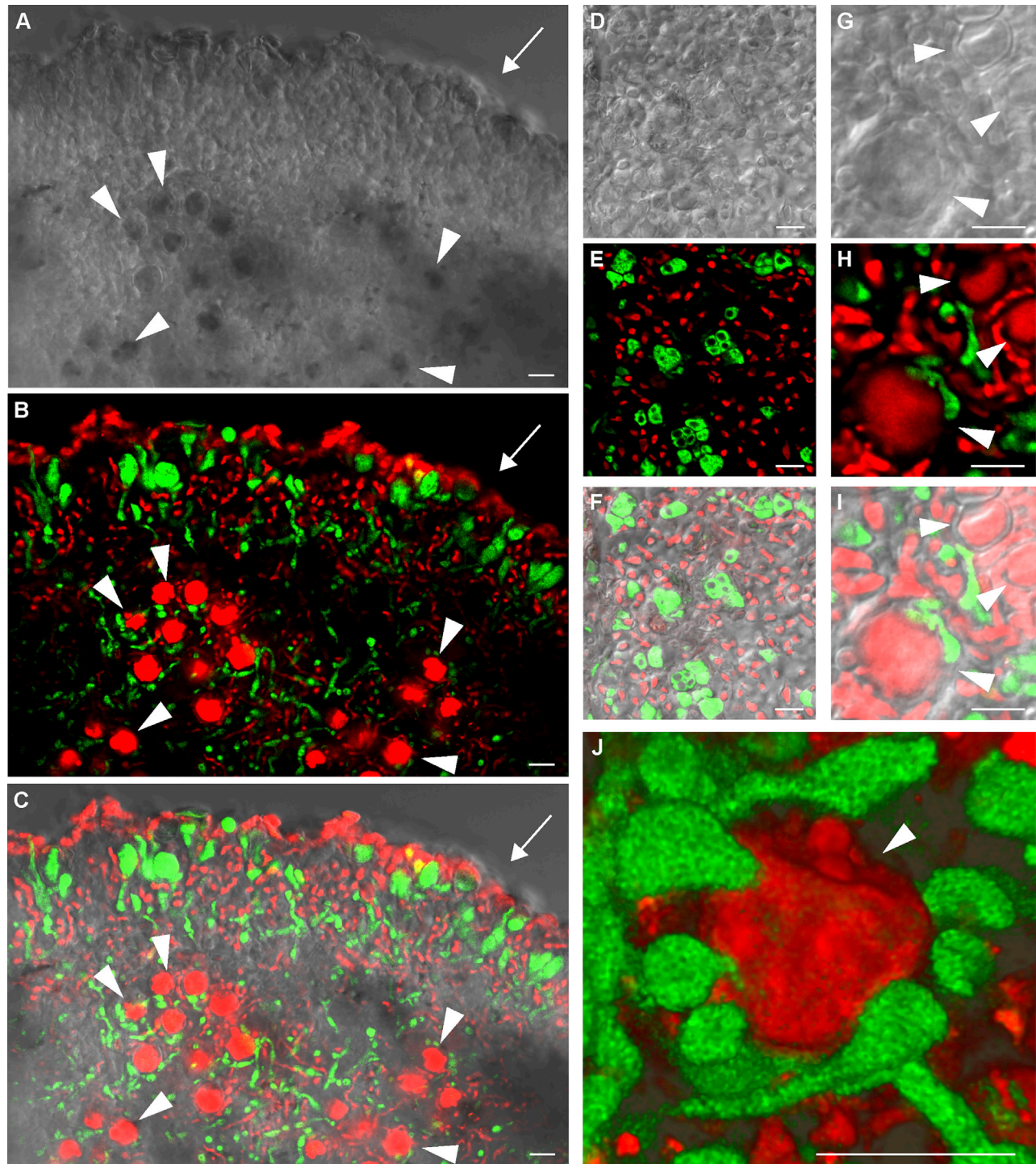


Figure 2. *Tremella lethariae* in Galls on *Letharia vulpina*

Pseudocolored confocal laser scanning microscopy images of fluorescent *in situ* hybridized *Tremella lethariae* galls on *Letharia vulpina* thalli. Green, *Tremella lethariae*; red, *Letharia vulpina* and algal autofluorescence.

(A–C) A cross section of a gall. The *Tremella* basidia are situated near the gall surface and surrounded by *Letharia* cells. Both *Tremella* and *Letharia* hyphae surround the algae beneath the layer of basidia inside the gall. Arrow points to autofluorescence of the outermost, cortical layer of the gall. Arrowheads point to algae (round, red cells >8 μm in diameter), but not all are marked. (A) Transmitted light (differential interference contrast [DIC]), (B) fluorescence, and (C) DIC and fluorescence are shown.

(D–F) Transverse section of the surface layer of a gall as shown from above, with mature four-celled and immature basidia of *Tremella* and *Letharia* cells in between. (D) Transmitted light (DIC), (E) fluorescence, and (F) DIC and fluorescence are shown.

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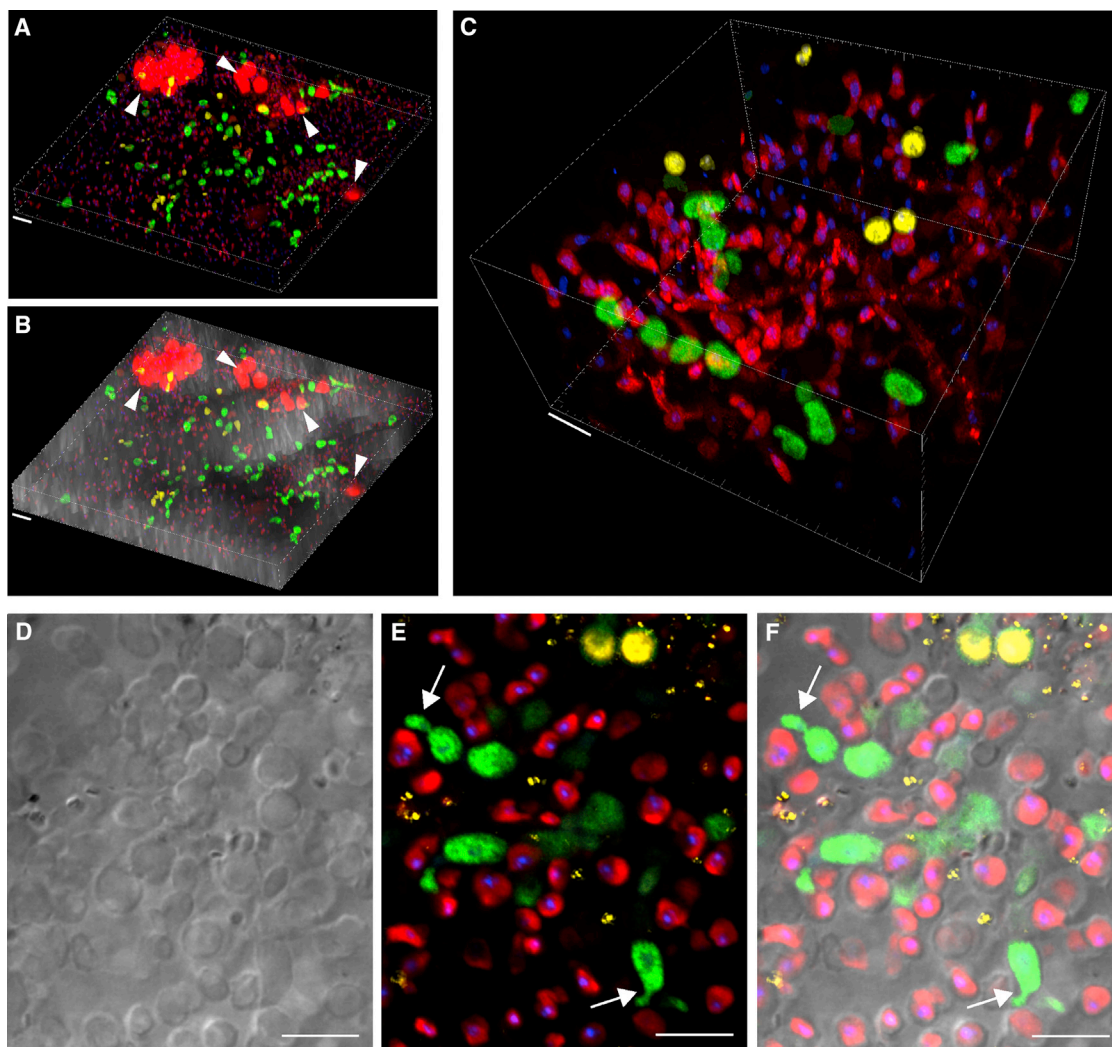


Figure 3. The Cortex of *Letharia* Lichens Contains Three Embedded Fungi

Pseudocolored confocal laser scanning microscopy images of fluorescent *in situ* hybridized *Letharia vulpina* cortex. Green, *Tremella lethariae*; red, *Letharia vulpina* and algal autofluorescence; yellow, *Cyphobasidium*; blue, nuclei.

(A–C) Maximum intensity projection of volume rendered cortex with *Tremella*, *Cyphobasidium*, and *Letharia*.

(A) Overview of the lower part of the cortex with some algae visible. Arrowheads point to algal groups (alga are round, red cells >5 μm in diameter).

(A) Fluorescence, (B) fluorescence, and transmitted light (DIC) are shown.

(C) Detail of the cortex, which always includes also nucleate cells that are not stained with the used probes. See also [Video S2](#).

(D–F) *Tremella* yeast cells budding (arrows) in the cortex surrounded by two *Cyphobasidium* yeast cells and numerous *Letharia* cells. (D) Transmitted light (DIC), (E) fluorescence, and (F) DIC and fluorescence are shown.

Scale bars, 10 μm. The color balance has been adjusted for the clarity of presentation.

nucleate cells of unknown identity that do not stain with any of the specific probes used in this study ([Figure 3C](#); [Video S2](#)). These yet unidentified cells may be quiescent (or beneath the detection level) or dead *Tremella*, *Cyphobasidium*, or *Letharia* cells, as previously hypothesized based on SEM results [7] and propidium iodide staining (data not shown).

DISCUSSION

As a species group, wolf lichens have been among the most intensively studied of all lichens. They were among the first for which phylogenetic species delimitation was implemented [12, 17–21] and for which the genetic structures of the both dominant fungal

(G–I) A close up of algal cells surrounded by *Tremella* and *Letharia* hyphae. Arrowheads point to algae. (G) Transmitted light (DIC), (H) fluorescence, and (I) DIC and fluorescence are shown.

(J) Blend projection of volume rendered algal layer with a possible *Tremella* haustorium intruding into an alga, with surrounding *Letharia* cells; see also [Video S1](#). The arrowhead points to an alga.

Scale bars, 10 μm. The color balance has been adjusted for the clarity of presentation.

(ascomycete) and algal symbionts were mapped for much of their range [17–19, 22]. As with the previous discovery of *Cyphobasidium* yeasts [7], our discovery of *Tremella* in 95% of *Letharia* thalli raises the question how a fungal species could evade detection for so long. In this case, the specific ITS reverse primer used in previous studies of *Letharia* [12, 17–22] has several mismatches to the corresponding *Tremella* sequence, excluding the latter fungus *a priori* from amplification using PCR.

Distantly related fungal yeasts, belonging to the genus *Cyphobasidium*, were previously reported in 98% of the same sample of *Letharia* lichens [7]. In that study, as in the present one, yeasts were detected in the cortex, the extracellular polymer matrix that forms the external “shell” of the lichen and imparts stability to the thallus [23, 24]. Combined with the ascomycete, which penetrates the cortex from within the thallus, this finding means that most *Letharia* lichens globally have a predictable suite of at least three fungal species. These results contrast with previous work on the hair lichen genus *Bryoria* [7]. Although *Tremella* species have been detected in *Bryoria* [13], we consistently found only a single basidiomycete, a species of *Cyphobasidium*, in 15 metatranscriptomes of *B. fremontii* and *B. tortuosa* [7], with which *Letharia* regularly co-occurs in North America. We have no evidence to suggest that any *Tremella* is a constituent component of these *Bryoria* species. Though only a few lichen symbioses have so far been sampled with this intensity, our data suggest that not only the identity but also the number of fungal species embedded in the lichen cortex may be lichen specific.

Our findings highlight the limitations of bright-field microscopy to infer biology and life history traits of unculturable fungi. Yeasts, the previously unknown life cycle stage of lichen-associated *Tremella* [25], in fact appear to be the form that is most common in nature and are likely to have been handled by anyone who has ever held a wolf lichen. Furthermore, it was previously assumed that lichen-associated *Tremella* species are mycoparasitic [9, 10, 26]. The microscopic evidence for the mycoparasitic nature of *Tremella* species relies largely on observations of fungal-fungal haustoria [27, 28]. We did not observe these in *T. lethariae*. Instead, we observed wall-to-wall contact with algal cells, which has not previously been reported for lichen-associated *Tremella*. The contacts are strikingly similar to hyphal-algal enmeshing that is often taken as the basis for recognizing successful lichen resynthesis *in vitro* [29–31]. The contacts formed by *Tremella* resemble intraparietal haustoria formed by the dominant ascomycete on algal cell walls [32], but ultrastructural studies are needed to verify the extent and type of penetration into the algal cell.

Our metatranscriptome data would seem to bring the demonstration of the function of *Tremella* within wolf lichens tantalizingly close. However, our limited knowledge of the biochemical recognition patterns involved in establishing lichen symbiosis means there are hard limits to what can be inferred from gene expression data at this time. For example, the conversion of algal ribitol to fungal mannitol, considered the foremost goods transfer of ascomycete-alga symbiosis [33], is thought to be catalyzed by the multifunctional pentose phosphate pathway [34]. However, all four eukaryotes in wolf lichens possess this pathway, and the assumption that *Tremella*, like the ascomycete, converts algal-derived polyols into mannitol is itself untested. Approaching this question with transcriptome data would also require

sampling from galls. Furthermore, symbiosis-specific lectins have been hypothesized as part of a recognition mechanism for lichen symbionts [35, 36], but even if a cellular communication pathway were known, it would likewise presuppose that the mechanism is the same between the ascomycete and *Tremella*. Surprisingly, few other examples of molecular give and take have been advanced. Analysis of our metatranscriptome data reveals that the most highly expressed tremellomycete genes are either unassignable to any known protein or relate to common cell functions. The exercise highlights the present limits of metatranscriptome data from lichen symbioses, for which few reference genomes are unavailable, >30% of putative proteins have no known function [37], conserved proteins cannot unambiguously be mapped to one of multiple fungi, and the biosynthetic pathways that could reveal function are themselves not known.

Our results have far-reaching implications for what we think we know, and what we know we do not know, regarding lichen-associated fungi. Over 1,800 species of fungi have been described from lichens that are now considered to be “commensalistic,” “pathogenic,” “parasymbiotic” [2], “asymptomatic,” “endolichenic” [4], or otherwise “lichenicolous” [8, 26]. Each of these terms implies a degree of certainty regarding either the interaction of the fungus in question with the lichen symbiosis or its extrinsicity to the lichen (the two latter terms, notwithstanding their neutral etymology, are never used for the dominant ascomycete). The results from our current study highlight at least two reasons why application of these adjectives to lichen fungi may with few exceptions be premature and encumber scientific inquiry. First, all descriptions of fungi from natural lichens have until now relied on bright-field microscopy, typically on sections. However, hyphae distant from a fruiting structure quickly become indistinguishable, leaving the extent of a fungus in the lichen thallus, its interactions with other fungi, or its absence a matter of conjecture [38]. Second, we can only know the phenotype of a thallus without a given fungus if we can either certify its absence or experimentally and specifically kill it and watch the effect on the phenotype. The latter makes use of the word “asymptomatic” for yeast-containing thalli in the best case presumptuous; even referring to *Tremella* basidiomata as “symptoms” implies bias concerning their extrinsicity. If we ultimately find that *Tremella* is required for the formation of a lichen, it could reasonably be expected to modify the thallus surface in its sexual stage, much as the ascomycete does. It may be more honest to concede we do not know the function of a fungus, even if this temporarily impinges on long-standing assumptions regarding the minimum components of a lichen [39].

Letharia lichens are the first and so far only lichens in which the extent of occurrence of *Tremella* has been studied over a global sample with molecular methods. Species of *Tremella*, occurring in a sexual stage in galls, have however been described from over 60 different lichen species [8] and together constitute the most widespread basidiomycete lineage documented based on morphological data from ascomycete lichens (Cyphobasidiales yeasts are now known from many lichens but mostly from DNA) [7]. *Tremella* galls are usually thought to be restricted to a fraction of the overall lichen species population, and some are globally rare [9]. For most species, the assumption that the galls represent the extent of *Tremella* occurrence will now need to be revisited. In addition to our present results, the

possibility that other lichen-specific *Tremella* species include a yeast stage is supported by the detection of *Tremella* DNA in barcoding studies conducted on thalli of *Cetrariella*, *Cladonia*, *Flavocetraria*, *Hypogymnia*, and *Peltigera* lichens [40, 41].

Based on current data, *Tremella* in wolf lichens is limited to two lineages, compared to six species of ascomycete that form the hyphal core of the six corresponding wolf lichen species. Remarkably, even in North America, where some species of *Letharia* frequently reproduce sexually [42] and where the only *Tremella* galls have been encountered, identical *Tremella* ITS variants were shared between thalli. The presence of identical *Tremella* ITS variants in transatlantic populations, similar to *Cyphobasidium* in *Letharia* [7], suggests that these fungi can be co-dispersed in their yeast stage in the symbiotic propagules (isidia, soredia, and thallus fragments) of *Letharia* lichens, similar to what has been suggested for bacteria co-dispersing on lichen isidia [43]. Co-dispersal in asexual propagules may explain the observed geographic pattern of the two *Tremella* species. It may also explain the single batch of negative screening results in which no *Tremella* was detected by PCR. Certifying the absence of *Tremella* in these 16 samples, all from a concentrated area of central Sweden, is non-trivial. These samples consisted only of the thallus tips and were thus among the smallest assays used in this study, from a part of the range of *Letharia* in which the species is so rare the lichens are legally protected. Resampling these populations with metagenome sequencing could clarify whether *Tremella* yeasts are ever absent from *Letharia* lichens.

Conclusions

Shotgun DNA- and RNA-based approaches have so far returned results inconsistent with long-standing assumptions regarding the number of eukaryotes present in a given lichen. We still do not know the function of these newly discovered players in lichen symbiosis, but the fact that we were able to achieve *in situ* rRNA hybridizations of *Cyphobasidium* and *Tremella* cells in the *Letharia* cortex shows that these cells are physiologically active components of that layer. One approach to the discovery of fungal yeasts in lichens has been to continue to assert their extrinsic nature relative to the lichen symbiosis [44]. Another approach may be to incorporate the new information into an alternate model in which macrolichen symbiotic outcomes are shaped by the microbial composition of the cortex as biofilm [39]. It is still unknown what the composition of this biofilm is in most lichens, what organisms give and take, and how they potentially affect its specific shape, chemistry, and biophysical properties. Future studies will tell and allow us to test competing models of what makes a macrolichen.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, two tables, one data file, and two videos and can be found with this article online at <https://doi.org/10.1016/j.cub.2018.12.022>.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.T., T.S., and H.J.; Methodology, V.T. and D.V.; Formal Analysis, S.E. and T.S.; Investigation, V.T., G.T., T.S., and H.J.; Resources, G.T., T.S., and H.J.; Data Curation, V.T.; Writing – Original Draft, V.T., T.S., and H.J.; Writing – Review & Editing, V.T., S.E., G.T., T.S., and H.J.; Visualization, V.T.; Supervision, T.S. and H.J.; Funding Acquisition, V.T., G.T., T.S., and H.J.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
For lichen specimens used in this study, See Data S1	UPS, this study	Data S1
Chemicals, Peptides, and Recombinant Proteins		
SlowFade Diamond	Thermo Fisher	Cat#S36972
Chitinase from <i>Streptomyces</i>	Sigma	Cat#C6137-25UN
Critical Commercial Assays		
DNeasy Plant Mini Kit	QIAGEN	Cat#69106
TruSeq Nano DNA Sample Preparation Kit	Illumina	Cat#15041110, rev. B
Ambion Ribopure Yeast Kit	Life Technologies	Cat# AM1926
TrueSeq Stranded RNA Kit	Illumina	Cat# 20020594
Scientific Green Phusion High-Fidelity DNA polymerase kit	Thermo Fisher	Cat#F534S
DreamTaq polymerase	Thermo Fisher	Cat#EP0705
TOPO TA Cloning kit for sequencing	Thermo Fisher	Cat#K457502
Exonuclease I	New England BioLabs	Cat#M0293S
SAP (Shrimp Alkaline Phosphatase)	USB, Affymetrix	Cat#70092Y
BigDye XTerminator™ Purification Kit	Thermo Fisher	Cat#4376486
Deposited Data		
Raw sequencing reads	This paper	https://www.ncbi.nlm.nih.gov/sra/SRP149293
<i>Tremella</i> ITS sequences	This paper	GenBank: MG774334–MG774390
<i>Letharia</i> ITS sequences	This paper	GenBank: MG645014–MG645052
Alignments	This paper	http://purl.org/phylo/treebase/phyloWS/study/TB2:S23378
Oligonucleotides		
ITS1F	[45]	N/A
BasidLSU3-3	[14]	N/A
ITS1Tm1F (5'-TTAGTGATTGGCCCTTGG-3')	This paper	N/A
LSUTm1R (5'-CGTCAAGTACGGGATTGTC-3')	This paper	N/A
TmSpB1F (AATGATTGGCCTACGTGG)	This paper	N/A
TmSpB1R (GTCAAGTACGGGATTATCACC)	This paper	N/A
Probes and helper probes for FISH, see Table S2	This paper [7]	Table S2
Software and Algorithms		
Trimmomatic 0.32	[46]	http://www.usadellab.org/cms/?page=trimmomatic
FastQC	[47]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
SPAdes	[48]	http://cab.spbu.ru/software/spades/
Trinity 2.0.6	[49]	https://www.hpc.science.unsw.edu.au/software/trinity/206
blastn	[50]	https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE=TYPE=BLASTHome
Fiji	[51, 52]	https://fiji.sc
Huygens Professional v. 17.04	Scientific Volume Imaging, the Netherlands	https://svi.nl
IMARIS v. 9.2	ImarisXT, Bitplane AG	https://bitplane.com
MAFFT 7.305	[53]	https://mafft.cbrc.jp/alignment/software/
PASTA 1.6.4	[54]	https://github.com/smirarab/pasta
OPAL 2.1.3	[55]	https://omictools.com/opal-tool

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RAxML 7.2.6	[56]	https://cme.h-its.org/exelixis/web/software/raxml/
IQ-TREE 1.6.6	[57–59]	https://embnet.vital-it.ch/raxml-bb/
MrBayes 3.2.6	[60, 61]	http://mrbayes.sourceforge.net/download.php
Tracer 1.7.1	[62]	http://tree.bio.ed.ac.uk/software/tracer/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Veera Tuovinen (tuovinen.veera@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

A total of 809 specimens of *Letharia* were included in this study. Of these, 369 were previously deposited in the herbarium UPS and included 82 North American, 263 European, 18 African and 6 Asian specimens (Data S1, Sheet 2). The remaining 440 were specimens collected in the United States (n = 147), Canada (118), Italy (60), Switzerland (17), and Sweden (98) specifically for this study (Data S1, Sheets 1 and 3). In Sweden, only a small piece of each thallus was sampled because *Letharia vulpina* is red-listed in this country. 316 of the newly collected specimens were used for molecular studies (Data S1, Sheet 1). Based on a combination of morphological and ITS sequence variation, we assigned these new specimens to the following species: *L. 'barbata'*, *L. columbiana*, *L. gracilis*, *L. lupina*, *L. vulpina*, *L. 'rugosa'* [12, 18, 20, 21].

METHOD DETAILS**Brightfield microscopy**

All thalli were visually inspected under a dissecting microscope for the presence of *T. lethariae* galls. Two specimens with galls were investigated in more detail: hand-cut sections of the outermost layer of the gall were pre-treated with KOH (5%), stained with phloxine (1% in water) and observed under a compound microscope.

DNA and RNA extraction

The total DNA was extracted from the 316 newly collected thalli using the DNeasy Plant Mini kit (QIAGEN). A small piece of each thallus was placed in a 2 mL safe lock tube (Sarstedt) together with two sterile 3 mm tungsten carbide beads (QIAGEN), dried at +46°C for 30 min, then frozen in –80°C for 30 min to overnight and finally ground with a TissueLyser II (QIAGEN) with 25 r/sec for 1 min or until the sample was completely pulverized. Subsequently, the manufacturer's instructions were followed with a prolonged incubation at 65°C for 1 hr. For RNA extraction, a fresh piece from each thallus was frozen with beads in liquid nitrogen and grinded as above. The total RNA was extracted with the Ambion Ribopure Yeast Kit (Life Technologies).

Molecular identification of *Letharia* species

Letharia ITS was amplified with the primer pair ITS1F [45] and ITS4A [18] and the Scientific Green Phusion High-Fidelity DNA polymerase kit (Thermo Fisher; used for all PCR reactions in this study if not otherwise mentioned) with annealing at 57.5°C for 30–35 cycles. The PCR products were cleaned with Exonuclease I (New England BioLabs) and SAP (Shrimp Alkaline Phosphatase, USB). Samples were prepared for sequencing with BigDye® X Terminator™ Purification Kit (Applied Biosystems) and sequenced with an ABI 3730 XL DNA Analyzer (Thermo Fisher) using the PCR primers.

Metagenome and metatranscriptome sequencing

Both metatranscriptomes and metagenomes from one thallus each of four *Letharia* species, *L. columbiana*, *L. lupina*, *L. vulpina* and *L. 'rugosa'*, were sequenced. Libraries for sequencing were prepared using the TruSeq Nano DNA Sample Preparation Kit (#15041110, rev B). Each of the samples were sequenced on 1/5 lane of Illumina HiSeq with 100bp paired-end sequencing by the SNP&SEQ Technology Platform, Science for Life Laboratory at Uppsala University. Libraries for the mRNA sequencing were prepared using the Illumina TruSeq Stranded RNA Kit and sequenced on an Illumina HiSeq at the University of Utah sequencing core, each sample on 1/6 of a lane.

Identification of tremellomycete contigs

The raw reads were trimmed and quality controlled using Trimmomatic 0.32 [46] (with options ILLUMINACLIP:/adapters.fa:2:20:10:4:true LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:25 HEADCROP:0) and the results were visually inspected using FastQC [47]. The genomes were *de novo* assembled with SPAdes [48] with raw reads and k-mer lengths of 55, 75, 85, 95. The quality-controlled cDNA reads from metatranscriptomes, excluding the orphan reads, were assembled using

Trinity 2.0.6 [49]. Taxon identities were assigned to the transcript contigs using protein predictions as in [7]. Open reading frames (ORF) were calculated from the assembly using the Trinity plug-in transdecoder_r2012-08-15. Contigs containing nuclear ribosomal DNA corresponding to tremellomycete fungi from all four assembled metagenomes were extracted using blastn [50]. These contigs were subsequently used for both phylogenetic analyses and FISH probe design.

Molecular identification of *T. lethariae*

Total DNA from eight galls (from three specimens; Data S1, Sheet 1) fitting the description of *T. lethariae* [9, 11] was extracted as above. Parts of the tremellomycete ITS and nrLSU were PCR amplified using the primer combination ITS1F and BasidLSU3-3 [14] with annealing at 53°C for 30–35 cycles. In addition, to verify that only one tremellomycete taxon was present in each gall, four of these galls were subjected to PCR using the same general primers and DreamTaq polymerase (Thermo Fisher). This polymerase creates a 3'-A overhang that facilitates cloning and sequencing of multiple PCR-products from the same gall. The PCR products were cloned with the TOPO TA Cloning kit for sequencing (Thermo Fisher), and twenty-three clones from each gall were sequenced. The non-cloned PCR amplicons from the galls were sequenced with BasidLSU3-3 and ITS1Tm1F (Key Resources Table), a specific primer designed in this study based on sequences of the tremellomycete fungus found in the metagenomes. The clones were sequenced from one direction using the general primer ITS1F.

PCR screening for tremellomycete fungi

Multiple thalli were sampled at each of 12 sites in Europe and six sites in western North America, as outlined in Data S1, Sheet 1. Sampling was stratified by subregions in which *Letharia* lichens occur (Sweden, Switzerland, Italy, western North America); within subregion sites were sampled that were a minimum of 20 km distant from each other; and each thallus gathered was taken randomly from a different tree. In addition, we used singleton samples provided by colleagues from 16 further sites on both continents. We were unaware of the presence of *Tremella* at the time of collection; all sampling was blind with respect to *Tremella* yeast occurrence in thalli, and all samples were used. The occurrence of tremellomycete fungi in the DNA extractions from the 316 *Letharia* thalli collected for this study (Data S1, Sheet 1) was investigated using *Tremella*-specific PCR primers. First, the primer combination ITS1F and BasidLSU3-3 was used to screen all samples. Thereafter, since both *T. lethariae* and *Tremella* sp. B. [13] (identical ITS variant) were frequently amplified and sequenced with the general primers from the European specimens, we designed specific primers to target these *Tremella* species separately; while *T. lethariae* was screened for with the primer pair ITS1Tm1F and LSUTm1R, *Tremella* sp. B. was amplified with the primer pair of TmSpB1F and TmSpB1R (Key Resources Table). The amplifications were performed with annealing at 53°C, 59.9°C or 59°C for the respective primer pair. PCR products were sequenced with the same primers used in the PCR reaction. Samples resulting in negative PCR were given three attempts with all primer combinations and changes in DNA concentration in the reaction. Then, the samples still resulting in negative PCR reaction were subjected to new DNA extractions if enough thallus material was available. These extractions were subjected to PCR with both specific primer pairs. In cases ($n = 27$) with an unambiguous or failed sequencing reaction but a PCR amplicon of the right size (resulting from reaction with the specific primers and assessed on 1% agarose gel) the results were counted as positive.

Taxon and marker selection for phylogenetic analyses

In order to place *T. lethariae* into a phylogenetic context, a subset of publicly available *Tremella* sequences of three markers was gathered: the nuclear ribosomal small subunit (nrSSU), the internal transcribed spacer (ITS) region (including the 5.8S) and the nuclear ribosomal large subunit (nrLSU) [13–15] (Table S1). As these markers are physically consecutive and different principles for delimiting those markers have been applied, we concatenated the data and carried out preliminary alignments to establish consistent borders between the markers and finally added the four sequences extracted from the metagenomes to this alignment. We used the GATCATN motif to define the 3' end of nrSSU, defined the 5.8S Rfam borders as in [63], and used the 5' GACCT motif to define the 5' end of nrLSU. Subsequently, we divided the data into nrSSU, ITS1, 5.8S, ITS2, and nrLSU.

Fluorescent *in situ* hybridization

We conducted FISH in order to simultaneously visualize and locate different fungi in *Letharia* thalli, and to determine whether yeasts were consistently associated with gall-free and hyphae with gall-bearing thalli. We sampled thalli for FISH from nine replicate localities in Montana and western Canada as stated in Results. Wherever possible, we imaged pairs of gall-bearing and non-gall-bearing thalli from the same locality (Data S1, Sheet 3), but were limited by the total available localities of gall-bearing thalli. A total of 124 thalli were collected for screening for fresh galls for FISH imaging in 2018. We designed specific probes and helper probes that match *Tremella*, but discriminate against *Letharia*, *Cyphobasidium* [7] and *Lichenostigma maureri* (NCBI accession KF176953), the latter an ascomycete that frequently occurred on *Letharia* thalli (Table S2). Probes were fluorescently monolabeled at the 5' end and targeted 18S or 28S rRNA. We tested and optimized the probes and protocols directly on fresh lichen thalli, as no pure cultures of the investigated fungi were available. The probe that gave the strongest signal for *Tremella* (Tm28S1) was further used in subsequent experiments, together with the probes specific to Lecanoromycetes and *Cyphobasidium* (Table S2). Hoechst 33342 was used for staining nuclei (Table S2). Hand-cut fragments of fresh *Letharia* branches from 20 individuals without galls (1–6 branches/individual) and galls from six individuals (25 galls in total) were fixed in 4% formaldehyde at +4°C for 2 h (Data S1, Sheets 1 and 3). The fragments were permeabilized at +36°C for 1 h in 1x PBS with 0.1 mg/ml (0.04 U) chitinase from *Streptomyces* (Sigma) and 1% SDS. The fragments were then washed 3 × 10 min in 1 × PBS, followed by 3 min dehydrations in 50%, 80% and 99.7% EtOH, and air-dried.

Hybridization was carried out in 1.5 mL test tubes in a solution of 1 μ M of each probe and helper probe, 0.5 μ M Hoechst and hybridization buffer consisting of 10% formamide, 0.01% SDS, 0.9 M NaCl and 20 mM Tris-HCl (1 M pH 7.2) at 46°C for 2–3 hr. After removal of the hybridization solution, fragments were incubated in a washing buffer (0.45M NaCl, 0.02M Tris-HCl, 0.01% SDS) for 20 min at 48°C, followed by a 10 min wash in 1 \times PBS at RT, air-dried and mounted on microscope slides with SlowFade Diamond (Thermo Fisher). A negative control without probes was included for each hybridization reaction.

Confocal laser scanning microscopy

We carried out CLSM of the hybridized slides by using a Leica SP5 microscope with a Leica HXC PL APO 63x NA 1.4 glycerol or 40x NA 1.25 oil immersion objective. The laser lines employed, and the detected emission wavelengths are listed in Table S2. Based on the negative controls, we selected laser intensities and gain values that prevented interpretation of false positives in samples with probes. Probes labeled with FAM and CY5 were imaged simultaneously and the rest sequentially. Z stacks of lichen thalli were acquired using $0.8 \times 0.8 \times 0.71 \mu\text{m}$ (Figure 2J, Video S1), $0.19 \times 0.19 \times 1.5 \mu\text{m}$ (Figures 3A and 3B) and $0.08 \times 0.08 \times 0.21 \mu\text{m}$ (Figure 3C, Video S2) sampling (x/y/z) for volume rendering of algal layer inside a gall and non-gall bearing cortex.

Image processing

We processed the 2D-images using Fiji [51, 52]. The z stacks were deconvolved with Huygens Professional v 17.04 (Scientific Volume Imaging, the Netherlands, <https://svi.nl>) using the classic maximum likelihood estimation algorithm, 40 iterations and with signal-to-noise ratio set to 15–20. Volume rendering was prepared with IMARIS v9.2 (ImarisXT, Bitplane AG, <https://bitplane.com>). Noise reduction was performed with Gaussian or median filtering and we adjusted color balance for the clarity of presentation. For the Video S2, some of the noise and autofluorescence from different channels was masked for the clarity of presentation.

QUANTIFICATION AND STATISTICAL ANALYSIS

The 316 thalli screened for *Tremella* by PCR derived from samples gathered 2013–2015. A total of 124 thalli were collected for screening for fresh galls for FISH imaging in 2018 but not additionally screened by PCR. In all analyses, n refers to individual thalli as listed in Data S1 Sheets 1, 2 and 3.

Phylogenetic analyses

Alignment

Sequences from each of the five markers were aligned separately. We used the E-INS-i algorithm of MAFFT 7.408 to align the nrSSU and nrLSU, and the G-INS-i algorithm for the 5.8S data [53]. The ITS1 and ITS2 were aligned using PASTA 1.8.4 [54] using MAFFT E-INS-i as aligner, OPAL 2.1.3 [55] as merger, and RAXML 7.2.6 [56] with a GTR+I+ Γ model as tree estimator. PASTA was set to run for a maximum of 100 iterations, stopping when there was no likelihood improvement for 10 iterations, and keeping the best tree and alignment. No alignment sites were excluded from downstream analyses.

Model selection, conflict assessment, and phylogeny estimation

We used IQ-TREE 1.6.6 for model selection, including partitioning, and maximum likelihood (ML) phylogeny estimation [57–59], including an assessment of potential topological conflicts between the nrSSU, ITS region, and nrLSU. Each marker was subjected to preliminary phylogeny estimation by use of the Bayesian Information Criterion to select the best 1-, 2-, or 6-rate likelihood model in the general time-reversible family. The gamma distribution on rate heterogeneity across sites was approximated with four discrete categories. We performed 1000 non-parametric bootstrap replicates with default search settings. As we detected no conflicting consensus topologies supported by bootstrap proportions at, or above, 70%, we concatenated the markers and repeated the model selection and non-parametric bootstrap, this time also including a partitioning scheme. IQ-TREE selected a partitioned model consisting of four independently parameterized subsets with proportional rates: (1) nrSSU and 5.8S with a GTR+I+ Γ model, (2) ITS1 with a GTR+ Γ model, (3) ITS2 with a SYM+G model, and (4) nrLSU with a SYM+I+ Γ model. Bayesian phylogeny estimation was carried out using MrBayes 3.2.6 [60, 61] with the same partitioned model as in the ML analysis. Priors included a uniform distribution on topology, and (when applicable) an exponential distribution with mean 1 on the gamma shape parameter describing rate heterogeneity across sites, a uniform (0, 1) distribution on the proportion of invariable sites, a (1, 1, 1, 1, 1) Dirichlet on the rate matrix, a (1, 1, 1, 1) Dirichlet on state frequencies, and a (1, 1, 1, 1) Dirichlet on the proportional subset rates. We assumed a compound Dirichlet prior on branch lengths [64, 65]. For the gamma distribution component of this prior, we set $\alpha = 1$ and $\beta = 0.2$, as the expected tree length (from the maximum likelihood analysis) was approximately $\alpha/\beta \approx 5$. The Dirichlet component of the distribution was set to the default (1, 1). Five parallel Markov chain Monte Carlo runs were performed, each with four chains and the temperature increment parameter set to 0.15 [66]. The appropriate degree of heating was determined by observing swap rates between the cold and hot chains in preliminary runs. Every 1000th tree was sampled. Topological convergence was assessed every 10^6 generations, removing the first 50% of the tree sample as burn-in. The analysis was halted when the average standard deviation of splits (with frequency 0.1) between runs fell below 0.01, which was achieved after 11×10^6 generations. Effective sample sizes of model parameters in the posterior sample were estimated using Tracer 1.7.1 [62] and found to range from 8449 to 23570, indicating that posterior sampling was sufficient. Potential scale reduction factors deviated from 1 by a maximum of 8×10^{-3} for branch lengths and 9×10^{-4} for model parameters, indicating a stable posterior sample.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the acquired ITS sequences of *Tremella* and *Letharia* reported in this paper are GenBank: MG774334–MG774390 and GenBank: MG645014–MG645052, respectively. The accession number for the raw reads reported in this paper is SRA: SRP149293 ([Data S1](#), Sheet 1). The accession number for the alignments reported in this paper is TreeBASE: S23378.