Tremella macrobasidiata and Tremella variae have abundant and widespread yeast stages in Lecanora lichens

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Summary

Dimorphism is a widespread feature of tremellalean fungi in general, but a little-studied aspect of the biology of lichen-associated Tremella. We show that Tremella macrobasidiata and Tremella variae have an abundant and widespread yeast stage in their life cycles that occurs in Lecanora lichens. Their sexual filamentous stage is restricted to a specific lichen: T. macrobasidiata only forms basidiomata on Lecanora chlarotera hymenia and T. variae only on Lecanora varia thalli. However, the yeast stage of T. macrobasidiata is less specific and can occur in L. varia lichens, whilst all life stages of T. variae may be specific to L. varia. Contrary to the hyphal stages, the yeasts are distributed across the thalli and hymenia of Lecanora lichens, and not limited to specimens with basidiomata. Tremella macrobasidiata was present in all studied L. chlarotera, and in 59% of L. varia specimens. Only in 8% of the L. varia thalli could none of the two Tremella species be detected. Our results indicate that lichen-associated Tremella may be much more abundant and widespread than previously assumed leading to skewed estimations about their distribution ranges and lichen specificity, and raise new questions about their biology, ecology and function in the symbiosis.

Introduction

The era of molecular studies has shown that most of the diversity of life is microbial (Pace, 1997; Castelle and Banfield, 2018). Lichens, symbiotic consortia formed by at least two different microorganisms, are a great manifestation of microbial diversity in a unique package; several studies in the last decade have continued to demonstrate the since long recognized presence of diverse fungal communities within lichen thalli (U’Ren et al., 2010, 2012; Fleischhacker et al., 2015; Grube and Wedin, 2016; Noh et al., 2020). In recent years, attention has been drawn especially to the presence of previously undetected basidiomycete yeasts in different macrolichens (S.Gridley et al., 2016; Černajová and Škaloud, 2019, 2020; Tuovinen et al., 2019; Mark et al., 2020). However, little is still known about the ubiquity of most of the individual organisms and their potential contributions or consequences for the lichen symbiosis (S.Gridley et al., 2016, 2020; Š Gridley, 2018).

Yeasts as a life cycle stage have originated in multiple distantly related clades of Fungi, and potential for yeast growth evolved early in their evolution (Kurtzman et al., 2011; Nagy et al., 2014). Dimorphic fungi have the ability to switch between an unicellular yeast form and a filamentous stage, sometimes in response to environmental cues (Sánchez-Martínez and Pérez-Martín, 2001; Lin, 2009). To date, one of the most studied and species rich group of lichen-associated basidiomycetes is Tremellomyces (Agaricomycotina), with over 60 described species belonging to Tremella s. lat. (Diederich et al., 2018) (http://www.lichenicolous.net). Many Tremellomyces have a dimorphic life cycle (Bandoni, 1995), but lichen-associated Tremella species have been mainly studied when they form sexual fruiting bodies (basidiomata) on the lichen thalli or on/in the fruiting bodies (apothecia) of the ascomycete lichen symbiont. Thus, for most species only the filamentous life stage is well known and has been studied in detail (Diederich, 1996; Millanes et al., 2021). However, in a recent study Tuovinen and colleagues (2019) showed...
that *Tremella lethariae* has a common and widespread yeast stage in thalli of *Leptaria* and seems to enter the sexual cycle only rarely. It is probable, that other lichen-associated *Tremella* species likewise have a yeast stage in their life cycle, but it has not been detected due to technical difficulties. Observations of basidiomeres germinating by repetition or budding, presence of conidia in *Tremella* basidiomata (Diederich, 1996; Zamora et al., 2011; Millanes et al., 2015), *Fellomyces* yeasts isolated from lichens (Prillinger et al., 1997), as well as Tremellomycete DNA records from lichen thalli without visible basidiomata (Ekman, 1999; Lindgren et al., 2015; Zhang et al., 2015; Wang et al., 2016; Fernández-Mendoza et al., 2017; Banchi et al., 2018), support this hypothesis. Nevertheless, complete life cycle and distribution of the different life stages and their prevalence in lichens remain unknown for most *Tremella* species.

Lichens as symbiotic entities do not have names (Goward, 2008), and in the following text we use the name of the ascomycete fungal symbiont followed by the term ‘lichen’ when referring to a whole lichen thallus including all its symbionts. *Letharia* lichens are macrolichens with a stratified thallus, in which *Tremella* cells are embedded in a thick polysaccharide matrix in the cortex (Tuovinen et al., 2019). However, several *Tremella* species have been described from crustose lichens that lack a clear thallus stratification and a thick, well-defined cortex, such as some representatives of *Lecanora* s. lat. and *Lecidea* s. lat (Diederich, 1996; Zamora et al., 2011, 2016, 2018). *Tremella* species associated with these two genera are nested within a group of *Tremella* species associated with stratified macrolichens in the Parmeliaceae, including also *T. lethariae* (Zamora et al., 2011, 2016, 2018; Tuovinen et al., 2019). For this study, we chose two *Tremella* species that produce basidiomata on different and well-defined parts of two different *Lecanora* lichens: *Tremella macrobasidiata* forms basidiomata only on the apothecial hymenium of *Lecanora chlorotera*, and *Tremella variae* only on the thallus of *Lecanora varia*, including the thalline margin of the apothecium (Zamora et al., 2011, 2016). This divergent location on the two lichens is remarkable, particularly as the two *Tremella* species are phylogenetically very closely related. On the other hand, the studied *Lecanora* species are part of different species complexes and phylogenetically not very closely related with each other (Zhao et al., 2016).

We wanted to study how, and at which life cycle stage, *Tremella* occurs in the thalli of these crustose lichens. *Tremella macrobasidiata* basidiomeres have been observed to germinate by ballistoconidia and blastoconidia in basidiomata formed on *L. chlorotera*, but for *T. variae* asexual stages have not yet been observed (Zamora et al., 2011, 2016). In this study, more specifically, we aimed to investigate if (i) also *T. variae* has a yeast stage, (ii) the yeast stage is restricted to occur in the hymenium or thallus parts of *L. chlorotera* and *L. varia* lichens, respectively, (iii) the yeast stage of both *T. macrobasidiata* and *T. variae* is restricted to lichen specimens that have *Tremella* basidiomata and (iv) the yeast stage is restricted to the respective lichen. We also aimed to study whether the different *Tremella* species differ in their physical interactions with other symbionts in the thalli.

**Results**

**Life cycle stages of Tremella observed by FISH and CLSM**

We used fluorescent *in situ* hybridization (FISH) to specifically stain *Tremella* cells in the *L. chlorotera* and *L. varia* thalli with and without basidiomata and visualized the cells in different parts of the specimens with confocal laser scanning microscope (CLSM; Fig. S1). FISH and CLSM verified that *Tremella* species occur and are abundant in a yeast form in both studied *Lecanora* lichens, also in specimens without basidiomata (Figs 1–3, Figs. S2 and S3), we observed them in every studied specimen. *Tremella* yeasts were intermixed with *Lecanora* hyphae in the areolae and often occurred in patches. The yeasts were often concentrated, but not restricted, to the upper parts of the thallus (Figs 1A, B and 2A, C, G, Fig. S2 G). We observed *Tremella* yeasts also in the apothecium thalline margin (Fig. 1B and C, Fig. S3G) as well as in the upper parts of the hymenium of the apothecia (Fig. 1C and D, 2B, H, Fig S2F, Fig. S3F and G) in both *Lecanora* species. In *L. chlorotera* thalli without basidiomata, we observed germinating *Tremella* yeasts, often with multiple projections (Fig. 1E–G, Videos S1–S2). In one of the *L. varia* specimens with *T. variae* basidiomata, in a thallus part where basidiomata were not yet formed, we observed *Tremella* hyphae (Fig. 2C–E, Video S3). We observed germinating *Tremella* yeasts in *L. varia* specimens with basidiomata, both in the thalli (Fig. 2F) and hymenia (Fig. 2H).

In basidiomata of both *Tremella* species, we observed basidia, basidiomeres, yeasts and hyphae of *Tremella* (Fig. 3, Fig. S2A–E, Fig. S3A–E). *Tremella* hyphae of both studied species grow around and between algal cells in the basidiomata, but we did not observe them penetrating the algal cell walls (Fig. 3F, H, Fig. S3E). We observed tremelloid haustoria in both species, but never clearly attached to any other cells (Figs 3E and 4E, Videos S4, S5). In basidiomata of *T. macrobasidiata*, we observed conidiogenous cells (Fig. 3C) similar to those described by Zamora et al., 2011, which stained with the *Tremella* specific probe, verifying that these cells belong to *Tremella* and not to any other fungus in the thalli.

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Fig 1. *Tremella in Lecanora chlarotera* specimens without basidiomata, volume rendering except for C, which is a maximum intensity projection with transmitted light. Green: *Tremella*, magenta: algal autofluorescence and *Lecanora*.

A. Thallus with three different areoles with *Tremella* yeasts and algae (*).

B. *Tremella* yeasts in thallus (arrowhead) and thalline margin (TM) of an apothecium. Woody substrate (W), cross section.

C. Apothecium thalline margin (TM) with *Tremella* yeasts, *Tremella* yeasts also in hymenium (H). Arrow points to dead algal cells that autofluoresce on green wavelengths, live algae (*).

D. Hymenium of *L. chlarotera* apothecium seen from above with *Tremella* yeasts, paraphyses in magenta, and algae (*).

E–G. *Tremella* yeasts germinating in thallus (arrows), live algae (*). E Related to Video S1. F related to Video S2. Scale 30 μm in A, B, D, 10 μm in C, E–G.

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Fig 2. *Tremella* in *Lecanora varia* specimens, volume rendering. A–B: specimens without basidiomata, C–H: specimens with *T. variae* basidiomata, parts of thallus without basidiomata. Green: *Tremella*, magenta: algal autofluorescence and *Lecanora*.

A. Thallus with *Tremella* yeasts and algae (*), woody substrate (W).

B. *Tremella* yeasts in the hymenium of *L. varia*, some of them budding. Asci and paraphyses clearly visible.

C–D. Thallus with *Tremella* yeasts, hyphae and algae (*), C overlay of both channels, D showing only the green channel. The white rectangle shows the *Tremella* hyphae.

E. Close-up of a subset of focal plains of the white rectangle in C and D, showing clamped hyphae with a tremelloid haustorium; the filament (arrow) not attaching to *L. varia* hyphae. Related to Video S3.

F. Germinating *Tremella* yeast (arrow) in a thallus, surrounded by *L. varia* hyphae, alga (*).

G. Thallus with *Tremella* yeasts and algae (*).

H. Hymenium of *L. varia* with paraphyses (magenta and green autofluorescence), asci, and *Tremella* yeasts, one of which is germinating (arrow). Scale 30 μm in A–D; 3 μm in E; 4 μm in F; 20 μm in G–H.
However, we did not observe asteroconidia in the basidiomata. In the only studied T. variae basidiomata that was formed on the thalline margin of a L. varia apothecium, both basidia and asci were observed next to each other, and some of the basidia were clearly formed inside the hymenium surrounded by paraphyses (Fig. 3G, Video S5). No Tremella hyphae was observed in samples from specimens without basidiomata.

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In addition to the specific staining with probes, we used the universal cell wall stain calcofluor white, which binds to β-(1,3) and β-(1,4) polysaccharides, e.g., chitin, for better separation of fungal interactions. Our tests revealed that calcofluor white binds only weakly or not at all to Tremella cell walls and was hence not usable for the study of the interspecies cell wall–cell wall contacts in this study system.

**Molecular evidence for the presence of Tremella in the Lecanora lichens**

In addition to the observations with FISH, we could verify the presence of both *Tremella macrobasidiata* and *T. variae* in Lecanora specimens without basidiomata by sequencing PCR amplicons produced with *in silico* species-specific primers (Fig. 4, Fig. S1, Table 1). We conclude that these DNA records correspond to occurrence of Tremella yeasts, since we observed only the yeast stage and no hyphae in samples without basidiomata using CLSM. DNA of *T. macrobasidiata* was present in each studied *L. chlarotera* specimen, but also in 73% of the studied *L. varia* specimens without basidiomata and even in 27% of the *L. varia* specimens that had *T. variae* basidiomata. DNA of *T. variae* was present in 58% of the *L. varia* specimens without basidiomata. We could not verify the presence of either of the two studied Tremella species in three *L. varia* specimens (i.e., 92% of *L. varia* specimens had at least one of the studied Tremella species), nor could we detect *T. variae* in any of the *L. chlarotera* specimens. In two of the *L. varia* specimens without basidiomata, that we studied by FISH and CLSM, *T. macrobasidiata* and *T. variae* were both present according to PCR. The identity of the visualized Tremella structures in those specimens could unfortunately not be verified, since the probe stains both Tremella species. However, in one of the *L. varia* specimens without *T. variae* basidiomata, and in two specimens with *T. variae* basidiomata, only *T. variae* was detected with PCR and Tremella yeasts were observed in all these specimens with CLSM further indicating that also *T. variae* occurs in a yeast form in *L. varia* thalli.

The 18 ITS haplotypes of *L. chlarotera* were mostly not structured by geographic location, although some were only found in Spain and some only in Sweden (Fig. S4A). Our *L. varia* samples were more variable in their ITS (33 haplotypes of which most were singletons) than our *L. chlarotera* samples (Fig. S4B). The presence or absence of *T. macrobasidiata* in *L. varia* specimens did not strongly depend on the *L. varia* ITS haplotype. However, the six *L. varia* specimens where we could detect only *T. macrobasidiata*, and the three *L. varia* specimens where neither of the targeted Tremella species could be detected, had a unique *L. varia* ITS haplotype (Fig. S4B, Table S1). At least in some of these specimens, the lack of detection of *T. variae*, or any of the two *Tremella* spp., respectively, could be because of divergent Lecanora lineages, as Tremella are assumed to be more or less species specific (Fig. S4B). Lack of detection could also be because of limited sample size for DNA extraction and low template concentration for PCR. Despite the *in silico* species-specific Tremella primers, several PCR reactions resulted in multiple bands and mixed signal in sequencing, especially in extractions from *L. varia* specimens, suggesting that even other Tremella species may be present in these thalli.

**Discussion**

Our study gives new and important insights into earlier overlooked aspects of the life cycle of two Tremella species associated with crustose Lecanora lichens. Our results show that both *T. macrobasidiata* and *T. variae* indeed have a widespread and abundant yeast stage in their life cycle, and that this life stage is not limited to Lecanora specimens, nor lichen parts, with Tremella basidiomata. These and previous results by Tuovinen et al., 2019 support the hypothesis that dimorphism is common also in lichen-associated Tremella species, consistent with interpretation of yeasts as a typical life cycle stage of tremellalean fungi in general (Chen, 1998; Millanes et al., 2011). Following the hypothesis that other lichen-associated Tremella species may have a prevalent yeast stage in their life cycles, they may be much more common and abundant symbionts of lichens than assumed based on observations of basidiomata only, and their distribution ranges may be severely underestimated. Furthermore, assumptions about their lichen specificity and ecology will need to be revisited.

The association with different, evolutionary distant groups of lichens has evolved several times in the Tremellales (Millanes et al., 2011; Liu et al., 2015). The studied Lecanora lichens are non-stratified crusts and lack the thick cortex layer in which basidiomycete yeasts have previously been observed to be concentrated in some stratified macrolichens (Spribille et al., 2016; Tuovinen et al., 2019). In the studied Lecanora lichens, the Tremella yeast cells are not restricted to the top or upper parts of thallus but occur vertically distributed within the areoles and can be found even close to the woody substrate. Thus, the space for interaction between Tremella yeasts and other organisms within the thallus is less limited in these crusts than what has been suggested for macrolichens (Spribille et al., 2020). Bergmann and Werth (2017) studied the intrathalline distribution of Tremella lobariacerum in three Lobaria lichens by quantitative real time PCR, but could detect Tremella DNA only inside or close to basidiomata and
**Fig 4.** ML tree from ITS sequences of *Lecanora*, showing the occurrence of associated *Tremella* species in each sample verified by sequencing. Grey labels represent sequences derived from GenBank for reference of the molecular identity of *Lecanora*. Samples with visible *Tremella* basidiomata are marked with a star. Black labels refer to samples where none of the target *Tremella* could be amplified. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. Maximum likelihood bootstrap percentages are shown above the branches when > 55%.

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not in thalli without visible basidiomata. Their results indicate that either T. lobariacerum lacks a yeast stage, the yeast stage is restricted to the vicinity of basidiomata, or the non-species specific primers used for qPCR could not detect Tremella in these specimens due to, e.g., low template concentration. In our study, we needed species-specific primers to amplify T. variae and T. macrobasidiata by PCR. Microscopic studies, together with molecular methods, are essential for interpreting the location and life cycle stage of fungal symbionts within the lichen thallus.

As reported here and by Tuovinen et al. (2019), more than one Tremella species can co-occur in the same thallus. Sequences belonging to Tremellales have been detected from lichen thalli in many metabarcoding studies (Fleischhacker et al., 2015; Zhang et al., 2015; Wang et al., 2016; Zhao et al., 2016; Fernández-Mendoza et al., 2017; Banchi et al., 2018), but these studies have focused on describing the lichen-inhabiting fungal communities only at higher taxonomic levels. Deep, targeted high throughput amplicon sequencing and detailed taxonomic assignments could give a more comprehensive picture of the amount of lichen-associated Tremella species in each lichen specimen, and further shed light on the frequency and abundancy of Tremella in lichen symbiosis in general.

Regarding the location and distribution of the filamentous stage in the lichen thallus, Grube and de los Ríos (2001) studied microscopically the anatomy and development of the basidiomata of Biatoropsis usnearum (Tremellales) on the stratified macrolichen Usnea usnearum, and could not detect any hyphae connecting the different basidiomata in Usnea thalli. This observation is in line with ours. In the light of our results, and those of Tuovinen and colleagues (2019), it seems probable that at least the studied Tremella species disperse within and between thalli as basidiospores, conidia or yeasts, and that hyphae are restricted to the basidiomata and its vicinity and formed only when the sexual cycle is induced.

We could observe T. variae basidia intermixed with asci and paraphyses in the hymenium of one L. varia, indicating that the basidium formation is not strictly restricted to the thalline parts of L. varia, although the major portion of the basidiomata is formed on the thalline parts based on macroscopic observations (Zamora et al., 2016). In addition, Tremella yeasts were present in L. varia hymenium, suggesting that these may germinate after conjugation and eventually the development of basidiomata may start even in the hymenium. The properties of a lichen that guide the place for development of Tremella basidiomata remain unknown, but different secondary metabolite and polysaccharide composition in different parts of the thallus, and different lichens, may play a role in this. Particularly, the concentration of secondary metabolites has been previously reported to correlate with the presence of a lichen-associated Ascomycete Plectocarpon scrobiculatae (Merinero et al., 2015).

Many Tremellomycetes species are either known or suspected mycoparasites (Spatafora et al., 2017), but the nature of the interactions between Tremella and associated lichens remains elusive. The presence of tremellloid haustoria in the basidiomata has been considered indicative of a mycoparasitic lifestyle in lichen-associated Tremella, but not all lichen-associated Tremella species form haustoria (Diederich, 1996). Haustoria-hyphae interactions have been studied in detail in Tremellomycetes associated with non-lichenized fungi (reviewed in Bauer and Oberwinkler, 2008), but rarely in lichens. However, in B. usnearum basidiomata, Grube and de los Ríos (2001) observed B. usnearum haustorial filaments reaching to U. rigida cell walls. While we also did observe typical tremellloid haustoria inside the basidiomata of both studied Tremella species, we could not trace them clearly attaching to Lecanora hyphae when inspecting the 3D reconstructions. Notably, the cell walls of the fungi are not stained in our study design, which complicates interpretation of wall-wall contacts in cases where the distance between the stained cytosols is small. In mycoparasitic Tremella, haustorial filaments attach to the

Table 1. The identity of Tremella in the studied specimens verified by PCR and sequencing.

| Specimens                      | Tremella macrobasidiata | Tremella variae | Both |
|--------------------------------|--------------------------|-----------------|------|
| **Without basidiomata**        |                          |                 |      |
| Lecanora chlorotera (n = 23)   | 100%                     | 0%              | 0%   |
| Lecanora varia (n = 26)        | 73%                      | 58%             | 42%  |
| **With basidiomata**           |                          |                 |      |
| Lecanora chlorotera (n = 10)   | 100%                     | 0%              | 0%   |
| Lecanora varia (n = 11)        | 27%                      | 100%            | 27%  |
| **In total**                   |                          |                 |      |
| Lecanora chlorotera (n = 33)   | 100%                     | 0%              | 0%   |
| Lecanora varia (n = 37)        | 59%                      | 70%             | 38%  |
cell wall of their host, and a micropore connection between the cytosols of the two fungi is formed (Zugmaier et al., 1994; Bauer and Oberwinkler, 2008). Transmission electron microscopy studies are needed to determine whether such connections between Tremella and Lecanora cells occurs, which would shed light on the functionality of the haustoria in the studied Tremella species.

Interestingly, in basidiomata of both studied Tremella species, Tremella hyphae were regularly observed growing around algae, similar to observations of T. lethariae in wolf lichens (Tuovinen et al., 2019). However, we could not observe Tremella penetration into the algal cell similar to Lecanora hyphae. Yet again, ultrastructural studies would be needed for the interpretation of the wall-wall interactions between these organisms. Since Tremella and Lecanora are indeed distantly related fungi, the type of contact between each of them and the alga can be expected to be different. We cannot verify or discard whether there is acquisition and utilization of sugars from the algae by the hyphal stage of Tremella, and to test such a hypothesis more specific studies of the nutrient transfer are needed. Moreover, the nutrition source of the Tremella yeasts remains unstudied. In many dimorphic species, the nutritional strategy of the yeast and hyphal stage is different (Begerow et al., 2017), and may be that also for lichen-associated Tremella.

The modern view of symbiotic relationships in nature in general is that they are often observed to be dynamic on a continuum from mutualistic to parasitic, affected both by external factors and by the life cycle stage of the organism (Johnson et al., 1997; Klironomos, 2003; Jones and Smith, 2004; Sapp, 2004; Johnson, 2010; Zook, 2015). Furthermore, lichen-associated Tremella lineages form several unrelated groups that may potentially have sister taxa with life strategies other than mycoparasitism (Millanes et al., 2011; Liu et al., 2015). It is possible that also the nature of symbiotic relations of different Tremella species associated with divergent lichens, which neither form a natural group, varies more than previously thought. The individual evolutionary paths of all symbiotic partners of any given lichen may have contributed to and resulted in different outcomes of the symbiotic relationships between Tremella and the other organisms present in a lichen thallus.

The study of microorganisms in their natural environments is complicated in part due to their tiny size, and lichen-associated Tremella species are no exception in this regard. They have most often been studied by conventional bright field microscopy and disruptive sample preparation with KOH, which has provided morphological information useful for taxonomical purposes, but hinders the study of spatial structure and physical interactions between different organisms in the lichen. Specific staining and CLSM that allows non-disruptive sampling and observation of the structures in 3D has shown to be a powerful tool in the study of lichen-associated microorganisms in their natural environment (de los Rios et al., 2002; Cardinale et al., 2008; Aschenbrenner et al., 2016; Spribille et al., 2016; Tuovinen et al., 2019). The method is especially valuable for the study of lichen-associated Tremellomycetes, as they are generally missing from the cultivable microbiome extracted from lichen thalli (Muggia et al., 2017; Oh et al., 2020), except for Fellomyces species (Prillinger et al., 1997; Lopandic et al., 2005). Unfortunately, our attempt to use calcofluor white for identification of cell–cell interactions of different fungi and alga failed, since Tremella cell walls did not stain with this widely used fungal cell-wall stain. Notably, Koch and Pimsler (1987) reported the lack of, or un-uniform, binding of calcofluor white to Cryptococcus (Tremellales). Cryptococcus is known to secrete an exopolysaccharide capsule of glucuronoxylomannans (GXM) around its cell walls (Martinez and Casadevall, 2015), and Harrington and Hagege (2003) reported that this polysaccharide capsule did not stain with calcofluor white. Spribille et al. (2020) hypothesized that also lichen-associated Tremella species could secrete GMXs to the extracellular interaction matrix in macrolichens. Interestingly, Grube and de los Rios (2001) observed acidic polysaccharides on the outer parts of hyphae of lichen-associated B. usnearum. The lack of calcofluor white binding in the cell walls of the studied Tremella species may be indicative of presence of such polysaccharides, which may hinder the access of the stain to the cell wall. The presence of relatively thick extracellular material around the Tremella hyphae was clearly visible in CLSM in basidiomata. Alternatively, the cell walls of the studied Tremella species may be poor in β-(1,3) and β-(1,4) polysaccharides, and rather consist of α(1,3) and β-(1,3), (1,6) polysaccharides as in T. mesenterica (Reid and Bartnicki-Garcia, 1976), leading to bad staining with calcofluor white.

Another intriguing aspect of the association between Tremella and lichens is specificity. Lichen-associated Tremella species have been considered to be rather specific regarding their ascocyte lichen symbionts, a trait that has been used as an initial character for the species identification of Tremella basidiomata occurring on a certain lichen (Diederich, 1996; Millanes et al., 2014, 2015, 2016; Diederich et al., 2018). To our knowledge, lichen-associated Tremella for which reference sequences are available are largely lacking from environmental sequence data (Li et al., 2020), which supports the hypothesis that at least some of them indeed are restricted in their occurrence to the lichen symbiosis and do not likely occur freely in the surrounding environment. In the B. usnearum species complex, the sexual stage of
some species is specific to a certain Usnea/Protousnea species whilst some are generalists within the genera (Millanes et al., 2014). Genus-level specificity was also observed for T. lethariae, which associates in the yeast stage with all described Letharia species and for which basidiomata has been observed on thalli of several of the taxa (Tuovinen et al., 2019). On the other hand, previous molecular studies have detected the yet formally undescribed species Tremella sp. B in both Bryoria and Letharia lichens without visible basidiomata (Lindgren et al., 2015; Tuovinen et al., 2019), indicative of the presence of a yeast stage of this species in those lichens. The yeast stage of Tremella sp. B thus seems to be a generalist not restricted to a certain lichen genus but may still be restricted to lichens belonging to Parmeliaceae. No basidiomata has yet been observed for this species, and conclusions about the specificity of its sexual cycle can hence not be made.

Basidiomata formation in the two Tremella species studied here seems limited to the respective Lecanora species, suggesting that the environment provided by a certain Lecanora lichen is needed for the completion of the sexual life cycle. However, our results suggest that as the yeast stage of T. macrobasidiata is common even in L. varia lichens, it is less specific than T. variae, which appears truly specific to L. varia. Our observation that some of the L. varia specimens where we could not detect T. variae were divergent in the Lecanora ITS sequence may further indicate the higher species specificity of T. variae. Millanes and colleagues (2014) showed that switching association from one Usnea species to another has promoted speciation in B. usnearum species complex, but the factors driving speciation in other lichen-associated Tremella are still largely unknown. The lack of species-specificity observed for the yeast stage of T. macrobasidiata could be related to a relatively recent speciation history within the group of Lecanora- and Lecidea-associated Tremella species (Zamora et al., 2018). Increased screening for these Tremella species in other Lecanora lichens and populations will help to better understand their specificity and general distribution patterns. Taken together, it seems that some lichen-associated Tremella species are species specific, whilst others are generalists associated with several related species.

Conclusions

In general, the ecology of the yeast stage of dimorphic basidiomycetes with conspicuous fruiting bodies – species traditionally studied by mycologists and lichenologists instead of microbiologists – is much less understood compared with their filamentous stages, since investigations on the yeast stage have mainly been undertaken using cultures produced in the lab (Begerow et al., 2017). Understanding an organism requires, however, knowledge of its whole life cycle under natural conditions. Our results add to the growing evidence of the presence of basidiomycete yeasts in lichens in general (Spribille et al., 2016; Černajová and Škaloud, 2019, 2020; Tuovinen et al., 2019; Mark et al., 2020), showing that their occurrence is more common and widespread than previously thought, and the dimorphic life cycle of lichen-associated Tremella species in particular. We have further shown that, despite growing inside a lichen in seemingly similar microenvironments, the ecological requirements of the yeast and filamentous stages are not necessarily the same, as both the lichen specificity and location within the thallus can vary depending on the life cycle stage in different Tremella species. While it is not possible to make conclusions about the ecological function of the studied Tremella species or their different life stages in lichens with the data at hand, it is clear that the frequency and abundancy at which tremellalean yeasts are being recovered in lichen symbiosis deserves deepened attention in future studies.

Experimental procedures

Lichen material

We collected seven L. chlarotera specimens with Tremella macrobasidiata basidiomata and 12 specimens without basidiomata from Spain. In all of these localities specimens with and without basidiomata were present. In addition, we collected three L. chlarotera specimens with T. macrobasidiata basidiomata and 11 without basidiomata from Sweden. No basidiomata were observed on other L. chlarotera lichens in the Swedish localities where specimens without basidiomata were collected, but no thorough inventory was made. All L. varia specimens were collected from Spain, as T. variae is not known from Sweden, 11 of them with and 26 without T. variae basidiomata. In one of the localities only specimens without basidiomata were observed but in all others both specimens with and without basidiomata were present. Detailed information on the sampling design and the specimens can be found in (Fig. S1, Table S1).

In order to verify the identity of the studied Lecanora and Tremella species, we extracted DNA from all of the Lecanora specimens and used it for PCR and Sanger sequencing, detailed below. From specimens without Tremella basidiomata, we used part of the thallus together with apothecia for DNA extraction. From specimens with Tremella basidiomata, we made two separate DNA extractions: one with a basidioma and one with a thallus piece together with apothecia without basidiomata. For the study of the occurrence and location
of different life stages of *Tremella* in the *Lecanora* specimens, we used FISH and CLSM. For these, we chose six specimens of each lichen, three with basidiomata and three without. More specifically, for each *L. chlarotera* specimen with basidiomata we studied apothecia with and without basidiomata as well as thallus parts. For *L. chlarotera* specimens without basidiomata we studied both apothecia and thallus parts. For each *L. varia* specimen with basidiomata we studied basidiomata, thallus parts without basidiomata and apothecia, and for specimens without basidiomata thallus parts and apothecia (Fig. S1).

**Fixation**

Directly after collection, we placed the specimens for FISH on moisturized filter paper on Petri dishes, sprayed them with $\alpha$H2O, and covered loosely with a lid. We let the specimens dry, and moisturized them again, repeating this cycle for up to 5 days in order to make the lichens physically active. Then, we cut pieces of moist thalli with attached woody substrate by hand and placed them in 1× PBS (pH 7.4) in 0.2 ml test tubes in a vacuum desiccator. We applied the vacuum for c.a. 10 s at a time, removed the tubes and gently tapped them against a bench, and repeated the procedure until the air from the tissues was removed and the pieces sunk to the bottom of the tubes. We replaced the PBS with 4%, methanol free, formaldehyde for fixation at +4°C for 2 h. After fixation, we washed the samples in 1× PBS three times, 10 min each. We then incubated the samples in acetone for 2–8 h in order to dissolve secondary metabolites with strong autofluorescence, followed with PBS wash as above. The PBS was removed, and the sample frozen at −20°C until FISH.

**FISH probe design**

We designed a FISH probe matching the studied *Tremella* species based on the available LSU sequences (*T. macrobasidiata*: GenBank: KT334594.1, KT334595.1 and *T. variae*: KT334598.1, KT334599.1), with no match for *Lecanora* (Table S2). Note that this probe matches several other, but not all, Tremellales species, and cannot differentiate between *T. macrobasidiata* and *T. variae*, for which purpose we used PCR and sequencing. The probe was first labelled with 6-FAM at 5’ end, but once the function of the probe was verified it was labelled also with FITC at the 3’ end in order to enhance the fluorescent signal. We evaluated the performance of the *Tremella* probe in both *T. macrobasidiata* and *T. variae* basidiomata and could achieve a high enough signal intensity on green wavelengths with the double-labelled *Tremella* probe to detect hybridized cells. We designed four different probes for the two *Lecanora* species, each mono labelled with Cy5 at 5’ end, and evaluated their performance in apothecia of both *Lecanora* species. Unfortunately, the intensity of the autofluorescence of hyphae of both *Lecanora* species on red wavelengths (based on comparisons with negative controls with no probes) varied considerably between different parts of thalli, as well as between different specimens, and in most cases separation between the weak signal intensity from the probe and the autofluorescence with certainty was not possible. Notably, hyphae close to algae are most intensely autofluorescent on red wavelengths. Also secondary metabolites, basidia, and *Tremella* yeasts to some degree, autofluoresce red. *Lecanora variata* hyphae and apothecia have stronger autofluorescence than *L. chlarotera*. The woody substrate is highly autofluorescent on both the green and red wavelengths. In addition, most of the fungal cells, and especially the paraphyses, are autofluorescent on green. We used each probe in combination with helper probes (Table S2).

**FISH and cell wall staining**

We performed the permeabilization, hybridization and washing steps for the fixed and acetone treated samples in 0.2 ml test tubes as described in Tuovinen *et al.*, 2019. We included a negative control without probes for each hybridization reaction and each species. In addition to the specific staining with probes, we tried the universal stain calcifluor white (Sigma-Aldrich) on seven thalli (three *L. chlarotera* with *T. macrobasidiata* and four *L. variata* with *T. variata*). Calcifluor white binds to β-1, 3 and β-1, 4 polysaccharides like chitin and cellulose, staining in general both fungal and algal cells, and we aimed for better separation of wall-wall interactions between the different fungi. We added calcifluor white to samples after the washing step after FISH, incubated for 10 min in darkness at room temperature, and washed the samples with 1X PBS. We mounted all samples on microscopy slides with SlowFade Diamond (Thermo Fisher).

**CLSM**

We studied the hybridized slides by Zeiss LSM710 confocal microscope, with Plan-Apochromat 63x NA 1.4 oil DIC M27 objective. We used 488 and 633 laser lines for excitation of 6-FAM + FITC labelled and Cy5-labelled probes, respectively. The detection wavelengths were 493–598 nm for green and 638–797 nm for red wavelengths. We used one Airy unit pinhole and simultaneous scanning. We acquired Z stacks of lichen thalli with
optimal settings calculated by the Zen blue software of the microscope (Zeiss).

**Image processing**

We processed the 2D-images using Fiji (Schindelin et al., 2012, 2015) or Zen blue (Zeiss), and prepared the volume rendering with Zen blue. We used median filtering for noise reduction and adjusted colour balance for the clarity of presentation.

**Molecular methods**

We pulverized apothecia together with a small piece of thallus, and a basidioma in separate test tubes with a pestle after freezing in liquid nitrogen. Subsequently, we extracted the total DNA using the DNeasy Plant Mini kit (QIAGEN) following the manufacturer’s instructions with a prolonged incubation at 65°C for 1 h and elution of the DNA in 50 μl of the elution buffer.

We used primers ITS1F (Gardes and Bruns, 1993), ITS4 (White et al., 1990), LR3 (Vilgalys and Hester, 1990) and TellLSU3-3 (Table S3) for PCR and sequencing of the Lecanora ITS. We used HotStart RTG beads (Illustra™) for the PCR with the following touch down program: four cycles of initial annealing at 60°C, four cycles at 58°C and 32 cycles at 56°C. We used the PCR primers as sequencing primers.

We designed specific PCR and sequencing primers for each Tremella species (Table S3). The most successful primer pairs were TmM_ITS_970F for T. macrobasidiata and TmV_ITS_1008F for T. variæ in combination with Basid-LSU3-3 (Millanes et al., 2011) (Table S3). From samples that resulted in multiple bands, we extracted the band of correct size with MinElute Gel Extraction kit (Qiagen). Based on comparison with band size from samples that resulted in multiple bands, we extracted the total DNA using the DNeasy Plant Mini kit (QIAGEN) following the manufacturer’s instructions and subsequently, we processed the DNA in 50 μl of the elution buffer.

We sent the samples for Sanger sequencing to Macrogen in the Netherlands.

**Sequence analyses**

We used Geneious 10.2.3 or R11.1.5 (Biomatters) to check the chromatograms and trim the sequence ends, and Aliview for initial sequence alignment (Larsson, 2014). For the Lecanora ITS, we aligned our newly generated sequences with closely related sequences available in GenBank (Table S4) with MAFT v.7 online service (Kuraku et al., 2013; Katoh et al., 2019), using the G-INS-I algorithm and otherwise default parameters. We performed a ML analysis of the alignment with raxmlGUI 2.0.0-beta.6 (Stamatakis, 2014; Edler et al., 2021) with the GTRGAMMA1 substitution model, 100 runs, and 1000 bootstrap replicates. We used Lecidella patawina as an outgroup based on the phylogeny by Zhao and colleagues (2016). We constructed ITS-haplotype networks for the Lecanora species with haploNet in Pegas (Paradis, 2010) in R. The newly generated sequences are available in GenBank with accession numbers MW374997-MW375029 (Table S1).

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**Author contributions**

AMM, MW, and VT designed the project. VT, AMM and SF-R collected the specimens. VT and SF-R conducted sample preparation, and VT conducted FISH, CLSM, the data analyses and wrote the original draft. MW, AMM and AR financed and supervised the project, and all co-authors contributed with writing to the final version.

**References**

Aschenbrenner, I.A., Cernava, T., Berg, G., and Grube, M. (2016) Understanding microbial multi-species symbioses. *Front Microbiol* 7: 180.

Banchi, E., Stankovic, D., Fernández-Mendoza, F., Gionechetti, F., Pallavicini, A., and Muggia, L. (2018) ITS2 yeasts are abundant in Lecanora lichens 2495

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metabarcoding analysis complements lichen mycobiont diversity data. Mycol Prog 17: 1049–1066.

Bandoni, R.J. (1995) Dimorphic heterobasidiomycetes, taxonomy and parasitism. Stud Mycol 38:13–27.

Bauer, R., and Oberwinkler, F. (2008) Cellular basidiomycete–fungus interactions. In Plant Surface Microbiology, Varma, A., Abbott, L., Werner, D., and Hamp, R. (eds). Berlin: Springer, pp. 267–279.

Begerow, D., Kemler, M., Feige, A., and Yurkov, A. (2017) Parasitism in yeasts. In Yeasts in Natural Ecosystems: Ecology, Buzzini, P., Lachance, M.-A., and Yurkov, A. (eds). Cham: Springer International Publishing, pp. 179–210.

Bergmann, T.C., and Werth, S. (2017) Intrathalline distribution of two lichenicolous fungi on Lobaria hosts — an analysis based on quantitative real-time PCR. Herzogia, 30: 253–271.

Cardinale, M., Vieira de Castro, J., Müller, H., Berg, G., and Grube, M. (2008) In situ analysis of the bacterial community associated with the reindeer lichen Cladonia arbuscula reveals predominance of Alphaproteobacteria: lichen-associated bacterial community. FEMS Microbiol Ecol 66: 63–71.

Castelle, C.J., and Banfield, J.F. (2018) Major new microbial groups expand diversity and alter our understanding of the tree of life. Cell 172: 1181–1197.

Černajová, I., and Škaloud, P. (2019) The first survey of Cystobasidiomycete yeasts in the lichen genus Cladonia; with the description of Lichenozyma pisutiana gen. nov., sp. nov. Fungal Biol 123: 625–637.

Černajová, I., and Škaloud, P. (2020) Lessons from culturing lichen soreidia. Symbiosis 82: 109–122.

Chen, C.J. (1998) Morphological and molecular studies in the genus Tremella. Bibi Mycol 174: 1–225.

de los Ríos, A., Ascaso, C., and Grube, M. (2002) Infection mechanisms of lichenicolous fungi studied by various microscopic techniques. Bibi Lichenol 82: 153–161.

Diederich, P. (1996) The lichenicolous heterobasidiomycetes. Bibi Lichenol 61: 1.

Diederich, P., Lawrey, J.D., and Ertz, D. (2018) The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. The Bryologist 121: 340.

Edler, D., Klein, J., Antonelli, A., and Silvestro, D. (2021) raxmGUI 2.0 beta: a graphical interface and toolkit for phylogenetic analyses using RAxML. Methods Ecol Evol 12: 373–377.

Ekman, S. (1999) Pcr optimization and troubleshooting, with special reference to the amplification of ribosomal dna in lichenized fungi. The Lichenologist 31: 517–531.

Fernández-Mendoza, F., Fleischhacker, A., Kopun, T., Grube, M., and Muggia, L. (2017) ITS1 metabarcoding highlights low specificity of lichen mycobionts at a local scale. Mol Ecol 26: 4811–4830.

Fleischhacker, A., Grube, M., Kopun, T., Hafellner, J., and Muggia, L. (2015) Community analyses uncover high diversity of lichenicolous fungi in alpine habitats. Microb Ecol 70: 348–360.

Gardès, M., and Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol 2: 113–118.

Goward, T. (2008) Nameless little things. Evansia 25: 54–56.

Grube, M., and de los Ríos, A. (2001) Observations on Biatoropsis usnearum, a lichenicolous heterobasidiomycete, and other gall-forming lichenicolous fungi, using different microscopic techniques. Mycol Res 105: 1116–1122.

Grube, M., and Wedin, M. (2016) Lichenized fungi and the evolution of symbiotic organization. Microbiol Spectr 4: 6.

Harrington, B.J., and Hageage, G.J. (2003) Calcofluor white: a review of its uses and applications in clinical mycology and parasitology. Lab Med 34: 361–367.

Johnson, N.C. (2010) Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. New Phytol 185: 631–647.

Johnson, N.C., Graham, J.H., and Smith, F.A. (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol 135: 575–585.

Jones, M.D., and Smith, S.E. (2004) Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Can J Bot 80: 1089–1109.

Katoh, K., Rozewicki, J., and Yamada, K.D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20: 1160–1166.

Kilronomos, J.N. (2003) Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84: 2292–2301.

Koch, H.H., and Pimsler, M. (1987) Evaluation of Uvitex 2B: a non-specific fluorescent stain for detecting and identifying fungi and algae in tissue. Labmedicine, 18: 603–606.

Kuraku, S., Zmasek, C.M., Nishimura, O., and Katoh, K. (2013) aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Res 41: W22–W28.

Kurtzman, C.P., Fell, J.W., and Boekhout, T. (2011) The Yeasts: A Taxonomic Study, 5th ed. San Diego: Elsevier Science.

Larsson, A. (2014) AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics 30: 3276–3278.

Li, A.-H., Yuan, F.-X., Groenewald, M., Bensch, K., Yurkov, A.M., Li, K., et al. (2020) Diversity and phylogeny of basidiomycetous yeasts from plant leaves and soil: proposal of two new orders, three new families, eight new genera and one hundred and seven new species. Stud Mycol 96: 17–140.

Lin, X. (2009) Cryptococcus neoformans: morphogenesis, infection, and evolution. Infect Genet Evol 9: 401–416.

Lindgren, H., Diederich, P., Goward, T., and Myllys, L. (2015) The phylogenetic analysis of fungi associated with lichenized ascmycete genus Bryoria reveals new lineages in the Tremellales including a new species Tremella huuskonenii hyperparasitic. Fungal Biol 119: 844–856.

Liu, X.-Z., Wang, M. Q.-M., Gökér, M., Groenewald, M., Kachalkin, A.V., Lumbsch, H.T., et al. (2015) Towards an integrated phylogenetic classification of the Tremellomycetes. Stud Mycol 81: 85–147.
Noh, H.-J., Molnár, O., and Prillinger, H. (2005) *Fellomyces mexicanus* sp. nov., a new member of the yeast genus *Fellomyces* isolated from lichen *Cryptothecia rubrocincta* collected in Mexico. *Microbiol Res* **160**: 1–11.

Mark, K., Laanisto, L., Bueno, C.G., Niinemets, Ü., Keller, C., and Scheidegger, C. (2020) Contrasting co-occurrence patterns of photobiont and symbiosymbiosiomyxylect yeast associated with common epiphytic lichen species. *New Phytol* **227**: 1362–1375.

Martínez, L.R., and Casadevall, A. (2015) Biofilm formation by *Cryptococcus neoformans*. *Microbiol Spec* **3**: 1–11.

Merinero, S., Bidussi, M., and Gausaula, Y. (2015) Do lichen secondary compounds play a role in highly specific fungal parasitism? *Fungal Ecol* **14**: 125–129.

Millanès, A.M., Diederich, P., Ekmán, S., and Wedin, M. (2011) Phylogeny and character evolution in the jelly fungi (*Tremellomycetes, Basidiomycota, fungi*). *Mol Phylogenet Evol* **61**: 12–28.

Millanès, A.M., Diederich, P., Westberg, M., Pippola, E., and Wedin, M. (2015) *Tremella cetariellae* (*Tremellales, Basidiomycota, Fungi*), a new lichenicolous fungus on *Cetraria delisei*. *Lichenologist* **47**: 359–368.

Millanès, A.M., Diederich, P., Westberg, M., and Wedin, M. (2016) Three new species in the *Biotropopsis usnearum* complex. *Herz* **29**: 337–354.

Millanès, A.M., Diederich, P., Westberg, M., and Wedin, M. (2021) *Crittendenia* gen. nov., a new lichenicolous lineage in the *Agarciostilbomycetes* (*Pucciniomycotina*), and a review of the biology, phylogeny and classification of lichenicolous heterobasidiomycetes. *Lichenologist* **53**: 103–116.

Millanès, A.M., Truong, C., Westberg, M., Diederich, P., and Wedin, M. (2014) Host switching promotes diversity in host-specialized mycoparasitic fungi: uncoupled evolution in the *Biotropopsis-Usnea* system. *Evolution* **68**: 1576–1593.

Muggia, L., Kopun, T., and Grube, M. (2017) Effects of growth media on the diversity of culturable fungi from lichens. *Molecules* **22**: 824.

Nagy, L.G., Ohn, R.A., Kovács, G.M., Floudas, D., Riley, R., Gácser, A., et al. (2014) Latent homology and convergent regulatory evolution underlying the repeated emergence of yeasts. *Nat Commun* **5**: 4471.

Noh, H.-J., Lee, Y.M., Park, C.H., Lee, H.K., Cho, J.-C., and Hong, S.G. (2020) Microbiome in *Cladonia squamosa* is vertically stratified according to microclimatic conditions. *Front Microbiol* **11**: 268.

Oh, S.-Y., Yang, J.H., Woo, J.-J., Oh, S.-O., and Hur, J.-S. (2020) Diversity and distribution patterns of endophytic fungi in Jeju Island, South Korea. *Sustainability* **12**: 3769.

Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.

Paradis, E. (2010) pegas: an R package for population genetics with an integrated—modular approach. *Bioinformatics* **26**: 419–420.

Prillinger, H., Kraepelin, G., Lopandic, K., Schweigkofler, W., Molnár, O., Weigang, F., and Dreyfuss, M.M. (1997) New species of *Fellomyces* isolated from epiphytic lichen species. *Syst Appl Microbiol* **20**: 572–584.

Reid, I.D., and Bartnicki-Garcia, S. (1976) Cell-wall composition and structure of yeast cells and conjugation tubes of *Tremella mesenterica*. *J Gen Microbiol* **96**: 35–50.

Sánchez-Martínez, C., and Pérez-Martin, J. (2001) Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis*—similar inputs, different outputs. *Curr Opin Microbiol* **4**: 214–221.

Sapp, J. (2004) The dynamics of symbiosis: an historical overview. *Can J Bot* **82**: 1046–1056.

Schindelka, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**: 676–682.

Schindelka, J., Rueden, C.T., Hiner, M.C., and Elcock, K.W. (2015) The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol Reprod Dev* **82**: 518–529.

Spatafoura, J.W., Aime, M.C., Grigoriev, I.V., Martin, F., Stajich, J.E., and Blackwell, M. (2017) The fungal tree of life: from molecular systematics to genome-scale phylogenies. *Mol Phylogenet Evol* **58**: 1–32.

Sprüthli, T. (2018) Relative symbiont input and the lichen symbiotic outcome. *Curr Opin Plant Biol* **44**: 57–63.

Sprüthli, T., Tagirdzhanova, G., Goyette, S., Tuovinen, V., Case, R., and Zandberg, W.F. (2020) 3D biofilms: in search of the polysaccharides holding together lichen symbioses. *FEMS Microbiol Lett* **367**: 1–17.

Sprüthli, T., Tuovinen, V., Resli, P., Vanderpool, D., Wolinski, K., Aime, M.C., et al. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. *Science* **353**: 488–492.

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.

Tuovinen, V., Ekmán, S., Thor, G., Vanderpool, D., Sprüthli, T., and Johannesson, H. (2019) Two basidiomycete fungi in the cortex of wolf lichens. *Curr Biol* **29**: 476–483.e5.

U’Ren, J.M., Lutzoni, F., Miadlikowska, J., and Arnold, A.E. (2010) Community analysis reveals close affinities between endophytic and endolithic fungi in mosses and lichens. *Microb Ecol* **60**: 340–353.

U’Ren, J.M., Lutzoni, F., Miadlikowska, J., Laetsch, A.D., and Arnold, A.E. (2012) Host and geographic structure of endophytic and endolithic fungi at a continental scale. *Am J Bot* **99**: 898–914.

Vigály, R., and Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* **172**: 4238–4246.

Wang, Y., Zheng, Y., Wang, X., Wei, X., and Wei, J. (2016) Lichen-associated fungal community in *Hypogymnia hypotrypa* (Parmeliaceae, Ascomycota) affected by geographic distribution and altitude. *Front Microbiol* **7**: 1231.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenetics. In *PCR Protocols*, Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds). San Diego: Academic Press, pp. 315–322.

Zamora, J.C., Millanès, A.M., Etayo, J., and Wedin, M. (2018) *Tremella mayrhoferi*, a new lichenicolous species on *Lecanora allophana*. *Hertogia* **31**: 666–676.

Zamora, J.C., Millanès, A.M., Wedin, M., Rico, V.J., and Pérez-Ortega, S. (2016) Understanding lichenicolous heterobasidiomycetes: new taxa and reproductive innovations in *Tremella* s.l. *Mycologia* **108**: 381–396.

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Table S4. Reference sequences acquired from the GenBank for the verification of the identity of the studied Lecanora specimens.

**Fig. S1.** Schematic of the sampling for DNA extractions used for PCR, and fluorescent in situ hybridization (FISH). The arrows in the macrophotographs point to the basidiomata of Tremella macrobasidiata on Lecanora chlorotera apothecium and T. variae on L. varia thallus. Scale bars 1 mm.

**Fig. S2.** Lecanora chlorotera specimens with Tremella macrobasidiata basidioma, volume rendering. Green: Tremella, magenta: algal autofluorescence and Lecanora. A-B) Cross section of a basidiomata with basidia (arrow), basidiospores (arrowhead), Tremella hyphae intermixed with L. chlorotera hyphae. B) As A, only the green channel shown. C) Basidiomata shown from above, slightly tilted, showing plenty of basidiospores (arrowhead). D) A spore germinating to a hypha, which gives rise to a ballistoconidium (arrowhead), yeasts possibly conjugating (arrow) inside a basidioma, basidia (B). Only the green channels shown for clarity. E) Germinating yeast inside a basidiomata. F) L. chlorotera hymenium without T. macrobasidiata basidiomata with Tremella yeasts and Lecanora asci (arrow). G) L. chlorotera apothecium and adjacent thallus in cross section. Woody substrate (W), thalline margin (TM), hymenium (H), Tremella yeasts (arrow), algae (*). Scale 30 μm in A-C, F-G; 10 μm in D; 2 μm in E.

**Fig. S3.** Lecanora varia specimens with Tremella variae basidiomata A-F, specimens without basidiomata G, volume rendering, except for E. Green: Tremella, magenta: algal and secondary metabolite autofluorescence and Lecanora. A-B) Cross section of a basidiomata with basidia (arrow), basidiospores, Tremella hyphae (arrowhead) intermixed with L. chlorotera hyphae and close to algae (*). Note the heavy red autofluorescence from the secondary metabolites on the basidiomata surface. B) As A, only the green channel shown. C) Basidiomata shown from above slightly tilted, showing, basidia (arrow), plenty of basidiospores (arrowhead) and the heavy autofluorescence from secondary metabolites. D) As C, only the green channel shown. E) One optical section of algal layer in T. variae basidiomata, related to Fig. 4F. L. varia haustoria (arrow) penetrates the algae (*). Large amount of polysaccharides surround the Tremella hyphae (arrowhead). F) L. varia hymenium with Tremella yeasts, Lecanora asci (arrowhead) and paraphyses (PF). G) L. varia apothecium with Tremella yeasts both in hymenium (H) and thalline margin (TM), algae. Scale 30 μm in A-B, F-G; 20 μm in C-D; 10 μm in E.

**Fig. S4.** Lecanora-ITS haplotype networks with the identity of the observed Tremella species in the same specimen and the presence of basidiomata. The size of the circle reflects the amount of identical ITS haplotypes. A: Lecanora chlorotera specimens from Spain and Sweden. All specimens had T. macrobasidiata. Individuals with visible basidiomata are marked with a star. B: All L. varia specimens came from Spain. We could not detect any of the two studied Tremella species in three L. varia specimens.