Universidad Rey Juan Carlos

TESIS DOCTORAL

Diversity, coupled evolution and interactions in lichen-inhabiting Tremellales

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Programa de Doctorado en Conservación de Recursos Naturales

Escuela Internacional de Doctorado

2023

Sandra Freire Rallo
Tesis doctoral

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Resumen/Summary

Resumen

Antecedentes

Los hongos son un grupo de organismos que comprenden una gran diversidad de formas de vida, desde hongos de vida libre hasta especies que necesitan vivir asociadas a otros organismos, ya sean animales, plantas, otros hongos o líquenes. Los hongos liquenícolas son aquellos que forman asociaciones obligadas con los líquenes y pueden ser tanto Ascomycetes como Basidiomycetes. Los Basidiomycetes liquenícolas son minoritarios y representan aproximadamente el 4% de todas las especies de hongos liquenícolas conocidas. De entre ellos, los Tremellomycetes son el grupo con el mayor número de especies liquenícolas conocidas. Este grupo de hongos mantiene una relación muy estrecha y especializada con los líquenes en los que viven, creciendo en o sobre el talo o el himenio de sus huéspedes y entremezclando sus propias hifas con el fotobionte y las hifas del micobionte. Estos rasgos de alta interrelación entre el hongoliquenícola y el liquen, y gran especificidad respecto al huésped, los convierten en sistemas apropiados para realizar estudios macroevolutivos conjuntos de ambos organismos. Los hongos liquenícolas han sido tradicionalmente menos estudiados que otras especies de hongos, probablemente debido a su rareza o a que es complicado para los no expertos observarlos en la naturaleza. Además, son organismos aparentemente simples morfológicamente y es difícil encontrar caracteres morfológicos diagnósticos útiles para la identificación de especies. La combinación de estas dos características, bajos números de recolección y escasez de caracteres morfológicos, con frecuencia han resultado en el diagnóstico impreciso de especies y la descripción de complejos de especies como especies únicas. En las últimas décadas se ha producido un gran aumento en el interés de micólogos y liquenólogos por los hongos liquenícolas Basidiomycetes. Sin embargo, todavía existen importantes lagunas de conocimiento en cuanto a su diversidad, evolución y ciclo de vida. Esta tesis estudia la historia evolutiva conjunta entre un grupo de hongos Tremellales

liquenícolas y sus hospedantes Teloschistaceae y su implicación en la diversificación del grupo, aumenta el conocimiento de la diversidad real que comprenden y examina las características de su ciclo de vida.

Objetivos

El objetivo general de esta tesis es desentrañar la diversidad en un grupo de Tremellomycetes liquenícolas, estudiar los patrones coevolutivos que han tenido lugar durante el proceso evolutivo conjunto con sus huéspedes Teloschistaceae y examinar el desarrollo de su ciclo de vida y su distribución en los líquenes en los que viven. En particular, en el capítulo 1 se estudiará si *Tremella caloplacae* s. l. es un complejo de especies, la diversidad potencial que engloba y la relación evolutiva conjunta de las nuevas especies putativas de *T. caloplacae* s. l. con sus huéspedes Teloschistaceae utilizando un enfoque macroevolutivo. En el capítulo 2 se realizará la caracterización morfológica y filogenética, y la descripción formal de las nuevas especies de *T. caloplacae* s. l. Finalmente, en el capítulo 3 se analizará el ciclo de vida de *T. caloplacae* s. l. y se determinará su presencia y ubicación en especímenes de líquenes de la familia Teloschistaceae.

Métodos

Recolección de muestras y datos moleculares

Se recolectaron muestras de líquenes Teloschistaceae que fueran potenciales huéspedes de *T. caloplacae* s. l. Para obtener el mayor número posible de muestras se realizó un muestreo exhaustivo en herbario y se recolectaron especímenes frescos directamente en el campo. Las secuencias de ADN nuclear ribosomal pertenecientes a las regiones nSSU,

ITS y nLSU se produjeron durante el desarrollo de la tesis y se combinaron con otras descargadas de GenBank.

Delimitación de especies

La diversidad de especies en *T. caloplacae* s. l. se analizó aplicando métodos basados en 1) la topología de los árboles filogenéticos y 2) distancias genéticas. Se estudió la topología del árbol ultramétrico de *T. caloplacae* s. l. utilizando los métodos "generalized mixed Yule coalescent" (GMYC), que se basa en la teoría de coalescencia neutral y en el modelo de especiación de Yule, y "Poisson Tree Process" (PTP), que tiene en cuenta el número de sustituciones entre secuencias. Las distancias genéticas fueron analizadas con los métodos "Automatic Barcode Gap Discovery" (ABGD) y "Probability of Correct Identification" (PCI). Las hipótesis de especies obtenidas fueron, además, comprobadas con "Bayesian Phylogenetics and Phylogeography" (BPP) un método paramétrico basado en el modelo coalescente multiespecie.

Análisis cofilogenéticos

La congruencia filogenética entre las especies de *Tremella* y sus huéspedes Teloschistaceae, para identificar patrones en su historia evolutiva conjunta, se estudió utilizando métodos con distintos enfoques. ParaFitGlobal es un método que analiza las distancias filogenéticas entre las dos filogenias estudiadas y evalúa si las asociaciones entre ambas son o no aleatorias. La dependencia entre las filogenias fue analizada con PACO ("Procrustean Approach to Cophylogeny"), que evalúa la contribución individual de cada interacción parásito-huésped a la congruencia global de ambas filogenias. La congruencia topológica entre el árbol de *Tremella* y el de sus huéspedes Teloschistaceae se analizó por medio del índice I_{cong}. Por último, se utilizó el programa informático Jane,

que reconstruye la historia evolutiva conjunta de las filogenias estudiadas. Para ello considera diferentes eventos evolutivos a los que atribuye un coste particular.

Análisis de reloj molecular

Las dataciones de ambos grupos se utilizaron para evaluar la congruencia temporal entre los momentos de diversificación en las especies de *Tremella* y de los líquenes Teloschistaceae que habitan. Se utilizaron fósiles para establecer puntos de calibración y los tiempos de divergencia de las especies fueron calculados con BEAST ("Bayesian evolutionary analysis by sampling trees").

Caracterización morfológica de las especies

Los estudios morfológicos de los especímenes de *T. caloplacae* s. l. muestreados, fueron realizados tanto a nivel macroscópico como microscópico. Se tomaron fotografías y medidas de todas las estructuras morfológicas utilizadas para la caracterización de las nuevas especies. Se realizaron estudios moleculares para analizar las relaciones filogenéticas entre las nuevas especies. Se utilizaron datos procedentes de distintas líneas de estudio, y los datos morfológicos y moleculares obtenidos se combinaron con otros ecológicos para caracterizar a las nuevas especies.

Análisis del ciclo de vida

Se estudió el ciclo de vida de *T. caloplacae* s. l., su presencia y distribución en los líquenes Teloschistaceae que habitan. Se realizaron análisis moleculares para detectar la presencia de la especie objetivo en los líquenes huésped. Se realizó hibridación fluorescente *in situ* combinada con microscopía confocal de barrido láser (FISH-CLSM) para determinar las fases del ciclo de vida de *T. caloplacae* s. l. y en qué partes del liquen

huésped se desarrollan las mismas. Para estudiar si existen diferencias en la presencia de *Tremella* que estén relacionadas con el desarrollo de su basidioma, se analizaron ejemplares que presentaban agallas de *Tremella* así como ejemplares aparentemente carentes de agallas.

Resultados y discusión

Capítulo 1

T. caloplacae s. l. es un complejo de especies, cada una de las cuales se corresponde con una especie o género independiente de liquen de la familia Teloschistaceae. La diversidad de especies dentro del complejo de *T. caloplacae* cambia dependiendo del método de delimitación de especies aplicado, variando entre cinco y 17 especies putativas. Ninguno de los análisis cofilogenéticos realizados muestra la existencia de congruencia entre las filogenias de las especies de *Tremella* y la de sus huéspedes Teloschistaceae, por lo que se descarta la coespeciación como evento evolutivo principal en este sistema. Del total de reconstrucciones basadas en coste de eventos ofrecidas por Jane, sólo una fue congruente con los tiempos de divergencia de las especies. Esta reconstrucción muestró que el cambio de huésped era el evento evolutivo que mejor explica la historia evolutiva conjunta de *T. caloplacae* s. l. con sus huéspedes Teloschistaceae. Los resultados de las dataciones establecen el origen de la diversidad conocida en *T. caloplacae* s. l. hace aproximadamente 30 Ma y el de sus líquenes huésped hace aproximadamente 42 Ma, concordando con la literatura previa.

Capítulo 2

Se han descrito formalmente cinco nuevas especies dentro del complejo *T. caloplacae*. Se detectaron diferencias morfológicas entre los especímenes de *T. caloplacae* s. l.

estudiados, que están estrechamente relacionados con los diferentes huéspedes Teloschistaceae en los que habitan. Estas diferencias morfológicas, junto con los datos ecológicos y moleculares han permitido realizar la caracterización y descripción formal de las especies. Todas inducen la formación de agallas en el himenio de sus huéspedes, excepto una que lo hace en el talo. *Tremella elegantis* desarrolla su basidioma en el himenio de *Rusavskia elegans*, *T. nimisiana* en el himenio *Xanthocarpia* spp., *T. parietinae* en el himenio de *X. parietina*, *T. pusillae* en el himenio de *Calogaya pusilla* y *T. sorediatae* en el talo de *R. sorediata*. *T. caloplacae* s. str. se ha circunscrito a crecer exclusivamente en el himenio de *Variospora* spp. Otras tres especies de *Tremella* se dejaron sin nombrar por falta de datos debido al bajo número de especímenes recolectados, *Tremella* sp. 13 en *Cg. biatorina*, *Tremella* sp. 14 en *Cg. decipiens* y *Tremella* sp. 15 en *Polycauliona* sp.

Capítulo 3

Tremella parietinae es una especie dimórfica que alterna su ciclo de vida entre una fase de levadura unicelular y monocariótica, y otra fase filamentosa dicariótica, durante la cual desarrolla su basidioma para reproducirse de forma sexual. Mediante las técnicas de FISH-CLSM se ha comprobado que la fase filamentosa de *T. parietinae* está restringida a desarrollarse en el himenio, con o sin agallas, de especímenes de *X. parietina* con agallas, mientras que la fase levadura puede encontrarse tanto en el himenio como en el margen talino y el talo de especímenes de *X. parietina* con y sin agallas. Estos resultados obtenidos con FISH-CLSM están respaldados por los datos moleculares, mediante los cuales se confirmó la presencia e identidad de *T. parietinae* en las muestras analizadas. Asimismo, en el estudio piloto realizado para detectar la presencia de *T. caloplacae* s. l. en líquenes Teloschistaceae, tanto con agallas de *Tremella* como sin ellas, se ha podido

confirmar la presencia de hongos liquenícolas *Tremella* mediante técnicas moleculares. Sin embargo, la presencia de especies liquenícolas de *Tremella* en líquenes Teloschistaceae sin agallas sólo se ha podido confirmar en aquellos especímenes muestreados en localidades que también presentaban huéspedes con agallas. La fase levadura de algunas especies liquenícolas de *Tremella* parece presentar una menor especificidad respecto al huésped que su fase filamentosa.

Conclusiones

Tremella caloplacae s. l. es un complejo formado por al menos 9 especies, cada una de ellas asociada a un huésped Teloschistaceae específico. Esta delimitación de especies ha demostrado que las regiones ITS y LSU son una combinación efectiva que puede ser utilizada como barcode para hongos liquenícolas Tremellomicetes. La diversificación de las especies conocidas dentro del complejo *T. caloplacae* se produjo en el Oligoceno, como posible consecuencia de la rápida radiación sufrida por los líquenes Teloschistaceae en el Cretácico tardío, aunque probablemente el origen de *T. caloplacae* sea anterior. Esta diversificación en *T. caloplacae* s. l. estuvo promovida por eventos de cambio de huésped y una subsecuente especialización respecto a su huésped particular, que acabaría provocando la especiación de las especies liquenícolas de Tremella. Dentro de este complejo se han podido describir formalmente cinco nuevas especies de *Tremella* basándose en caracteres morfológicos, datos moleculares y ecológicos, tres especies más se han descrito, pero permanecen sin nombrar, mientras que *T. caloplacae* s. str. se ha circunscrito restringida a habitar diferentes especies de *Variospora*. Se ha demostrado el carácter dimórfico de *T. parietinae*, que alterna su ciclo de vida entre una fase levadura y otra fase filamentosa en la cual se reproduce sexualmente. La fase filamentosa está limitada a desarrollarse en el himenio de aquellos líquenes huésped que presentan agallas,

y se ha comprobado que estos basidiomas pueden crecer, al menos en fases iniciales, sin inducir la formación de agallas en *X. parietina*. La fase levadura de *T. parietinae* puede encontrarse, además de en el himenio, en el talo y el margen talino de especímenes de *X. parietina* con y sin agallas. También se ha comprobado la presencia de *T. caloplacae* s. str., *T. candelariellae*, T. *dendrographae* y *T. pusillae* en fragmentos sin agallas de líquenes Teloschistaceae con y sin agallas, evidenciando el posible carácter dimórfico de estas especies. Finalmente, se ha comprobado que existe una diferencia en el nivel de especificidad respecto al huésped entre las distintas fases del ciclo de vida de las especies liquenícolas de *Tremella*, con una fase filamentosa muy específica y una fase levadura más generalista.

Summary

Background

Fungi are a group of organisms that comprise a great diversity of life forms, from free-living fungi to species that need to live in association with other organisms, whether they be animals, plants, other fungi, or lichens. Lichenicolous fungi are those that form obligate associations with lichens and can be both Ascomycetes and Basidiomycetes. Lichenicolous Basidiomycetes are in the minority and represent approximately 4% of all known lichenicolous fungal species. Among them, the Tremellomycetes are the group with the largest number of known lichenicolous species. This group of fungi maintains a very close and specialized relationship with the lichens they inhabit, growing in or on the thallus or the hymenium of their hosts and intermixing their own hyphae with the photobiont and with the mycobiont hyphae. These traits of high interrelation between the fungus and lichen, and great specificity with respect to the host, make them appropriate systems for joint macroevolutionary studies of both organisms. Lichenicolous fungi have traditionally been less studied than other fungal species, probably due to their rarity or because they are difficult for non-experts to observe in nature. Furthermore, they are apparently morphologically simple organisms, and it is difficult to find useful diagnostic morphological characters for species identification. The combination of these two characteristics, low collection numbers and paucity of morphological characters, have often resulted in inaccurate species diagnosis and description of species complexes as single species. In recent decades there has been a great increase in the interest of mycologists and lichenologists in the Basidiomycetes lichenicolous fungi. However, there are still important knowledge gaps in terms of their diversity, evolution, and life cycle. This thesis studies the joint evolutionary history between a group of lichenicolous Tremellales fungi and their Teloschistaceae hosts and its implication in the diversification

of the group, increases the knowledge of the real diversity that they comprise and examines the characteristics of their life cycle.

Objectives

The general objective of this thesis is to unravel the diversity in a group of lichenicolous Tremellomycetes, to study the coevolutionary patterns that have taken place during the joint evolutionary process with their Teloschistaceae hosts and to examine the development of their life cycle in the lichens in which they live. Particularly, in chapter 1 it will be studied whether *Tremella caloplacae* s. l. is a species complex, the potential diversity it encompasses, and the joint evolutionary relationship of the putative new species of *T. caloplacae* s. l. with their Teloschistaceae hosts with a macroevolutionary approach. In chapter 2, it will be performed the morphological characterization and formal description of the potential new species of *T. caloplacae* s. l. will be carried out. Finally, in chapter 3 the life cycle of *T. caloplacae* s. l. will be examined, and its presence and location will be determined in specimens of Teloschistaceae lichens with and without *Tremella* galls.

Methods

Sample collection and molecular data

Samples of Teloschistaceae lichens that were potential hosts for *T. caloplacae* s. l. were collected. In order to obtain the largest possible number of samples, an exhaustive herbarium sampling was carried out and fresh specimens were collected directly in the field. The ribosomal nuclear DNA sequences belonging to the nSSU, ITS and nLSU regions were produced during the development of the thesis and were combined with others downloaded from GenBank.

Species delimitation

Species diversity in *T. caloplacae* s. l. was analysed applying methods based on 1) the topology of phylogenetic trees and 2) genetic distances. The topology of the ultrametric tree of *T. caloplacae* s. l. was studied using the methods "generalized mixed Yule coalescent" (GMYC), which is based on the neutral coalescence theory and the Yule speciation model, and "Poisson Tree Process" (PTP), which considers the number of substitutions between sequences. Genetic distances were analysed with the methods "Automatic Barcode Gap Discovery" (ABGD) and "Probability of Correct Identification" (PCI). The species hypotheses obtained were also verified with "Bayesian Phylogenetics and Phylogeography" (BPP), a parametric method based on the multispecies coalescent model.

Cophylogenetic analyzes

The phylogenetic congruence between *Tremella* species and their Teloschistaceae hosts, was studied using methods with different approaches to identify patterns in their joint evolutionary history. ParaFitGlobal analyses the phylogenetic distances between the two studied phylogenies and evaluates whether or not the associations between them are random. The dependence between the phylogenies was analysed with PACO ("Procrustean Approach to Cophylogeny"), which evaluates the individual contribution of each parasite-host interaction to the global congruence of both phylogenies. The topological congruence between the *Tremella* tree and that of its Teloschistaceae hosts was analysed with the I*cong* index. Finally, the software Jane was used to reconstruct the joint evolutionary history of the studied phylogenies. To do this, Jane considers different evolutionary events to which it attributes a particular cost.

Molecular clock analysis

The datings of both groups were used to evaluate the temporal congruence between the moments of diversification of the *Tremella* species and the Teloschistaceae lichens they inhabit. Fossils were used to establish calibration points and species divergence times were calculated with BEAST ("Bayesian evolutionary analysis by sampling trees").

Morphological characterization of the species

Morphological studies of the *T. caloplacae* s. l. specimens sampled, were carried out both at the macroscopic and microscopic level. Photographs and measurements of all the morphological structures used for the characterization of the new species were taken. Molecular studies were performed to analyse the phylogenetic relationships between the new species. Data from different lines of study were used, and the morphological and molecular data obtained were combined with other ecological data to characterize the new species.

Life cycle analysis

The life cycle of *T. caloplacae* s. l., its presence and distribution in the Teloschistaceae lichens they inhabit, was studied. Molecular analyses were performed to detect the presence of the target species in the host lichens. Fluorescence *in situ* hybridization combined with confocal laser scanning microscopy (FISH-CLSM) was carried out to determine the life cycle phases of *T. caloplacae* s. l. and in which parts of the host lichen they develop. To study whether there are differences in the presence of *Tremella* related to the development of its basidioma, specimens that had *Tremella* galls as well as specimens apparently devoid of galls were analysed.

Results and discussion

Chapter 1

T. caloplacae s. l. is a complex of species, each of which corresponds to an independent species or genus of lichen from the Teloschistaceae family. Species diversity within the *T. caloplacae* complex changes depending on the species delimitation method applied, ranging from five to 17 putative species. None of the cophylogenetic analyses carried out shows the existence of congruence between the phylogenies of *Tremella* species and that of their Teloschistaceae hosts, therefore cospeciation is ruled out as the main evolutionary event in this system. Of the total event cost-based reconstructions offered by Jane, only one was consistent with the divergence times of the species. This time-congruent reconstruction showed that host switching was the evolutionary event that best explains the joint evolutionary history of *T. caloplacae* s. l. with their Teloschistaceae hosts. The dating results establish the origin of the known diversity in *T. caloplacae* s. l. about 30 Mya and that of its host lichens about 42 Mya, agreeing with previous literature.

Chapter 2

Five new species within the *T. caloplacae* complex have been formally described. Morphological differences were detected between *T. caloplacae* s. l. specimens studied, which are closely related to the different Teloschistaceae hosts they inhabit. These morphological differences together with the ecological and molecular data, have allowed the characterization and formal description of the studied species. All induce gall formation on the hymenium of their hosts, except one that does so on the thallus. *Tremella elegantis* develops its basidioma in the hymenium of *Rusavskia elegans*, *T. nimisiana* in the hymenium of *Xanthocarpia* spp., *T. parietinae* in the hymenium of *X. parietina*, *T. pusillae* in the hymenium of *Calogaya pusilla*, and *T. sorediatae* in the thallus of

R. sorediata. *T. caloplacae* s. str. has been circumscribed to grow exclusively in the hymenium of *Variospora* spp. Three other *Tremella* species were left unnamed due to the lack of data and to the low number of specimens collected, *Tremella* sp. 13 in *Cg. biatorina*, *Tremella* sp. 14 in *Cg. decipiens* and *Tremella* sp. 15 in *Polycauliona* sp.

Chapter 3

Tremella parietinae is a dimorphic species that alternates its life cycle between a unicellular and monokaryotic yeast phase, and another dikaryotic filamentous phase, during which it develops its basidioma to sexually reproduce. Using FISH-CLSM techniques, it has been verified that the filamentous phase of *T. parietinae* is restricted to develop in the hymenium, with or without galls, of specimens of *X. parietina* with galls, while the yeast phase can be found both in the hymenium as in the thalline margin and thallus of *X. parietina* specimens with and without galls. These results obtained with FISH-CLSM are supported by molecular data, which confirmed the presence and identity of *T. parietinae* in the analyzed samples. Likewise, in the pilot study carried out to detect the presence of *T. caloplacae* s. l. in Teloschistaceae lichens, both with and without *Tremella* galls, it has been possible to confirm the presence of lichenicolous *Tremella* species using molecular techniques. However, the presence of *Tremella* in Teloschistaceae lichens without galls could only be confirmed in those specimens sampled in localities that also had potential hosts specimens with galls. The yeast phase of some lichenicolous *Tremella* species appears to have less host specificity than its filamentous phase.

Conclusions

Tremella caloplacae s. l. is a complex formed by at least 9 different species, each one of them associated with a specific Teloschistaceae host. This species delimitation has shown

that the ITS and LSU regions are an effective combination that can be used as a barcode for Tremellomycete lichenicolous fungi. The diversification of the known species within the *T. caloplacae* complex occurred in the Oligocene, as a possible consequence of the rapid radiation suffered by Teloschistaceae lichens in the late Cretaceous, although the origin of *T. caloplacae* is probably previous. This diversification in *T. caloplacae* s. l. was promoted by host switch events and a subsequent specialization with respect to its particular host, which would end up causing the speciation of these *Tremella* species. Within the complex, five new lichenicolous *Tremella* species have been formally described based on morphological characters, molecular and ecological data, three more species have been described but remain unnamed, while *T. caloplacae* s. str. has been circumscribed to inhabit different species of *Variospora*. The dimorphic character of *T. parietinae* has been demonstrated, as it alternates its life cycle between a yeast phase and another filamentous phase in which it reproduces sexually. The filamentous phase and the basidiomata formation is restricted to the hymenium of *X. parietina* specimens with galls, being able to grow, at least in early stages, without inducing galls. The yeast stage of *T. parietinae* can be found, additionally to in the hymenium, in the thallus and the thalline margin of specimens of *X. parietina* with and without galls. The presence of *T. caloplacae* s. str., *T. candelariellae*, *T. dendrographae* and *T. pusillae* in fragments without galls of Teloschistaceae lichens with and without galls, evidence the possible dimorphic character of these species. Finally, it has been verified that the level of specificity with respect to the host of the Tremella lichenicolous species vary depending on the phase of the life cycle: the filamentous phase is highly host-specific while the yeast phase is more generalist.

General introduction

General introduction

Fungal diversity and coevolution

Fungi are one of the largest groups of eukaryotes (Blakcwell 2011) whose origin occurred approximately 2 billion years ago (± 500 million years) (Berbee & Taylor, 2010). Since then, fungi have colonized all types of habitats, from soil and water to other organisms, interacting with most groups of organisms (Blackwell 2011), and playing an essential role in ecosystems (Dulla et al. 2016). Since the XIX century with Fries (1825), there have been many studies that have tried to estimate the number of fungal species that could exist on Earth (Bisby & Ainsworth, 1943; Martin 1951; Hawksworth 1991, 2001), reaching the number of 5.1 million species if data obtained from massive sequenced environmental samples is considered (O'Brien et al. 2005). Hawksworth and Lücking (2017) in their last review on this subject, estimated the fungal diversity in a more conservative way at 2.2 to 3.8 million species. Despite these estimates, only around 120,000 described fungal species are known (Hawksworth and Lücking, 2017). The majority of these species are encompassed within the two main groups of fungi, are the Ascomycetes and the Basidiomycetes. Fungal species in these two groups have a wide variety of habitat requirements, growing in soil, both fresh and marine water, associated with animals such as arthropods and invertebrates, with the roots, leaves, and stems of plants, and with lichens (Blackwell 2011). Approximately 20% of the fungi form symbiosis with green alga or cyanobacteria (Rikkinen 2003), that is, they are lichenized fungi. Lichens have a polyphyletic origin and this association between mycobionts and photobionts, has evolved and disappeared several times in various fungal groups in the evolutionary history of fungi (Gargas et al. 1995; Tehler et al. 2000; Lutzoni et al. 2001; Wedin et al.

2004). Likewise, some groups of fungi that are not currently lichenized have evolved from lichenized ancestors (Lutzoni et al. 2001).

Relationships between organisms of different species, such as those established between hosts and symbionts, play an important role in population diversification (Nunn et al. 2004). These interaction between species has historically been one of the objectives of study in the field of biodiversity (Thompson 1994, 2005). Coevolution is the reciprocal adaptation between two organisms over time, resulting in micro-evolutionary changes in both organisms (Page 2003; Thrall et al. 2007; de Vienne et al. 2013). At large time scales, coevolution could be related to the ecological interaction between species, influencing their diversification, explaining the origin and maintenance of symbiosis in some organisms and the adaptive response in sexually reproducing organisms (Thrall et al. 2007; Montarry et al. al. 2012; Althoff et al. 2014; Koskella & Brockhurst, 2014; Wininger & Rank, 2017). The term coevolution is sometimes used as a synonym for cospeciation, but these two concepts should not be confused (Smith et al. 2008). Cospeciation is the process by which speciation in one symbiont is linked to speciation in the other (de Vienne et al. 2013), being a macroevolutionary pattern. According to Page (2003), coevolution results from a series of reciprocal adaptations that give rise to lineages that coevolve without cospeciating, although cospeciation does require a certain degree of coevolution. From early studies of organisms with very close relationships, such as that between an obligate parasite and its host, it was inferred that similar parasites associated with similar hosts, resulting in the phylogenies of both organisms being consistent with each other (Kellogg 1913; Fahrenholz 1913; Szidat 1940). However, speciation on symbionts can take place by host switching without the speciation of the host (Agosta et al. 2010; Giraud et al. 2010), and these events can also result in congruent

phylogenies (de Vienne 2007, 2013). Based on the congruence of the phylogenies of the interacting species, cophylogenetic methods have been developed to assess the effect of cospeciation on their joint evolutionary history. De Vienne et al. (2013) groups these methods into two main approaches: 1) methods based on evolutionary events, that compare the phylogenies of symbionts and hosts to reconstruct their joint evolutionary history and the nature and frequency of the coevolutive events, and 2) methods based on topology and distance, which assess the general congruence between phylogenies of the involved organisms.

Species concept and species delimitation

Defining what a species is, is one of the biggest debates that have been generated in biology and that has extended from the 18th century to the present day (Naomi 2011; Cao et al. 2021). The species has been considered a basic units for comparison in biology (Mayden 1997; de Queiroz 2005), allowing communication between biologists from different fields (Naomi 2011). The first definitions of the species concept, from the 18th century with Micheli (1729), Linnaeus (1753) or Fries (1821), were based mainly on the morphology of the organisms and species were defined based on their morphological differences. It was Mayr (1942) who first named the different approaches to the species concept that had emerged up to that time, marking the beginning of the modern era of species concepts (Hey 2006). From the beginning of the debate to the present, at least 32 different species concepts have been proposed (Mayr 1942; Wiley 1978; Mayden 1997; de Queiroz 2005; Naomi 2011; Cao et al. 2021). Of all the proposed species concepts, the most widely used and discussed have been:

1) The morphological concept of species, where species are defined as the smallest population or group that maintains constant certain morphological characteristics that make it different from other populations (Cronquist 1978).

2) The biological concept of species, which defines species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr, 1942, 1963).

3) The evolutionary concept of species, proposed by Simpson (1951) and defining species as "a single lineage of ancestor-descendant populations of organisms which maintains its identity from other such lineages (in space and time) and which has its own evolutionary tendencies and historical fate".

4) The phylogenetic concept of species, developed in parallel by Eldredge & Cracraft (1980) where species is defined as "a diagnosable cluster of individuals within which there is a parental pattern of ancestry and descent, beyond which there is not, and which exhibits a pattern of phylogenetic ancestry and descent among units of like kind" and by Nelson and Platnick (1981), who defined species as "simply the smallest detected samples of self-perpetuating organisms that have unique sets of characters".

The number of species that are described varies depending on the species concept with which they are studied, which may have had negative consequences for biodiversity. Mayden (1997, 1999, 2002) was the first to attempt to integrate the different species concepts by creating a hierarchical system with an evolutionary approach. He separated the concepts of species into primary (theoretical) and secondary (operational). He considered that the only purely theoretical species concept was the evolutionary species concept, and the rest of the concepts such as the morphological, the biological or the phylogenetic were secondary to the evolutionary concept (Taylor 2000). Afterwards, de

Queiroz (1998, 1999, 2005, 2007) tried to create a unified concept which he called "unified species concept". From the set of species concepts with evolutionary considerations, he eliminated the criteria that made them different and thus separated the common conceptual part that they all had (what he called the unified species concept) and the contingent or secondary properties. Both Mayden and de Queiroz separated into two different categories the conceptualization of the species and their recognition or delimitation.

For fungi, morphological characters and pairing tests have traditionally been used to delimit species. Although morphology has been widely used for species diagnosis, relying exclusively on one method could be problematic. Many fungi, such as unicellular yeasts, have few morphological diagnostic characters (Boekhout et al. 2021), which could lead to diagnosing as a single species what is actually a group of them (Cao et al. 2021). Pairing tests have also demonstrated their usefulness for species delimitation, being sometimes more precise than morphological analyses (Petersen & Hughes, 1999). However, they are not applicable in many types of fungi such as those that are morphologically asexual, those that produce meiospores without a partner (Taylor 2000), those that hybridize and undergo genetic isolation alone (Boekhout et al. 2021) or those that are not cultivable (Cao et al. 2021). For fungi, the species recognition method most consistent with the evolutionary species concept is phylogenetic analysis (Taylor 2000). This method can cause problems if the analysis is based on a single gene due to the subjectivity that introduces where to set the limit to diagnose species, but it is easily solvable if it is based on several unbound DNA regions. This system of species recognition based on phylogenies and proposed by Taylor (2000) is called Genealogical Concordance Phylogenetic Species Recognition (GCPSR). Its strength is relies mainly on the fact that

it compares the genealogies of more than one gene, but this could generate conflict between the genealogies of each gene due to recombination or clonal reproduction of some fungi, introgressions or structured populations (Cao et al. 2021).

Speciation is characterized by the interruption of gene flow (Coyne & Orr, 2004), and since genetic changes are detected before morphological or biological ones (Scorzetti et al. 2002), analyzing the genes of organisms it would be possible to perform species delimitations (Cao et al. 2021). Based on phylogenetic data, powerful species delimitation methods have been created by applying the principle of coalescence (Pons et al. 2006; Puillandre et al. 2012; Yang 2015) or by analyzing the number of substitutions to model speciation events (Zhang et al. al. 2013) on organisms where morphological methods or inbreeding tests may have limitations, such as fungi (Leavitt et al. 2011; Lumbsch & Leavitt, 2011; Millanes et al. 2014; Zamora & Ekman, 2020; Bhunjun et al. 2020; Freire-Rallo et al. 2023). Among the most accepted and applied methods that are based on neutral coalescence (Hudson 1990) are the General Mixed Yule Coalescent (GMYC) model (Pons et al., 2006; Fontaneto et al., 2007; Monaghan et al., 2009; Fujisawa and Barraclough, 2013), the Automated Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) and the Bayesian Phylogenetics and Phylogeography (BPP) (Yang 2015).

GMYC assumes that there is a specific moment in which the generation of species occurs and to determine it, it discriminates between nodes generated by a coalescence process or by a Yule process (Hudson 1990). It is a fast and computationally undemanding method that does not require previous hypotheses. However, it assumes the absence of lineage sorting, reciprocal monophyly in gene trees, and does not perform well when populations are small or speciation events occur in short time intervals (Fujisawa and Barraclough,

2013; Luo et al, 2018; Rannala & Yang, 2020; Chethana et al. 2021). The ABGD analyzes intra- and inter-specific distances to distinguish existing differences within a population from differences caused by species divergence through an automated process (Puillandre et al. 2012). It is a very computationally efficient method that can be used even when the barcode gap is overlapping (Chethana et al. 2021). In contrast, it does not work well when there is lineage sorting in the gene tree and, based only on pairwise distance calculations, it wastes the rest of the information provided by the data sequences (Rannala & Young, 2020). The main problem that both GMYC and ABGD present is that they are methods designed to operate on a single locus, although they can also be used on datasets with several concatenated genes (Rannala & Young, 2020). The BPP is a method based on coalescence, but in this case, it is based on information from multiple loci to model the evolution of the studied populations (Yang 2015; Luo et al. 2018). It is not very sensitive to the effects of gene flow, assumes the possible discordance between trees and obtains a lower number of false positives or negatives than other methods (Luo et al. 2018). On the other hand, it has the disadvantage that it needs the specification of good priors to operate correctly, and its performance is affected by the population size (Luo et al. 2018). Of the most widely used delimitation methods, PTP is the only one that is not based on coalescence, but delimits species based on the branch length distribution, that is, on substitutions, in the gene tree (Zhang et al. 2013). It can be used on large datasets and does not require calibrated trees to perform its analysis. Among the main problems it presents are that it has been designed for a single locus, although multiple loci can be concatenated, and its performance is affected by an unbalanced individuals sampling among species (Zhang et al. 2013; Luo et al. 2018; Rannala & Young, 2020).

Despite the drawbacks of multilocus methods, they have led to an advance in the knowledge and development of these methods and their application has resulted in an important source of data for the establishment of new taxa (Cao et al. 2021). Approaches based on the use of the whole genome have recently been developed and have been used in mycology to, among other things, solve resolution problems (Lücking et al. 2020). Because there are advantages and disadvantages to all species delimitation methods, it is not recommended to apply a single method, but to use a combination of them (Carstens et al. 2013), and to provide data from as many lines of evidence as possible following an integrative approach (Cao et al. 2021).

Lichenicolous fungi

Among the fungi that depend on other organisms for their survival, lichens are one of the best-known examples. In addition to the mycobiont and the photobiont that make them up, lichens host a great microbial biodiversity (Cengia Sambo 1926; Honegger 1990; Lawrey & Diederich, 2003; Petrzik et al. 2014; Grimm et al. 2021), which has found a suitable niche for its development within the lichen. Part of this lichen biota is made up of fungi that grow in or on the lichen, the mycobiota, and to which Arnold et al. (2009) called endolichenic fungi making an analogy with endophytic fungi of plants. Some authors have established an artificial differentiation between endolichenic fungi, those that cannot be detected morphologically in lichens because they do not form reproductive structures in them, and lichenicolous fungi, those that do develop their reproductive structures in or on lichens (Arnold et al. 2009; Lawrey & Diederich, 2003). Lichenicolous fungi are highly specialized fungi that inevitably live in association with lichens (Lawrey & Diederich, 2003; Diederich et al. 2018). Although the existence of endolichenic fungi has been recognized by scientists for some time there is evidence of the study of

Biatoropsis usnearum, a lichenicolous fungus, from the mid-18th century (Dillenius 1742; Acharius 1795). Throughout the 19th century and the beginning of the 20th century, various revisions and compilations on lichenicolous fungi were made, but it was from the publications by Clauzade & Roux (1976), Hawksworth (1981, 1983), Clauzade et al. (1989) and Diederich (1996), when interest in these fungi increased (Lawrey & Diederich, 2003; Diederich et al. 2018). Although, lichenicolous fungi were recognized even before the symbiosis of lichens (Grube & Wedin, 2016) little is still known about their diversity, life cycle, habitat, and ecology.

Since the first compilation by Clauzade & Roux (1976) with 457 recorded species, the number of lichenicolous fungal species has increased to 2000 at the last count (Diederich et al. 2018), to which new species are added each year. The species of lichenicolous fungi belong to both the Ascomycetes and the Basidiomycetes groups. Although the vast majority of lichenicolous fungi are Ascomycetes (around 95%), those species that belong to the Basidiomycetes (5%) are phylogenetically very diverse (Lawrey & Diederich 2003) and, of the total lichenicolous Basidiomycete species, around 50% belong to entirely lichenicolous genera (Diederich et al. 2018). This group is found within the dimorphic fungi, alternating between the yeast-like (monokaryotic, asexual) phase and the filamentous (dikaryotic, sexual) phase; Diederich (1996) was the first to observe the presence of their yeast stage. Lichenicolous Basidiomycetes are very specialized fungi towards their hosts, with a very narrow range of species on which they can live and develop their sexual structures, establishing relationships that range from parasitism to commensalism, saprotrophy and lately hypothesized symbiosis (Hawksworth 1982, 2003; Triebel et al. 1997; Lawrey & Diederich 2003; Asplund et al. 2016; Spribille et al. 2016; Diederich et al. 2018; Tuovinen et al. 2019, 2021; Tagirdzhanova et al. 2021).

Lichenicolous fungi can generate abnormal growths or galls on the thallus or hymenium of their hosts (Diederich 1986, 1996; Sérusiaux et al. 2003), causing lesions, discoloration and even serious damage to the host (Hawksworth 1982). Less frequently, the presence of asymptomatic lichenicolous fungi has been observed in their hosts (Fleischhacker et al. 2015; Fernández-Mendoza et al. 2017; Diederich et al., 2018; Millanes et al. 2021) and, recently, it has begun to be verified that this yeast phase is less specific and can be found in a broader spectrum of hosts (Spribille et al. 2016; Tuovinen et al. 2019, 2021). Little is known about the reasons behind the high host specificity displayed by lichenicolous Basidiomycetes. Lichens are great producers of secondary compounds, and lichenicolous fungi live in close interaction with them, so adapting the compounds generated by their host must be essential for their development. The adaptation and affinity of some lichenicolous fungi for the lichenic secondary metabolites of their host lichens has been experimentally verified (Lawrey 1993, 1997; Lawrey et al. 1999; Lawrey & Diederich, 2003; Werth et al. 2013; Asplund et al. 2018), and these compounds could play a role in the genetic structuring of the lichenicolous fungi (Pino-Bodas et al. 2018). In contrast, some studies suggest that the presence of lichenicolous fungi and their production of secondary metabolites could have a negative effect on the lichen host (Merinero et al. 2015; Merinero & Gauslaa, 2018; Asplund et al. 2016, 2018).

Traditionally, the different species of lichenicolous fungi have been considered from saprotrophs or commensals to parasites with highly variable virulence (Hawksworth 1982). Some species of lichenicolous fungi are very virulent and eventually kill their host (necrotrophic parasites), such as *Athelia arachnoidea* (Atheliaceae, Basidiomycota) (Hawksworth 1982; Lawrey & Diederich, 2003), others show barely apparent parasitism, keeping their host alive for long periods of time (biotrophic parasites) (Lawrey &

Diederich, 2003). Hawksworth (1982), classified gall-forming lichenicolous fungi as biotrophic parasites. Some gall-forming lichenicolous fungi, such as *Biatoropsis usnearum*, are known to parasitize the lichen mycobiont via haustoria (de los Ríos & Grube 2000; Grube & de los Ríos, 2001; de los Ríos et al. 2002), although they can also parasitize the alga or both (Diederich et al. 2018). Recently, other studies have detected the presence of lichenicolous Basidiomycetes yeasts in a great diversity of lichens (Muggia et al. 2016; Fernandez-Mendoza et al. 2017; Lendemer et al. 2019; Smith et al. 2020; Tagirzhanova et al. 2021; Cometto et al. 2022) and suggest that these species could play an important role in lichen symbiosis and in the phenotypic expression of lichen (Spribille et al. 2016). However, the nature of the relationship established by the vast majority of lichenicolous fungi is still unknown.

Tremellomycetes

The Tremellomycetes are a very diverse group of fungi (He et al. 2022). Many of the species in this group are dimorphic, alternating in their life cycle between a unicellular haploid yeast phase and a filamentous phase that is dikaryotic (Bandoni 1995). Of many species, only their yeast phase is known, in which they reproduce asexually, but when two compatible yeasts mate, the filamentous phase is produced. This filamentous phase is responsible for the formation of the basidioma, producing basidia and haploid basidiospores. Numerous Tremellomycete species can be parasites of a great variety of organisms, like *Cryptococcus neoformans* and *C. gattii*, both of them human parasitic fungi that can cause cryptococcosis in patients with a depressed immune system (Waters & Nelson, 2005). Most of the parasitic species of Tremellomycetes are mycoparasites of Ascomycetes or other Basidiomycetes. *Tremella mesenterica* ("jelly fungi") which is a fungicolous species that parasitizes fungi of the genus *Peniophora*. Other species are

lichenicolous, living in or on lichens parasitizing the lichen mycobiont, the photobiont or both (Lawrey & Diederich, 2003). As a whole, the Tremellomycetes have a wide range of potential hosts, but individually they are highly specialized species that show very high host specificity (Lawrey & Diederich, 2003), although the nature of their interactions is still unknown for most of them. Zugmaier et al. (1994, 1995) visualized *T. mesenterica* haustoria penetrating Peniophora laeta hyphae. De los Rios et al. (2000, 2002) and also Grube & de los Ríos (2001) showed how the haustoria of *Biatoropsis usnearum* and other lichenicolous fungi penetrated the hyphae of the mycobiont of their host lichens.

Since the early 1990s, with the increasing ease of generating and accessing molecular data, knowledge in mycology has undergone a great boost. The first phylogenetic studies placed the Tremellomycetes within the Basidiomycetes group (Swann & Taylor, 1995) and phylogenies were produced for yeast-like (Fell et al. 2000) and filamentous (Chen 1998) taxa, which would later be unified in more recent studies (Matheny et al. 2007; Liu et al. 2015a). Obtaining molecular data for lichenicolous species is an extra challenge. Since their hyphae grow intercrossed with the hyphae of the lichen mycobiont, it is necessary to use specific primers to amplify the DNA of the target organism. Two decades after the beginning of the molecular era, Millanes et al. (2011) obtained the first phylogeny that included lichenicolous taxa, placing them within the Tremellales. The beginning of molecular studies in the Tremellomycetes, which has resulted in essential advances for the correct classification of the species that make up this group. The work of Liu et al. (2015a,b) that allowed seven new families and 18 new genera to be included in this group, new studies have been added that have contributed to clarifying the relationships of the different Tremellomycetes species (Hawksworth et al. 2016; Wedin et al. 2016).

Lichenicolous Tremellomycetes species have traditionally been known to induce gall formation in their hosts, although these deformations often go unnoticed, and sometimes the presence of the lichenicolous fungus is inconspicuous (Diederich 1996; Diederich et al. 2022). These galls are the result of the development of their basidioma (filamentous phase), in which they develop sexual reproduction structures, the basidia and subsequently basidiospores. However, recently the presence of lichenicolous Tremellomycetes yeasts has been discovered both in their host lichen (in which they develop their filamentous phase) and in other closely related lichen species (Tuovinen et al. 2019, 2021). The function that this haploid unicellular form of lichenicolous fungi may develop in the lichen, or the relationship that it may have with it, is a matter that remains to be elucidated.

The *Tremella caloplacae* complex

Within the Tremellomycetes, we can find the genus *Tremella*, which species form conspicuous basidiocarps with gelatinous appearance (‘jelly fungi’) or develop gall like structures on the thallus or the apothecia of its hosts (Diederich 1986, 1996; Sérusiaux et al. 2003), although sometimes sexual reproductive structures are created without the formation of galls (Diederich 1996; Zamora et al. 2011). The genus *Tremella* encompass the higher number of lichenicolous species of the Basidiomycetes, with 117 (Diederich et al. 2022). Millanes et al. (2011) tested for the first time the phylogenetic relationships of lichenicolous *Tremella* species, placing them within the genus *Tremella*, and stablishing three different clades for the lichenicolous species (*Tremella* s. l. clades I, II and III).

One of these lichenicolous *Tremella* species is *Tremella caloplacae* (Zahlbr.) Diederich. This was first studied by Zahlbruckner in a specimen of *Variospora aurantia* (at that time *Caloplaca callopisma*) collected in 1904 in Crete, Greece, which presented deformations in the hymenium of its apothecia. In 1906 the species was described as a hypomycete under the name of *L. caloplacae* Zahlbr (Zahlbruckner 1906). Decades later, Diederich collected *Caloplaca* specimens from other European localities (Austria and Great Britain) and identified a species of lichenicolous *Tremella* growing on its hymenium, but which he did not name and published as *Tremella* sp. 1 in his compendium of lichenicolous heterobasidiomycetes (Diederich 1996). Almost a decade later, after studying the *L. caloplacae* type material, Sérusiaux et al. (2003) concluded that it was the same species as *Tremella* sp. 1 and that what Zahlbruckner interpreted as conidia were basidia instead, and consequently formally described *T. caloplacae.* The work by Millanes et al. (2011) was the first to evince the existence of numerous lichenicolous *Tremella* species complexes, such as that formed by *T. caloplacae* (circumscribed in *Tremella* s. l. clade I). The difficulty involved in the description of *T. caloplacae* s. l., and other lichenicolous *Tremella* species, is evident due to the scarcity of diagnostic morphological characters they possess. For this reason, it is essential to apply an integrative approach with data from as many lines of evidence as possible.

Thesis objectives and hypothesis

Objective 1 (Chapter 1): To study the diversity comprised within *Tremella caloplacae* potential species complex, the coupled evolution of the *T. caloplacae* s. l. putative different species with their Teloschistaceae lichen hosts and to date their origin. We hypothesize that the *T. caloplacae* complex is formed by several different species, each one of them developing in a particular host. We will also test the null hypothesis (H_0) that

each potential new species of *Tremella* is randomly related to its lichen hosts. The alternative hypothesis (H_1) is that these associations are not random but there is a correspondence between each potential new species of *Tremella* and the Teloschistaceae host.

Objective 2 (Chapter 2): To morphologically characterize and formally describe, or tentatively describe in those cases where the material is not enough, the new species within the *Tremella caloplacae* complex through an integrative approach, combining morphological, ecological and molecular data together with phylogenetic analysis.

Objective 3 (Chapter 3): To study the life cycle of *Tremella parietinae* and the location of the filamentous and yeast stages within its lichen host *Xanthoria parietina*, from specimens with and without galls of *Tremella* by using FISH-CLSM analysis. We hypothesize that the yeast phase of *T. parietinae* is widespread within the *X. parietina* hymenium and thallus, while the filamentous phase is restricted to the hymenium. We will also achieve a pilot study on potential Teloschistaceae lichen hosts of *T. caloplacae* s. l. to investigate how common are tremelloid yeast phase in samples without galls from lichen specimens with a without galls by phylogenetic analysis. Our hypothesis is that the tremelloid yeasts detected correspond to the yeast phase of the *Tremella* gall-inducing species.

Chapter 1

'To explore strange new worlds' - speciation by host shift in *Tremella caloplacae* followed the cretaceous adaptive radiation of the Teloschistaceae

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Abstract

Lichenicolous fungi are a heterogeneous group of organisms that grow exclusively on lichens, forming obligate associations with them. It has often been assumed that cospeciation has occurred between lichens and lichenicolous fungi, but this has been seldom analysed from a macroevolutionary perspective. Many lichenicolous species are rare or are rarely observed, which results in frequent and large gaps in the knowledge of the diversity of many groups. This, in turn, hampers evolutionary studies that necessarily are based on a reasonable knowledge of this diversity. *Tremella caloplacae* is a heterobasidiomycete growing on various hosts from the lichen-forming family Teloschistaceae, and evidences suggest that it may represent a species complex. We combine an exhaustive sampling with molecular and ecological data to study species delimitation, cophylogenetic events and temporal concordance of this association. *Tremella caloplacae* is here shown to include at least nine distinct host-specific lineages (=putative species). Host switch is the dominant and most plausible event influencing diversification and explaining the coupled evolutionary history in this system. This is also supported by the divergence times of *T. caloplacae* s. l., which are often subsequent to the divergence times of their Teloschistaceae hosts. Speciation in *T. caloplacae* would therefore have occurred after the rapid diversification – by adaptive radiation in the late Cretaceous – of their hosts. New species in *T. caloplacae* would have developed as a result of specialization on diversifying lichen hosts that suddenly offered abundant new ecological niches.

Keywords: Fungi, Teloschistales, Tremellales, species delimitation methods, coevolution, dating.

Introduction

Lichenicolous fungi are a diverse and heterogeneous assemblage of organisms that grow and develop exclusively on lichens, often forming obligate associations. The early literature on lichenicolous fungi is scattered, but the interest in these fungi increased much during the past decades with the production of many very useful reviews, monographs, and identification keys (Hawksworth, 2003; Clauzade et al., 1989; Diederich, 1996; Lawrey and Diederich, 2003; Ihlen and Wedin, 2008; Diederich et al., 2018; Millanes et al., 2021). The nature of these associations is frequently not known with certainty. Some lichen-inhabiting fungi are more or less clearly parasites of the mycobiont, the photobiont, or both, while others are potentially saprotrophs or symbionts (Hawksworth, 1982a, 2003; Triebel et al., 1997; Lawrey and Diederich, 2003; Asplund et al., 2016; Spribille et al., 2016; Diederich et al., 2018; Tuovinen et al., 2019, 2021; Tagirdzhanova et al., 2021). We therefore refer to 'hosts' and 'parasites' for simplicity in this manuscript, assuming that other kinds of interactions are possible. Independently of the character of the association, the long-term evolutionary partnership with lichens seems to drive speciation at least in some groups of lichenicolous fungi (Lawrey and Diederich, 2003, Millanes et al., 2014a)

In 2018, 2000 species of obligately lichenicolous fungi had been described, but it is estimated that the number of species could reach 5000 (Diederich et al., 2018). Approximately 95% of the lichenicolous fungi are supposed to be highly specialized towards their hosts (Diederich et al., 2018), although the degree of host-specificity can be concealed by uncertainties in species boundaries. The close and intimate relationship between lichenicolous fungi and their hosts, and the frequent high specificity, resulted in the widespread assumption that lichenicolous fungi cospeciated with lichens

(Hawksworth, 1982b; Lawrey and Diederich, 2003), an assumption that has only rarely been tested (Millanes et al., 2014a).

Organisms that live ecologically linked potentially affect each other's evolution (coevolution; Page, 2003), which results in the microevolutionary processes of mutual selection and reciprocal adaptation defined as coevolution (Page, 2003; Nunn et al., 2004; Thrall et al., 2007; de Vienne et al., 2013). Coevolution does not necessarily imply cospeciation, although it promotes it in some cases (Page, 2003; Smith et al., 2008). Cospeciation is, on the contrary, the macroevolutionary pattern observed when two symbionts speciate at the same time and can simply result from allopatric co-diversification in both linked organisms. In host-parasite systems, however, hosts can promote parasite speciation in sympatry by providing new ecological niches. When two symbionts undergo speciation at the same time (cospeciation) their joint evolutionary history results in congruent phylogenies (Wang et al., 2019). However, cospeciation is not the only process leading to matching topologies (Millanes et al., 2014a; Herrera et al., 2016; Zhao et al., 2016; Singh et al., 2017; Fecchio et al., 2018; Navaud et al., 2018; Layton et al., 2019; Lindgren et al., 2020). De Vienne et al., (2013) already pointed out that only 7% of the published cophylogenetic studies constituted convincing cases of cospeciation. Symbionts speciate more often by host switch, which implies specialization of a newly emerged parasite species on a new host (Buser et al., 2014; Fecchio et al., 2018; Mestre et al., 2020). Host switches can also result in congruent phylogenies, particularly when branch lengths are shorter in parasite phylogenies, indicating that speciation in the parasites occurred much later than that of the hosts (de Vienne et al., 2013). The first, and so far only cophylogeny study carried out on lichenicolous fungi and their hosts showed that speciation by host switch rather than cospeciation was prevalent

in this system (Millanes et al., 2014a). In organisms ecologically linked, 'cascading' or 'sequential' speciation has been proposed as a process in which associated organisms evolve by subsequent adaptive differentiation of one of them as a response to an initial divergence of the other. Diversification events in one group would then in turn act as drivers for further diversification (Feder and Forbes, 2010; Hood et al., 2015). Although sequential speciation has been linked to cospeciation derived from coevolution (Bracewell et al., 2018), sequential speciation could also result from host shifting, when new parasite species form as a result of new opportunities for hosts colonisation in scenarios of host diversification (Janz et al., 2006; Forbes et al., 2009). Such a situation would resemble parasites 'exploring strange new worlds', suddenly made available after speciation in the host group.

Within the fungal groups including lichenicolous representatives, the Tremellomycetes (Agaricomycotina, Basidiomycota) are particularly interesting in terms of their varied ecology. They comprise species with a remarkably diverse range of lifestyles, including lichenicolous fungi, saprotrophs, parasites of non-lichenized fungi, and parasites of animals, including humans (Millanes et al., 2011; Liu et al., 2015). Lichenicolous Tremellomycetes are also interesting in that they represent a reservoir of overlooked diversity. Diederich (1986) was the first to formally describe a lichenicolous *Tremella* species. Since then, more than 60 new species have been described, which indicates a great and still largely unexplored diversity in the Tremellomycetes, probably also hidden in several species complexes (Millanes et al., 2014a, 2015, 2016a; Diederich, 1996; Diederich et al., 2018). Species identification is crucial to a wide range of biological research, including cophylogeny studies, and several operational criteria are used to circumscribe species in empirical studies. With this purpose, a number of molecular-based

species delimitation methods had been developed in the past decades, founded on different assumptions and statistical theories (see Rannala and Yang, 2020, for a review). Different such approaches often yield different species delimitation hypotheses, and it is not uncommon that studies aimed at identifying species boundaries compare several methods in order to reach sounder taxonomic conclusions (Amador et al., 2018; Matos-Maraví et al., 2019; Košuthová et al., 2020; Maharachchikumbura et al., 2021). Moreover, to solely rely on genetic data to set species boundaries could lead to inaccurate species delimitations, and using different lines of evidence, including ecology is also recommendable (Yang and Rannala, 2010; Carstens et al., 2013; Luo et al., 2018; Rannala and Yang, 2020). Lichenicolous *Tremella* species are usually confined to a particular fungal host genus or species, and host selection has proved to be a good indicator of species boundaries (Millanes et al., 2015, 2016b; Zamora et al., 2016). The Tremellomycetes are in general a poorly known group where further studies are needed to disentangle their diversity, and where much is still uncertain on how host-specific different species are (Hawksworth, 2003; Lawrey and Diederich, 2003; Tuovinen et al., 2021).

Tremella caloplacae is an example of a lichenicolous tremellalean species with a presumably wide host range on different species of the lichen family Teloschistaceae (Sérusiaux et al., 2003; Diederich, 2007). It was first reported growing on several species of *Caloplaca* s. l. (*C. arenaria*, *C. arnoldii*, *C. aurantia*, *C. carphinea* and *C. saxicola*), and later on other hosts of the Teloschistaceae (Diederich, 2007). The generic classification of Teloschistaceae has since then progressed dramatically (Gaya et al., 2003, 2008; Arup et al., 2013; Kondratyuk et al., 2014; Bungartz et al., 2020; Wilk et al., 2021). Following an increased understanding of their phylogeny, *Caloplaca* has recently

been divided in many different genera, although some generic delimitations remain unsettled (Arup et al., 2013; Gaya et al., 2015; Bungartz et al., 2020; Wilk et al., 2021). The Teloschistaceae is nevertheless one of the most diverse families of lichen forming fungi with more than 1000 species known worldwide (Arup et al., 2013). Still, *Tremella caloplacae* s. l. is known from comparatively few hosts in the family, and galls induced by this fungus are not frequently observed in the species of the Teloschistaceae on which they are known to grow. Lawrey and Diederich (2003) already hypothesized that the number of species of lichenicolous fungi would prove to be roughly proportional to the number of lichen genera, and we would like to explore this hypothesis further. The macromorphology of *Tremella caloplacae* varies considerably depending on the host genus (Fig. 1), which suggests that *T. caloplacae* could constitute a species complex (Diederich, 2007). We have achieved a thorough search of *T. caloplacae* s. l. in a wide range of Teloschistaceae hosts (Table S1), which will provide a first overview of the diversity of this putative species complex.

In this work, we investigate the diversity and possible cophylogenetic patterns occurring in the system formed by *Tremella caloplacae* s. l. and their Teloschistaceae hosts, in the light of recent developments in the host phylogeny and classification. We will perform species delimitation studies to assess whether *Tremella caloplacae* s. l. constitutes a species complex. We will also evaluate the congruence between the tree topologies and the divergence times of *T. caloplacae* s. l. and their Teloschistaceae hosts to identify patterns resulting from their coupled evolution.

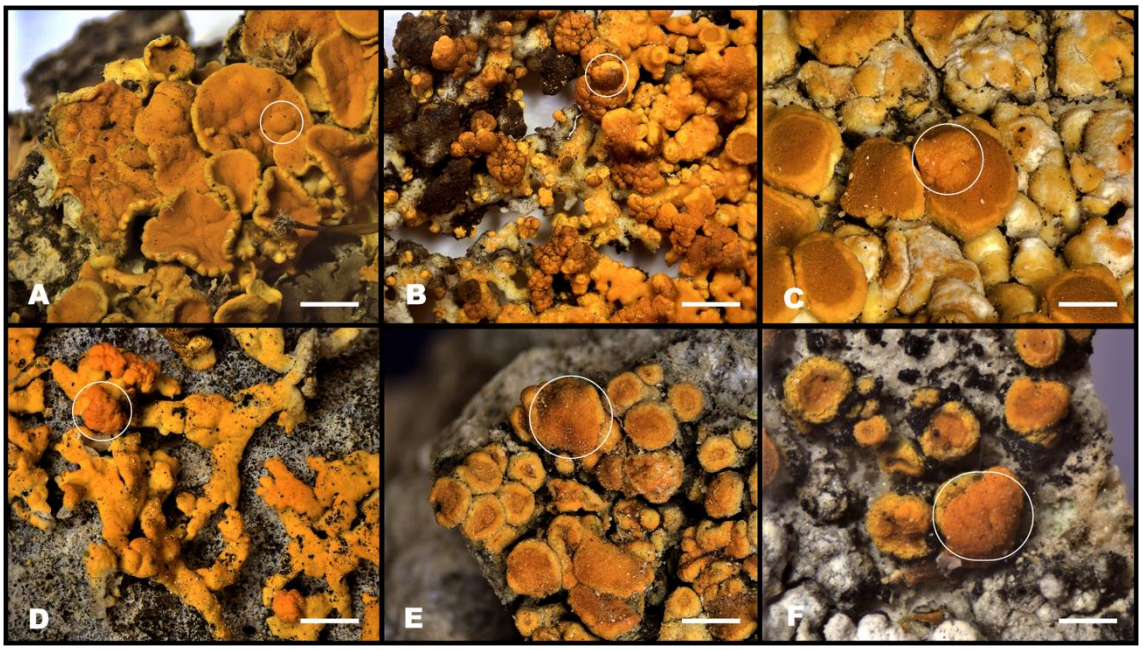

Fig. 1. *Tremella caloplacae* s. l. growing on different hosts: (A) on *Xanthoria parietina*, (B) on *Rusavskia elegans*, (C) on *Variospora flavescens*, (D) on *Rusavskia sorediata*, (E) on *Calogaya pusilla* and (F) on *Xanthocarpia* sp. White circumferences enclose *Tremella*-induced galls. Scale bars = 1 mm

Materials and Methods

Sampling and DNA amplification

We collected fresh specimens in different localities in Europe (Table S2). Herbarium samples from potential hosts in the Teloschistaceae in the collections in S and UPS (Thiers, 2016) were also thoroughly checked to detect *Tremella caloplacae* (Tables S1 and S2). The sequences resulting from these samples were used for our phylogenetic studies, together with sequences retrieved from GenBank (see Table S2 for detailed information).

Total DNA was extracted using the Qiagen DNeasy Plant MiniKit, following the manufacturer's instructions but using a final elution of 50 µl of ultrapure water. Because it is not possible to physically isolate parasites and hosts, these were selectively amplified using specific primers for a portion of ca. 1300 nucleotides of the nSSU rDNA gene, the internal transcribed spacer I, the 5.8S rDNA gene, the internal transcribed spacer II and a

portion of ca. 900 nucleotides of the nLSU rDNA gene. PCRs were performed by using a combination of general fungal primers (Vilgalys and Hester, 1990; White et al., 1990; Gargas and Taylor, 1992; Gardes and Bruns, 1993) and specific primers for *Tremella* or for the lichen host (Millanes et al., 2011; this study) (Table S3). Before sequencing, the PCR products were purified with Exo-sap-ITTM (USB Corporation, Cleveland, Ohio, USA). Samples were sent for Sanger sequencing to Macrogen Korea (Seoul, South Korea), Macrogen Europe (Amsterdam, the Netherlands) or Macrogen Spain (Barajas, Spain). For detailed information about DNA extraction primer design and DNA amplification, see the supplementary material (Materials and Methods).

Sequence alignment and phylogenetic analysis

We produced two phylogenies, one including *Tremella caloplacae* s. l. and the other including their lichenized fungal host in the Teloschistaceae. Outgroups chosen to root the trees were selected based on previous literature and preliminary trees (Millanes et al., 2011; Liu et al., 2015). For the phylogeny of the parasites we chose *Tremella candelariellae*, while *Megalospora tuberculosa* was selected for the phylogeny of the hosts. Analyses were performed using four loci in the nuclear ribosomal DNA repeat unit: ITS1, 5.8s, ITS2 and nLSU. Sequences were aligned using the Q-INS-I algorithm implemented in MAFFT (Katoh et al., 2002, 2005; Katoh and Standley, 2013). Misaligned positions, major insertions and ambiguous and/or divergent regions were identified and excluded by GBlocks v.0.91b (Castresana, 2000), with relaxed selection of blocks following Talavera and Castresana (2007). Terminal gaps were converted to missing data in Mesquite v.3.6 (Maddison and Maddison, 2018). To perform maximum likelihood analysis, we used RAxMLGUI v.1.5b1 (Silvestro and Michalak, 2012), a graphical front-end for RAxML (Stamatakis, 2006). We set a partition of two subsets for

this analysis, one grouping the ITS1, 5.8s and ITS2 regions, and another one with the nLSU partial region. Besides, we assessed possible conflicts between topologies by producing two different trees, the first one for the complete nuclear ITS rDNA region (combining the ITS1, 5.8s and ITS2 regions), and the second one with the nuclear LSU rDNA region. A thorough ML search was performed with 100 runs using 1000 bootstrap pseudoreplicates. Bayesian analyses were carried out with a Markov chain Monte Carlo (MCMC) approach as implemented by MrBayes v.3.40 (Ronquist et al., 2012). Substitution models were selected based on the corrected Akaike information criterion (AICc) in JModeltest v.2.1.10 (Guindon and Gascuel, 2003; Posada, 2008; Darriba et al., 2012). In the alignment including tremellalean samples, a K80+I model was selected for the nuclear ITS1 rDNA, a JC for the nuclear 5.8s rDNA, a K80+Γ for the nuclear ITS2 rDNA, and a GTR+Γ for the nuclear LSU rDNA. In the alignment including host species of the Teloschistaceae, a K80+I model was selected for the nuclear ITS1 rDNA, a JC for the nuclear 5.8s rDNA, a K80+Γ for the nuclear ITS2 rDNA and a GTR+I+Γ model for the nuclear LSU rDNA. The combined analyses treated the different gene regions as separate subsets with topology linked across partitions but separate model parameter values and proportional rates across them. The number of discrete gamma categories was kept at the default value four. For each combined dataset, three parallel runs were performed for the MCMC search with five chains, four of which were incrementally heated with a temperature of 0.15. The analyses were diagnosed for convergence every 100000th generation and were set to halt automatically when the average standard deviation of splits across runs in the last half of the analysis descended below 0.01. Every hundredth tree was saved, and the first half of each run was discarded as burn-in.

Ultrametric tree generation for species delimitation methods

Ultrametric trees were generated with BEAST v.2.0.2 (Drummond and Rambaut, 2007). To construct these trees, we used the same substitution models obtained by jModeltest, analyzing the ITS1, 5.8s, ITS2 and nLSU regions separately. A relaxed lognormal clock was chosen (Drummond et al., 2006) using a constant population coalescent prior and assuming constant population size to estimate branch lengths. Substitution models, rate heterogeneity and base frequencies were unlinked across partitions in the combined analysis. Three independent MCMC analyses were run for 100 million generations, sampling trees every 10000 generations, for the combined dataset. The 10% of the sample was eliminated to assess convergence (burn-in), and the likelihood plots were checked using Tracer v.1.7 (Rambaut et al., 2018) to confirm that all parameters have an effective sample size (ESS) over 200. A combination of tree files corresponding to the three independent runs was made with LogCombiner v.1.10.4 (Drummond and Rambaut, 2007). The posterior tree sample was summarized using TreeAnnotator v.1.10.4 (Rambaut et al., 2018) after discarding the first 5000 trees of each run as burn-in.

Species delimitation methods

In order to compare different approaches for species delimitation we chose four different heuristic methods based on tree topologies and on genetic distances to analyse the species delimitation of *Tremella caloplacae* s. l., by studying the ITS1, 5.8s, ITS2 and nLSU regions. We used the generalized mixed Yule coalescent (GMYC) method, under single (GMYCsingle) and multiple (GMYCmultiple) approaches (Pons et al., 2006; Fontaneto et al., 2007; Monaghan et al., 2009; Fujisawa and Barraclough, 2013), which takes into account the neutral coalescent theory (Hudson, 1990; Wakeley, 2006) and the Yule speciation models (Yule, 1924). We also completed the Poisson tree process model (PTP)

(Zhang et al., 2013), which considers the number of substitutions between sequences. Both GMYC and PTP methods, are based on topologies and require ultrametric trees. The genetic distances analysis was made with the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al., 2012). Probability of Correct Identification (PCI) was calculated based on uncorrected distances (p-Distance) (Suwannasai et al., 2013). We considered yet an additional species delimitation hypothesis consisting of a consensus of the hypotheses obtained by the methods mentioned above and including also ecology as line of evidence (this was called adopted consensus). All species delimitation hypotheses were further tested by a parametric statistic approach, based on the Bayesian multispecies coalescent model (MSC) implemented in the software Bayesian Phylogenetics and Phylogeography (BPP) (Rannala and Yang, 2003; Yang and Rannala, 2010; Yang, 2015; Rannala and Yang, 2017; Flouri et al., 2020). BPP estimated the posterior model probabilities of the species delimitations given by GMYC (single and multiple approaches), PTP, ABGD, PCI and the adopted consensus. We performed the so-called analyses A11 following Flouri et al., (2020), which estimates species delimitation and species tree without a fixed guide tree (unguided) (Yang and Rannala, 2010; Yang, 2015; Flouri et al., 2020), for each one of the species delimitations hypotheses above-mentioned. According to Yang (2015), the A11 analysis is recommended when there is lack of fully resolved phylogenies to avoid phylogenetic uncertainty on the species tree. Posterior probabilities above 0.90 were considered supported and highly supported when above 0.99.

Molecular Clock analyses

Based on our species delimitation results we produced two additional phylogenies to infer diversification times in *Tremella caloplacae* s. l. and in their Teloschistaceae hosts. Since

fossils are not available for any of these groups, we constructed larger phylogenies of the Basidiomycota and Ascomycota, respectively, which included our groups of interest. Datasets corresponding to *T. caloplacae* s. l. and the Teloschistaceae were aligned with MAFFT and subsequently treated with GBlocks and Mesquite. ML analyses were conducted for preliminary analyses (for more details, see Molecular clock analyses in Supplementary Materials).

To date *Tremella caloplacae* s. l., we selected 129 taxa, which represent seven classes of Basidiomycota and five classes of Ascomycota (Table S2), and as outgroup we used a taphrinomycete, *Taphrina deformans*. The nuclear gene datasets corresponding to the nSSU, ITS1, 5.8s, ITS2 and nLSU regions were analysed with BEAST v1.10.4 (Suchard et al., 2018), and the XML file for the analyses was constructed in BEAUTi v1.10.4. The substitution models, the rate heterogeneity and the base frequencies were unlinked across partitions, but clock and tree models remained linked. We set constraints to five nodes for fossil calibrations (Table S4). To select the substitution models, we based on the corrected Akaike Information Criterion (AICc) analysed with jModelTest v2.1.10 for each subset: (1) GTR+I+Γ for 18S and 28S, (2) GTR+Γ for the ITS1 and ITS2, and (3) SYM+Γ for the 5.8s region. We used the uncorrelated relaxed clock model with a lognormal distribution that allows independent rates of heterogeneity (Drummond et al., 2006). We selected the birth-death process speciation tree prior with a random starting tree (Gernhard, 2008). Three independent MCMC of 250 million generations were performed, logging every 10000 generations. Convergence was assessed with Tracer v1.7 (Rambaut et al., 2018), checking that all ESS values were higher than 200. After removing 10% of the trees as burn-in with LogCombiner v.1.10.4 (Suchard et al., 2018), the maximum-

clade-credibility (MCC) tree was summarized with TreeAnnotator v.1.10.4 (Suchard et al., 2018).

For the dating analyses of the Teloschistaceae hosts, we used 135 taxa from 12 different classes of Ascomycota (Table S2), and *Schizosaccharomyces pombe* was selected as outgroup. These divergence times were also estimated with BEAST v.1.10.4. To avoid errors at the start of the MCMC analysis, and convergence problems at the end of it, we first constructed a calibrated starting tree following the same procedure as in the previous *Tremella caloplacae* s. l. dating analysis. This initial tree was calibrated with the fossil *Palaeopyrenomycites devonicus* (Taylor et al., 2005) (Table S4). Substitution models were also obtained with jModelTest v2.1.10 and selected taking into account the AICc. A SYM+I+Γ substitution model was selected for the nuclear SSU rDNA region, a GTR+Γ model for the nuclear ITS1 and ITS2 rDNA regions, a SYM+Γ model for the nuclear 5.8s rDNA region and a GTR+I+Γ model for the nuclear LSU rDNA region. The resulting tree from this analysis was exported with Newick format to be used as starting tree for the dating analysis of the Teloschistaceae hosts. The final dating analysis was conducted with BEAST v.1.10.4 following the same protocol previously used to generate the calibrated Bayesian starting tree but performing the analysis with three independent MCMC of 550 million generations and setting the previously calibrated tree as starting tree. In addition to the *Paleopyrenomycites devonicus* fossil, we used ten other fossil calibrations (Table S4).

For more details about molecular clock analyses, see supplementary material (Materials and Methods).

Phylogenetic congruence and cophylogenetic analyses

We applied four different methods to test for congruence between host and parasite phylogenies, considering only the best supported species delimitation hypothesis. These were: (1) a distance method performed by ParaFitGlobal test (Legendre et al., 2002); (2) the Procrustean Approach to Cophylogeny (PACo), a statistical tool that evaluates dependence between phylogenies (Balbuena et al., 2013; Hutchinson et al., 2017), (3) the I_{cong} index (de Vienne et al., 2007), to assess topological congruence between hosts and parasite phylogenies, and finally (4) an event-cost based method as implemented in Jane v.4.0 (Conow et al., 2010). To perform these analyses, we based on the alignments of the ITS1, 5.8s, ITS2 and nLSU regions (Parafit) or on the topology of phylogenies previously obtained (PACo, I_{cong} and Jane). ParaFitGlobal test takes as null hypothesis that each parasite species is associated with hosts selected randomly along the host phylogenetic tree. The alternative hypothesis considers that the positions of each host–parasite association is not arbitrary but instead they are associated to phylogenetic distances between hosts and parasites. The analyses were performed using the *parafit* function from the "ape" v. 5.1 package implemented in R (Paradis et al., 2004). PACo evaluates the contribution of individual host-parasite associations to the global congruence between phylogenetic topologies by testing if the parasite phylogeny is constrained by the host phylogeny (Jousselin et al., 2009; Balbuena et al., 2013). PACo analyses were performed with the "paco" v.0.4.2 and "ape" v.5.5 packages implemented in R (Balbuena et al., 2013; Paradis and Schliep, 2019). I_{cong} evaluates topological congruence by means of the Maximum Agreement SubTree (MAST), which calculates the highest number of tips that are compatible with the two given trees. We performed this test at the on-line I_{cong} calculator interface (http://max2.ese.u-psud.fr/icong/index.help.html) for the tree topologies of *Tremella caloplacae* s. l. and the Teloschistaceae. Jane (Conow et al., 2010)

assumes as null hypothesis that the congruence between the topologies of the two trees is not higher than could be attributed to chance (Light and Hafner, 2008). The analysis considers five different evolutionary events: (1) cospeciation, (2) duplication, when the parasite speciates independently of the host and both new parasites remain on the same host (Page, 1990, 1996), (3) duplication and host switching, when a duplication event is accompanied by the switch of one of the two descendants to a different host (Conow et al., 2010), (4) loss or lineage sorting, when a host speciates and the parasite remains only on one of the new hosts species (Paterson and Gray, 1997; Paterson et al., 1999) and (5) failure to diverge, when a host speciates and the parasite remains on both new host species (Page, 2003). Each event is assigned a particular cost (Table 1), and the best solution is that with the overall lowest cost. Results obtained from the analyses were compared with the divergence times obtained from the dating analysis of *Tremella caloplacae* s. l.

For more information about cophylogenetic analyses, see supplementary material (Materials and Methods).

Table 1. Jane v.4.0 cost combinations used for the cophylogenetic analyses.

Cost combination	Cospeciation	Duplication	Duplication & Host switching	Loss	Failure to diverge
Combination 1	0	1	2	1	1
Combination 2	0	1	1	1	1
Combination 3	1	1	1	1	1
Combination 4	1	0	0	1	1
Combination 5	2	1	1	1	0
Combination 6	2	1	1	1	1
Combination 7	2	1	1	0	0
Combination 8	0	0	1	1	1
Combination 9	1	1	2	1	1

Results

Taxon sampling

More than 770 lichen specimens of Teloschistaceae were thoroughly examined at the UPS and S herbaria (Thiers 2016) (Table S1) from which seven *Tremella* specimens were found. In addition, 45 specimens were collected in the field (Table S2). A total of 52 specimens of *Tremella caloplacae* s. l. were selected for sequencing.

DNA sequences and phylogenetic analysis

We generated 202 new sequences, 9 of the nSSU, 99 of ITS and 94 of the nLSU region of *T. caloplacae* and its host species belonging to the genera *Xanthoria*, *Xanthocarpia*, *Calogaya*, *Rusavskia*, *Leproplaca* and *Variospora*, that were deposited in GenBank (Table S2). Sequences were concatenated into two datasets, one for *T. caloplacae* specimens and another one for their Teloschistaceae host species, as no incongruence among loci was found.

Phylogenetic analyses for *T. caloplacae* and the Teloschistaceae hosts were conducted using four loci: ITS1, 5.8S, ITS2 and nLSU. Data matrices consisted of 1358 (ITS1: 1-78; 5.8S: 79-233; ITS2: 234-373: nLSU: 374-1358) and 1005 (ITS1: 1-83; 5.8S: 84-237; ITS2: 238-365: nLSU: 366-1005) aligned positions respectively. ML and Bayesian Inference (BI) analyses gave similar topologies, and no incongruences were found between the ML and BI trees. Two 50% majority rule consensus Bayesian trees were constructed (Fig. 2) from the trees of the stationary tree sample: one for *T. caloplacae* based on 10503 trees and another one for its hosts, based on 18003 trees. The *T. caloplacae* specimens studied formed nine monophyletic groups, each of them growing on a single lichen species or genus.

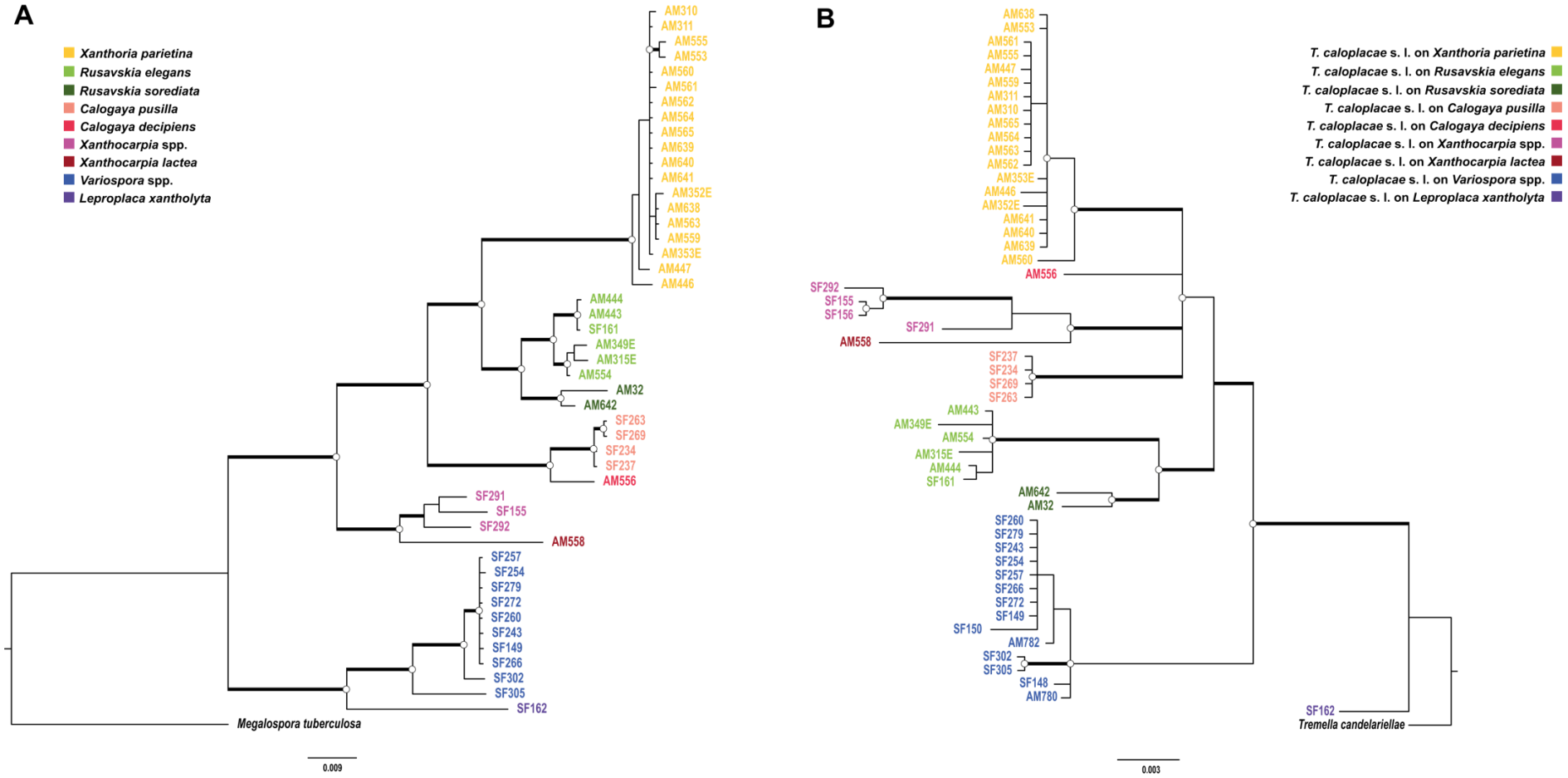

Fig. 2. Fifty percent majority rule Bayesian consensus trees of the concatenated ITS and nLSU regions for Teloschistaceae hosts (A) and lichenicolous fungi (*Tremella caloplacae* s. l.) (B). Colours indicate the host taxon, or the host selection. Supported nodes are indicated with thick branches for Bayesian analysis (BPP values ≥0.95) and white dots for maximum likelihood (bootstrap values ≥70%). Branch lengths are scaled to the expected number of substitutions per site.

Species delimitation within **Tremella**

The number of potential new species inferred varies depending on the species delimitation analysis used (Fig. 3). The single-threshold GMYC model delimited six potential species, while the multiple-threshold GMYC model delimited seven potential species, since the later approach divides samples on *Calogaya pusilla* in two lineages. The PTP model inferred the highest number of potential species, separating the samples growing on *Rusavskia elegans* from those on *R. sorediata*, and dividing each of the clades on *Xanthocarpia* sp., *Rusavskia elegans* and *Variospora* sp. in three different potential species. The ABGD method delimited 11 independent lineages. In this case, each clade growing on a different host species was delimited as a single species, except on *Xanthocarpia* sp. and *Rusavskia sorediata* where the method inferred two potential species on each host. The PCI model was applied only for those clades with two or more specimens and delimited a total of five potential species corresponding to *Tremella* species growing on *Xanthoria parietina*, *Xanthocarpia* sp., *Rusavskia elegans*, *R. sorediata* and *Variospora* sp. In the species delimitation hypothesis called 'adopted consensus' (1) we considered some singletons – recovered only by PTP and ABDG – as potential species as they were placed on a long branch and were associated with a different host (i. e., singletons on *Calogaya decipiens* and *Leproplaca xantholyta*), and (2) avoided over-splitting, when singletons grew on the same host species (i. e., singletons on *Rusavskia sorediata*). This resulted in the following working hypothesis: (1) *Tremella* sp. growing on *Xanthoria parietina*, (2) *Tremella* sp. growing on *Xanthocarpia lactea*, (3) *Tremella* sp. growing on *Xanthocarpia* sp., (4) *Tremella* sp. growing on *Calogaya decipiens*, (5) *Tremella* sp. growing on *Calogaya pusilla*, (6) *Tremella* sp. growing on *Rusavskia elegans*, (7) *Tremella* sp. growing on *Rusavskia sorediata*, (8) *Tremella* sp.

growing on *Variospora* sp., and (9) *Tremella* sp. growing on *Leproplaca xantholyta* (Fig. 3).

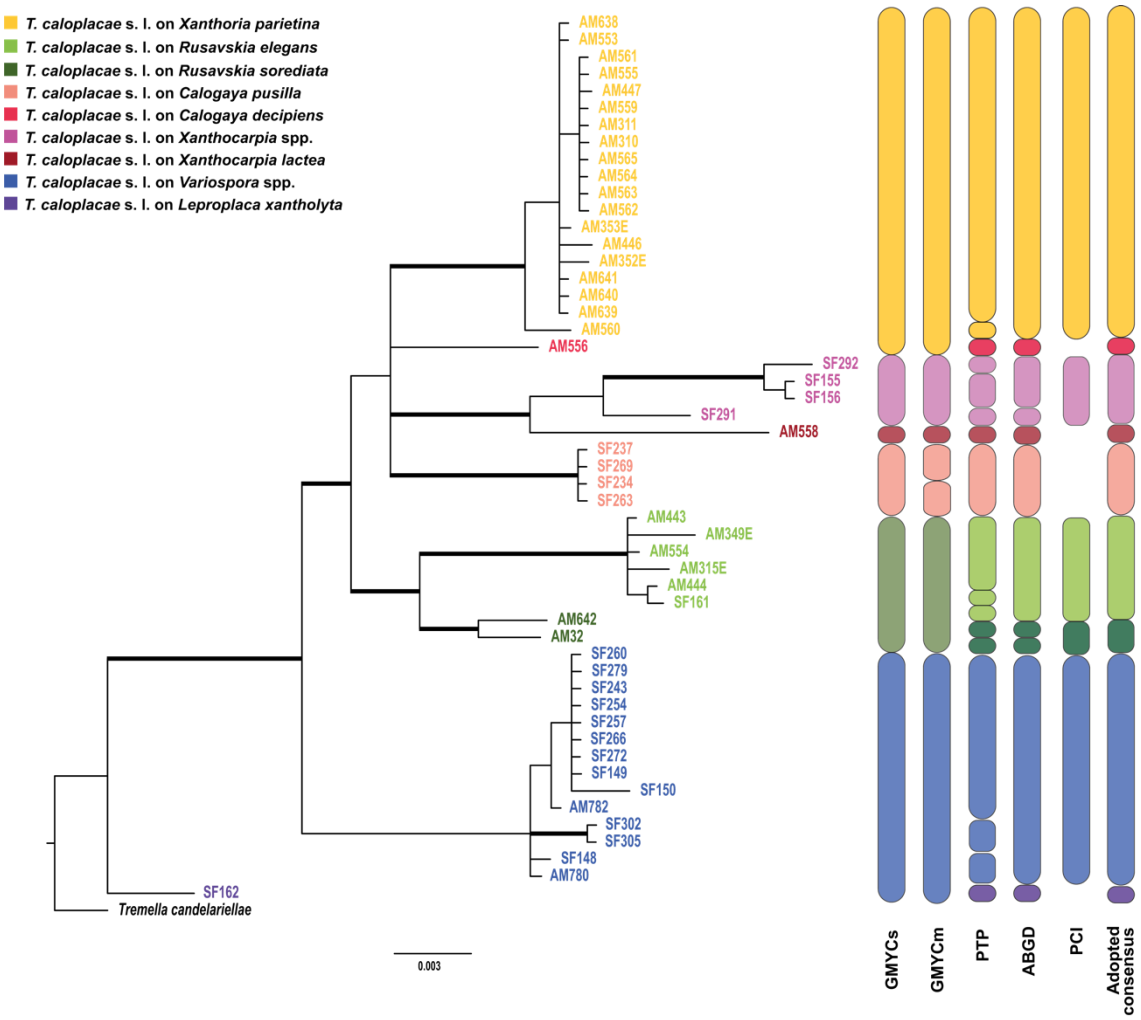

Fig. 3. Bayesian consensus tree of *Tremella caloplacae* s. l. from Fig. 2, with species delimitation hypotheses inferred by four different methods – GMYC single and multiple threshold, PTP, ABGD, PCI and the adopted consensus – indicated on the right side of the figure.

All six species delimitation hypotheses were further tested under BPP. Of the six BPP unguided species delimitation analyses, four obtained a posterior probability value above 0.95 (Table 2). The six potential new species delimited by GMYCm were not highly supported by the BPP analysis, which reached its best posterior probability PP = 0.9390 for a model of five species. The analyses based on the PTP species delimitation were not supported with PP = 0.3709, reached for a best model of 15 potential new species instead

of 17. The obtained posteriors for the species delimitations given by GMYCs, ABGD, PCI and the adopted consensus were highly supported, these last two delimitations having the two highest PP-values. We therefore considered only the adopted consensus – which obtained the highest value of posterior probability by BPP among the hypotheses allowing singletons – for subsequent cophylogenetic analyses.

Table 2. BPP results for the different species delimitations generated.

Method	Inferred species (Species delimitation method)	BPP Posterior probability (best model)	BPP accepted Species (best model)
GMYCs	6	0.9865	6
GMYCm	7	0.9390	6
PCI	5	0.9987	5
ABGD	11	0.9568	11
PTP	17	0.3709	15
Adopted consensus	9	0.9907	9

Molecular Clock analyses

The Bayesian trees generated with BEAST and the most probable divergence times for *T. caloplacae* s. l. and their Teloschistaceae hosts are represented in Fig. 4. The age intervals of the nodes, corresponding to the 95% highest posterior density (HDP), are shown in Table 4. The dating results for the complete datasets analysed are included in the Supplementary material (Fig. S1 and Fig. S2).

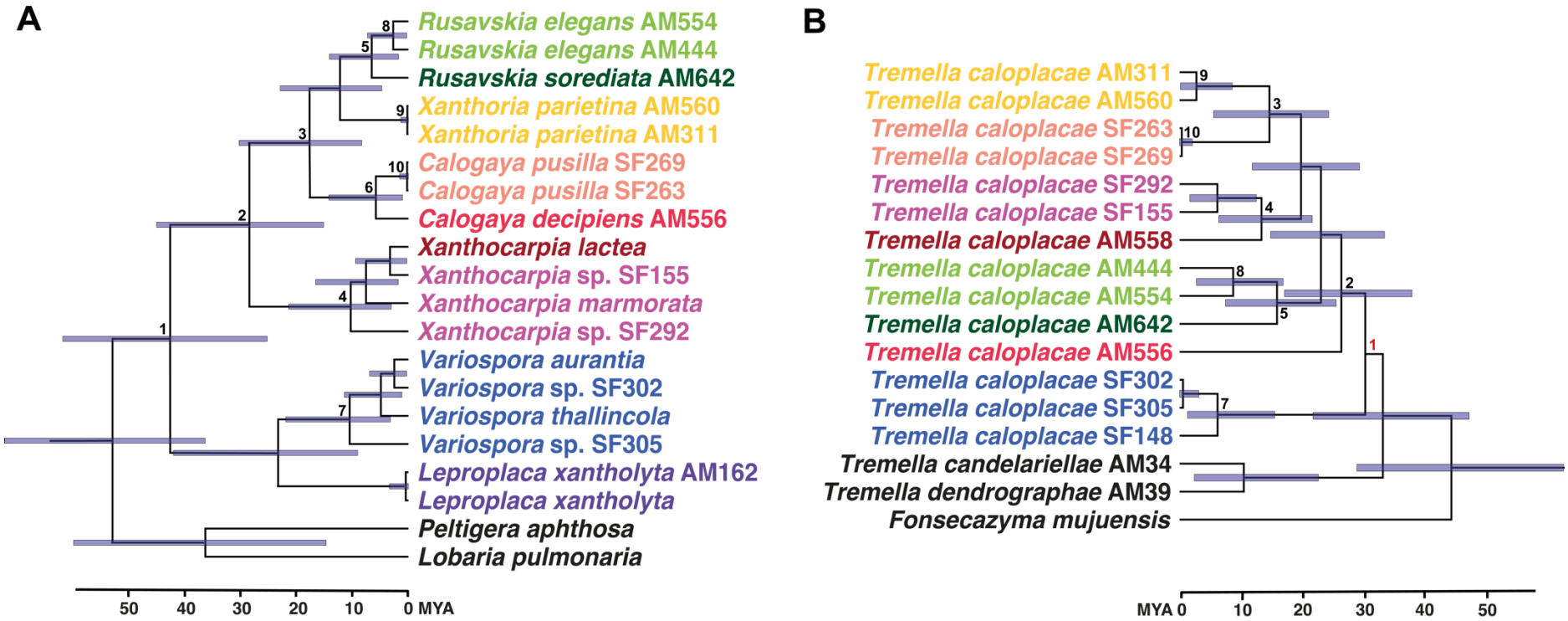

Fig. 4. Divergence times estimations for *Tremella caloplacae* s. l. (A) and their Teloschistaceae hosts (B) based on the five-markers dataset (nSSU, ITS1, 5.8s, ITS2 and nLSU). The most probable divergence time is indicated for each node. Numbers 1 – 10 correspond to selected nodes shown in Table 4. Bars correspond to the 95% highest posterior density (HPD). Red numbers indicate nodes without support. See Fig. S1 and Fig. S2 for complete dated trees.

Table 4. Most probable divergence times and 95% highest posterior density (HDP) of selected nodes in the dating analyses performed for *Tremella caloplacae* s. l. and their hosts in the Teloschistaceae. For details about node positions, see Fig. 4.

	Teloschistaceae		*T. caloplacae* s. l. on	
Node	**Age (Mya)**	**HDP (Mya)**	**Age (Mya)**	**HDP (Mya)**
1	42.15	24.99 – 61.14	29.74	–
2	28.14	15.02 – 44.54	25.91	16.82 – 37.22
3	17.49	8.25 – 29.95	14.39	5.44 – 23.87
4	10.21	3.06 – 21.10	13.11	6.26 – 21.25
5	6.49	1.74 – 13.98	15.60	7.35 – 25.08
6	5.76	1.06 – 14.07	–	–
7	10.40	3.20 – 21.69	6.08	1.25 – 15.22
8	2.69	0.31 – 7.25	8.58	2.70 – 16.60
9	0.20	0.00 – 1.35	2.69	0.14 – 8.38
10	0.23	0.00 – 2.00	0.34	0.00 – 1.55

Molecular clock dating and cophylogenetic tests

The dating analyses of *T. caloplacae* and its Teloschistaceae hosts were conducted using five loci: nSSU, ITS1, 5.8S, ITS2 and nLSU. Alignment data matrices consisted of 2929 (nSSU: 1-1244; ITS1: 1245-1809; 5.8S: 1810-1962; ITS2: 1963-2057: nLSU: 2058-2929) and 2693 (nSSU: 1-1149; ITS1: 1150-1309; 5.8S: 1310-1464; ITS2: 1465-1556: nLSU: 1557-2693) positions respectively. The Bayesian trees generated with BEAST and the most probable divergence times for *T. caloplacae* s. l. and their Teloschistaceae hosts are represented in Fig. 4. The age intervals of the nodes, corresponding to the 95% highest posterior density (HDP), are shown in Table 4. The dating results for the complete datasets analysed are included in the Supplementary material (Fig. S1 and Fig. S2).

General phylogenetic congruence between both trees was not supported by ParaFit (global test *P*-value = 0.123). ParaFitLink1 and ParaFitLink2 showed that only five individual events were supported for cospeciation in *Tremella caloplacae* s. l. growing on *Xanthocarpia* sp., *Rusavskia elegans* and three different specimens of *Variospora* sp.

PACo did not detected significant overall congruence between both *T. caloplacae* and their hosts phylogenies. The congruence index showed that there was no more congruence (I_{cong} = 1.155) between the tree topologies of *Tremella caloplacae* s. l. and their Teloschistaceae hosts than expected by chance. The test had no statistical significance with an associated P-value of 0.231 and a MAST result equal to 5. Considering different events, Jane reconstructions revealed a significant congruence between the tree topologies of *T. caloplacae* s. l. and their hosts (i.e., >95% of random solutions were worse than the solution reconstructed by Jane). The solutions obtained for the adopted consensus species delimitation hypothesis and event-costs had a final total cost that ranged from 0 to 11 (Table 3 and Table S5). The majority of associations could be explained by cospeciation or host switching, depending on the cost regime chosen (Table 3 and Table S5). Nevertheless, most of the cospeciation events inferred are not congruent with the most probable times of divergence given by the dating analyses for the same nodes (Table 3). The most frequent reconstruction obtained by Jane corresponds to cost combinations 1, 2, 8 and 9 (Table 1), and recovered 3-5 cospeciations, 0 duplications, 2-4 duplications + host switches, 2 losses, and 0 failures to diverge. The overall costs of this reconstruction varied from 4 to 11. Of the cospeciation events reconstructed by Jane, just one is compatible with the divergence times of the dating analysis (Tables 3 and 4).

Discussion

Tremella caloplacae comprises at least nine independently evolving clades, each of them restricted to grow on a different host genus or species, as revealed by the species delimitation analyses (Fig. 3). Based on these, and on morphological and ecological data, formal descriptions are being prepared and will be published in a separate manuscript.

Table 3. Results of Jane v.4.0 cophylogenetic analyses for the adopted consensus species delimitation hypothesis. Events compatible with divergence times are shown in bold numbers before the slash. *P*-values of the tests were <0.05.

	Cospeciation	Duplication	Duplication & Host switching	Loss	Failure to diverge	Total cost
Combination 1	**1**/5	0	2	2	0	6
Combination 2	**0**/3	0	4	0	0	4
Combination 3	0	0	7	0	0	7
Combination 4	0	0	7	0	0	0
Combination 5	0	0	7	0	0	7
Combination 6	0	0	7	0	0	7
Combination 7	0	0	7	0	0	7
Combination 8	**0**/3	0	4	0	0	4
Combination 9	**0**/3	0	4	0	0	11

Suitable diagnostic characters, other than host selection, are often scant in lichenicolous Tremellales (Diederich, 1996, 2007; Millanes et al., 2012, 2014b; Zamora et al., 2016) and molecular tools have proven very useful to investigate their diversity. In particular, the combination of the ITS and nLSU regions is useful for delimiting fungal species (Eberhardt, 2010; Schoch et al., 2012), including lichenicolous tremellalean fungi (Millanes et al., 2014a). Species delimitation methods provide objective tools to incorporate molecular information (other than reciprocal monophyly of clades) in the identification of species boundaries. It is not uncommon, however, that different species delimitation methods yield different hypotheses on species boundaries (Miralles and Vences, 2013; Vitecek et al., 2017; Becchimanzi et al., 2021). Methods based on coalescence could be showing the sample's own population structure, which would also be supported by short periods of divergence (Sukumaran and Knowles, 2017). GMYC (particularly the multiple threshold approach) has a tendency to overestimate the number of potential new species, and its performance and precision can be affected by sample size, geographic sampling, number of putative species, presence of singletons, ultrametric tree dating, and even by the number of markers chosen (Fujita et al., 2012; Tang et al., 2012, 2014; Ritchie et al., 2016; Pentinsaari et al., 2017; Luo et al., 2018; Mason et al.,

2020). On the other hand, ABGD tends towards more conservative results of under-estimation of potential new species although its precision depends to a great extent on the priors specifications given by the user and, like GMYC, is very sensitive to the presence of singletons (Puillandre et al., 2012; Pentinsaari et al., 2017). In this respect, BPP turns to be a useful tool that allows discriminating among the performance of the different analyses (Hotaling et al., 2016; Hurtado-Burillo et al., 2017; Flouri et al., 2020).

DNA regions used as barcodes can nevertheless fail to provide enough variation in some organisms, including fungi, particularly when incomplete lineage sorting, recombination or hybridization has occurred (Chambers and Hillis, 2020). Therefore, it is good to consider that delimiting species based on just one or a few DNA regions may be inaccurate, independently of the method applied, and we anticipate that further studies will be necessary to correctly interpret diversity and evolution in this system. One additional issue is the large diversity of lichens in the Teloschistales (Arup et al., 2013; Gaya et al., 2015; Bungartz et al., 2020; Wilk et al., 2021), which could harbour a much larger diversity of tremellalean fungi – both symptomatic and asymptomatic – than detected in our study. We have screened a considerable and representative number of species in the Teloschistaceae in search of galls, but a larger diversity of lichen-inhabiting tremellalean species in this complex is surely present. Despite being aware of these limitations, our approach constitutes a first and valid exploration of the diversity included in *Tremella caloplacae*.

Lichenicolous *Tremella* species are in general considered very host-specific, and species in the *Tremella caloplacae* complex are not an exception, although the factors governing host selection are unknown. Acquisition of carbon from the lichen host must be a critical

ability and must depend, at least in part, on the capacity of the lichenicolus fungus to produce appropriate cell wall-degrading enzymes (Lawrey and Diederich, 2003). Moreover, adaptation to secondary lichen compounds is most probably another key aspect of the interaction. Lawrey (1997) demonstrated experimentally that some unrelated lichenicolous fungi grew better on lichen thalli containing lichen compounds, than on thalli from which such compounds were removed. Some lichenicolous fungi tolerate the secondary compounds of their hosts, but not those of other lichens (Lawrey et al., 1999). Some highly specialized lichenicolous fungi are also adapted to the secondary metabolites of their hosts in the way that they are capable of degrading these carbon-based secondary compounds (CBSCs) (Lawrey, 1993; Lawrey and Diederich, 2003; Asplund et al., 2018). This, in turn, makes the hosts less effective in deterring invertebrate herbivory, contributing negatively to their fitness (Merinero et al., 2015; Merinero and Gauslaa, 2018; Asplund et al., 2016, 2018). In tremellalean lichenicolous fungi, secondary metabolites have also been suggested to create a selective environment that only adapted fungal strains could survive (Werth et al., 2013). Pino-Bodas et al., (2018) tested this hypothesis for the first time in a lichenicolous heterobasidiomycete (*Heterocephalacria bachmannii*, growing on *Cladonia*) and found that host species and host secondary metabolites were the most relevant factors influencing the genetic structure of *H. bachmannii*, although both effects were difficult to separate. Adaptation to new chemical environments still needs to be further investigated as a possible driving force in the diversification of lichenicolous fungi.

How host specialization can influence speciation in lichenicolous fungi is also not well understood (Diederich, 1996; Millanes et al., 2012, 2014a, 2015, 2016a; Zamora et al., 2016). The first evolutionary study of host-specific lichenicolous fungi focused on

Biatoropsis usnearum s. l. (Tremellaceae) and their *Usnea* and *Protousnea* (Parmeliaceae) hosts (Millanes et al., 2014a). That study showed that host switching, instead of cospeciation, was the evolutionary event that explained the high specificity of this system and the relationships between the two groups of organisms. Our study on *Tremella caloplacae* s. l. supports again predominant speciation by host switching. The lack of congruence between the phylogenies (Figs. 2 and 4), the non-significance of the Parafit and PACo tests, and the generally more recent dates of speciation events in *Tremella* compared to the Teloschistaceae (Table 4) suggest host switching as a prevalent phenomenon in this system. Species in the *Tremella caloplacae* complex seem to be specialists restricted to grow on a single genus or species in the Teloschistaceae. The fact that parasites are often specialists with restricted host ranges and that at the same time they commonly switch onto different hosts along their evolutionary history has been referred to as the 'parasite paradox' (Agosta et al., 2010). The so-called *'Stockholm Paradigm'* (e.g., McLennan and Brooks, 2002; Agosta et al., 2010; Janz, 2011; Hober and Brooks, 2015), solved this paradox suggesting that host switching and host range expansion are more often the consequence of taking advantage of opportunities offered by a changing host landscape than of the previous evolution of novel host-use capabilities of the parasite. The Stockholm paradigm lays, among others, on the concept of Ecological Fitting (Janzen, 1985). This, translated to host-parasites systems, suggests that phenotypic flexibility rather than evolution of genetic novelties related to increased fitness of the parasites, provides substantial opportunities for rapid host switching of parasites on suboptimal hosts (i.e., 'sloppy fitness', Agosta and Klemens, 2008). The *Oscillation Hypothesis* (Janz and Nylin, 2008) - also part of the *Stockholm Paradigm* - predicts that, along their evolution, specialists will become colonizing generalists when opportunities arise, and those generalists will then produce new isolated specialists on new hosts. In

lichenicolous *Tremella* species there is evidence suggesting that the asymptomatic yeast phase is less specific towards the lichen host than the filamentous phase (Tuovinen et al., 2021). It would be possible to speculate that yeast stages could then facilitate the permanence of the parasite in a 'sloppy fitness space', smoothing further the way for host switch. These are nevertheless hypotheses that would need further testing.

Previous studies suggested that the diversification of the Teloschistaceae hosts started 102.89 Mya (68.97-140.62 Mya) in the Cretaceous (Gaya et al., 2015). The adaptive radiation of the Teloschistaceae has been linked to a combination of abiotic (sun exposure or rock substrate) and biotic (chemical phenotypic innovations) ecological factors (Gaya et al., 2015). According to Gaya et al., (2015), the Teloschistaceae underwent rapid diversification approximately 100 Mya, as a consequence of their change to rocky sunny habitats and the appearance of anthraquinone pigments. New evolved anthraquinones in these lichens, in conjunction with an ecological switch to exposed, rocky environments, allowed them to colonize unexploited habitats worldwide and triggered an acceleration in their diversification (Gaya et al., 2015). Our study is the first one to incorporate times of divergence to cophylogeny studies in lichenicolous fungi and their hosts. Although we performed the dating analyses based on few loci, the divergence estimations obtained for the Teloschistaceae hosts included in our analyses and the other groups of Lecanoromycetes (Table 4) are congruent with those in the literature (Amo de Paz et al., 2011; Prieto and Wedin, 2013; Beimforde et al., 2014; Gaya et al., 2015). According to our analyses, at least part of the diversification of *Tremella caloplacae* s. l. would have occurred around 30 Mya in the Oligocene, although most probably the actual origin of *Tremella caloplacae* predates this age, if we consider potential unknown diversity. The divergence times of the new species of *T. caloplacae* s. l. are in the majority of cases

subsequent to the divergence of their corresponding hosts (Table 4), which further supports a host switch scenario. For the associated *Tremella* species, exposure to different chemical environments provided by the Teloschistaceae represented new niches to explore, probably through host switches, leading to processes of adaptation and specialization finally resulting in speciation. These patterns have been widely studied in host plants and phytophagous insects and their parasitoids and referred to as 'sequential' or 'cascade' speciation (Janz et al., 2006; Feder and Forbes, 2010; Forister and Feldman, 2011; Hood et al., 2015). The adaptive radiation of the Teloschistaceae associated to an ecological shift, followed by speciation of the associated Tremella species by specializing on new host-niches, could constitute a particular case of cascade speciation by sequential ecological adaptations.

In some particular cases, however, the time of divergence or the origin of particular putative new species of *T. caloplacae* s. l. – as the taxa growing on *Calogaya*, *Rusavskia* and *Xanthoria* – predates that of their hosts. The existence of parasite lineages that are much older than current host lineages has also been reported, resulting from persistence of the parasite after episodes of host colonization (Hober and Brooks, 2008). If a host lineage gets extinct but the parasite survives in an alternative host, extinction of the optimal host does not necessarily implies extinction of the parasite lineage (Araujo et al., 2015). An intriguing hypothesis to test further is that if, in such cases, host switches and continued establishment of tremellalean lichenicolous fungi onto suboptimal hosts could in turn have contributed to accelerate the speciation rate in the Teloschistaceae in the late Cretaceous (Gaya et al., 2015). In that case, emerging lichen species in the Teloschistaceae would have needed to adapt not only to previously identified factors promoting their diversification – as the ecological shift to sunny rocky habitats – but also

to the associated *Tremella* species, emerging by host switching. It is not impossible to imagine that groups of organisms that share tangled evolutionary paths could alternatively trigger or follow each other diversification (Forbes et al., 2009).

Conclusions

The *Tremella caloplacae* species complex is another example of tremellalean lichenicolous fungi in which host specialization is a driving force behind speciation. The common trend in our species delimitation hypotheses is host-related genetic structure. Additional and still undiscovered diversity within the *Tremella caloplacae* complex can of course provide a better picture of the shared evolutionary history between the Teloschistaceae and their associated tremellalean fungi, in the future. But based on the data at hand, speciation by host switch followed by specialization is the most plausible and common process in the evolution of the *T. caloplacae* species complex, according to tree topologies and divergence times of *T. caloplacae* s. l. and their Teloschistaceae hosts. Hypotheses on speciation modes associated to host shifts, including potential ecological fitting followed by reproductive isolation, chemical adaptation, and cascade speciation, need to be investigated further.

Supplementary material – Freire-Rallo et al. – Chapter 1

'To explore strange new worlds' - speciation by host shift in *Tremella caloplacae* followed the cretaceous adaptive radiation of the Teloschistaceae

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Materials and methods

DNA extraction and amplification

To design the specific primers, we identified highly conserved regions which at the same time differed considerably between parasites and hosts. General fungal primers NS19 (Gargas and Taylor, 1992), ITS1F (Gardes and Bruns, 1993), ITS4 (White et al., 1990), LR5 (Vilgalys and Hester, 1990); the specific primers for Teloschistales TelLSU3-3 (Tuovinen et al., 2021), TelLSU1-5 (this study); and the specific primers for basidiomycete fungi Tc2SSU1-3_XpXcsp, Tc2SSU1-3_Cgp, Tc2SSU1-3_Vsp1, Tc2SSU1-3_Vsp2, Tc3SSU1-3_Cgp, Tc3SSU1-3_Vsp (this study), BasidLSU3-3, BasidLSU1-5 (Millanes et al., 2011), TRMcal_R2 and TRMLSU_1F (this study) (Table S3) were used to amplify a portion of the nSSU rDNA gene in the tremellalean fungi, the internal transcribed spacer I, the 5,8S rDNA gene, the internal transcribed spacer II and a portion of the nLSU rDNA gene in both hosts and parasites (Table S2).

PCRs were carried out using Illustra^{TM} Hot Star PCR beads, according to the manufacturer's instructions, with the following settings for each primer combination: 1) for NS19/ Tc2SSU1-3_XpXcsp, NS19/ Tc2SSU1-3_Cgp, NS19/ Tc2SSU1-3_Vsp1, NS19/ Tc2SSU1-3_Vsp2, NS19/ Tc3SSU1-3_Cgp, and NS19/ Tc3SSU1-3_Vsp we run an initial denaturing at 95 °C for 3 min; 4 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 90 s; 30 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s; final extension step of 72 °C for 8 min 2) for ITS1F/BasidLSU3-3, ITS1F/TelLSU3-3, ITS1F/TRMcal_R2, TRMLSU_1F/LR5 we run an initial denaturing at 95 °C for 5 min; 4 cycles of 95 °C for 40 s, 53 °C for 40 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; 32 cycles of 95 °C for 30 s, 47 °C for

30 s and 72 °C for 90 s; final extension step of 72 °C for 10 min, and finally 3) for BasidLSU1-5/LR5 and TelLSU1-5/LR5 we used an initial denaturing at 95 °C for 5 min; 4 cycles of 95 °C for 40 s, 56 °C for 40 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s; 32 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; final extension step of 72 °C for 10 min.

Specific primers designed for protein-coding genes did not allow amplifying the target organism. We also performed experiments following a modification of the Meyer and Kircher (2010) next generation sequencing (NGS)-based method. In this case we amplified the desired markers with general fungal primers, obtaining a mix of DNA from different fungi in the PCR product. These PCR products were fragmented to get pieces of the desired length for Illumina® sequencing. The sequenced reads were aligned to reference sequences of non-lichenicolous Tremellales. However, most of the reads obtained were not from the expected targeted tremellalean fungi but from other fungi and no useful results came out. Therefore only ITS and nuLSU were used in the molecular studies.

Species delimitation methods

The GMYC analysis (Pons et al., 2006; Fontaneto et al., 2007; Monaghan et al., 2009; Fujisawa and Barraclough, 2013) was performed by using the ultrametric tree previously generated and the GMYC single and GMYC multiple analyses were run with the 'splits' package (http://r-forge.r-project.org/projects/splits, Ezard et al., 2009; Fujisawa and Barraclough, 2013) available for R v.3.0.2 (R Core Team, 2013). This package uses functions from the 'ape' v.5.1 library (Paradis et al., 2004). The dataset of *Tremella* samples comprises individuals with identical sequences, these samples were removed from the GMYC models. The PTP method (Zhang et al., 2013) was performed with

default settings at the Exilis Lab web server (https://species.h-its.org/ptp/). We used the maximum likelihood tremellalean phylogenetic tree, previously obtained with RAxML (Silvestro and Michalak, 2012), as input file for this analysis. For the ABGD analysis (Puillandre et al., 2012) we used the web server at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html and set a Pmax and Pmin of 0.001 and the Kiruma (K80) TS/TV distance according to the model that best suited our data, the rest of parameters were left as default. The input file for this analysis was the tremellalean alignment in fasta format. PCI (Suwannasai et al., 2013) was calculated by comparison of intraspecific and interspecific uncorrected distances of potential species with at least two specimens. We generated the uncorrected distance matrix of these specimens in Mesquite and compared those distances manually. If the intraspecific uncorrected distance between samples is lower than the interspecific one, we can set the species as correctly identified (Meier et al., 2006; Erickson et al., 2008; Suwannasai et al., 2013). To perform the BPP A11 analyses (Rannala and Yang, 2003; Yang and Rannala, 2010; Yang, 2015; Rannala and Yang, 2017; Flouri et al., 2020) we followed Flouri et al., (2020) and manually constructed six multiple sequence alignment files with concatenated ITS1, 5.8s, ITS2 and LSU sequences, and six Imap files corresponding to each one of the species delimitations previously specified. The six control files were created with Minimalist BPP (https://brannala.github.io/bpps/) for the species delimitation Algorithm 0 with a value of $\varepsilon = 2$ and we manually constructed the control files for the Algorithm 1 with $a = 2$ and $m = 1$. We assigned inverse-gamma diffuse priors Θ (3 0.0054) and τ (3 0.15). The MCMC was set to 20000 generations with a sampling frequency of 2 and 2000 of burn-in. All analyses were performed with cleandata = 0.

Molecular Clock analyses

The taxon sampling for the dating analyses of the Basidiomycota included representatives of 24 orders of basidiomycetes and six orders of ascomycetes, that allowed us to place the fossils selected for the time calibration. We conducted preliminary analyses including a lager representation of lichenicolous taxa, a larger sampling within *Tremella caloplacae* s. l., and closely related taxa that belong in Clade I designed by Millanes et al., (2011) (i.e., *T. christiansenii*). None of these taxa were nested within *T. caloplacae* s. l. and they are not included in the final analysis in order to obtain a more equilibrated matrix. Definitive analyses to estimate the diverging times of *T. caloplacae* s. l. were performed based on the five nuclear loci corresponding to SSU, ITS1, 5.8s, ITS2 and LSU, with a total length of 2929 characters. The alignment was conducted on the MAFFT (Katoh et al., 2002, 2005; Katoh and Standley, 2013) online server using the L-INS-I algorithm, which is advised for long sequences with gaps and one conserved region (Katoh et al., 2005). The alignment was treated with GBlocks v.0.91b (Castresana, 2000) and Mesquite v.3.6 (Maddison and Maddison, 2018). We inferred ML and Bayesian trees, in this case only to decide the sampling choice for the dating analysis. ML analyses were conducted in RAxMLGUI v.1.5b1, following the same procedures described for the phylogenetic analyses. For the Bayesian analyses, we selected the substitution models in JModeltest v.2.1.10 (Guindon and Gascuel, 2003; Posada, 2008; Darriba et al., 2012), using the corrected Akaike information criterion (AICc). A GTR + I + G selected for the nuclear SSU and the nuclear LSU rDNA regions and a SYM + I + G model for the complete nuclear ITS rDNA. We run the Bayesian analyses as previously described for the phylogenetic analyses of *T. caloplacae* and its Teloschistaceae hosts.

To date the diverging times of the Teloschistaceae hosts, we used as a base the same taxon sampling used in Prieto and Wedin (2013) but including some additional sequences produced for this study and others downloaded from GenBank (Table S1). A total of 133 taxa belonging to 11 classes of Ascomycota, and *Schizosaccharomyces pombe* as the outgroup, were included in the final alignment with a total length of 2693 characters corresponding to five nuclear gene loci: SSU, ITS1, 5.8s, ITS2 and LSU. Sequences alignment, edition, ML trees and choice of substitution models were achieved following the same protocols as for the dating analysis of *T. caloplacae* but performing the RAxML analysis using the combined nuclear genes dataset with five partitions, one for each nuclear gene used: SSU, ITS1, 5.8s, ITS2 and LSU. The starting tree for the dating analysis Teloschistaceae host was calibrated with the fossil *Palaeopyrenomycites devonicus* (Taylor et al., 2005) (Table S3). It was constructed by using an uncorrected relaxed clock model with a lognormal relaxed distribution. For the tree prior we selected a Birth-Death speciation process with a random starting tree. We run three independent MCMC runs with 200 million generations, logging every 10,000 generations. Convergence of runs was evaluated with Tracer v.1.7 (Rambaut et al., 2018), verifying that all the ESS values were greater than 200. The 10% of the trees were eliminated as burning with LogCombiner v.1.10.4 (Drummond and Rambaut, 2007) and the MCC tree was obtained with TreeAnnotator v.1.10.4 (Rambaut et al., 2018).

Cophylogenetic analysis

For the Jane v4.0 (Conow et al., 2010) analyses we selected the topologies of the parasite and host trees based on the Bayesian trees obtained with MrBayes (Ronquist et al., 2012) and for each of the species delimitation hypotheses, or with the BEAST (Suchard et al., 2018) dating tree in the case of the time constricted analyses. The topologies for the trees

were reconstructed manually with the Jane Tree Editor by selecting just one representative specimen of the different clades obtained. Each parasite was linked to its host using the Link Mode. The similarity of the parasite and host trees was evaluated by carrying out an analysis with 1000 replicates for a population size and a sample size of 1000. As input for ParaFitGlobal test (Legendre et al., 2002) we used three data matrices: two uncorrected distances matrices obtained with Mesquite for both *T. caloplacae* s. l. and their hosts and their interaction matrix, constructed manually. We also tested the individual link contribution of each interaction to the global analysis with ParaFitLink1 and ParaFitLink2. To perform the PACo cophylogenetic analyses (Balbuena et al., 2013; Hutchinson et al., 2017), we employed three data files: the two phylogenetic trees of *T. caloplacae* s. l. and their Teloschistaceae hosts, and an interaction matrix manually constructed We conducted Cailliez corrections in the transformation of phylogenetic trees to uncorrected distances and subsequently to Principal Coordinates to avoid negative eigenvalues. The jackknife method was applied to calculate the contribution of each individual interaction to the global sum of squares. For the I_{cong} analysis (de Vienne et al., 2007), we constructed newick trees manually, based on the dated phylogenies of hosts and parasites previously obtained. In addition to the I_{cong} index, the P-value is calculated to test the significance of the analysis.

Results

DNA sequences and phylogenetic analysis

The best tree obtained for *T. caloplacae* in the Maximum Likelihood (ML) analyses had an ln-likelihood of -3204.457280. The best tree obtained for the hosts in the ML analyses had an ln-likelihood of -3573.609030. The Bayesian analyses stopped after 700000 generations for *T. caloplacae* and after 1200000 generations in the case of the

Teloschistaceae hosts' alignment. At that time, the values of the average standard deviation of split frequencies were 0.009568 and 0.009525 for the analyses of *T. caloplacae* and its hosts respectively, indicating that convergence had been reached.

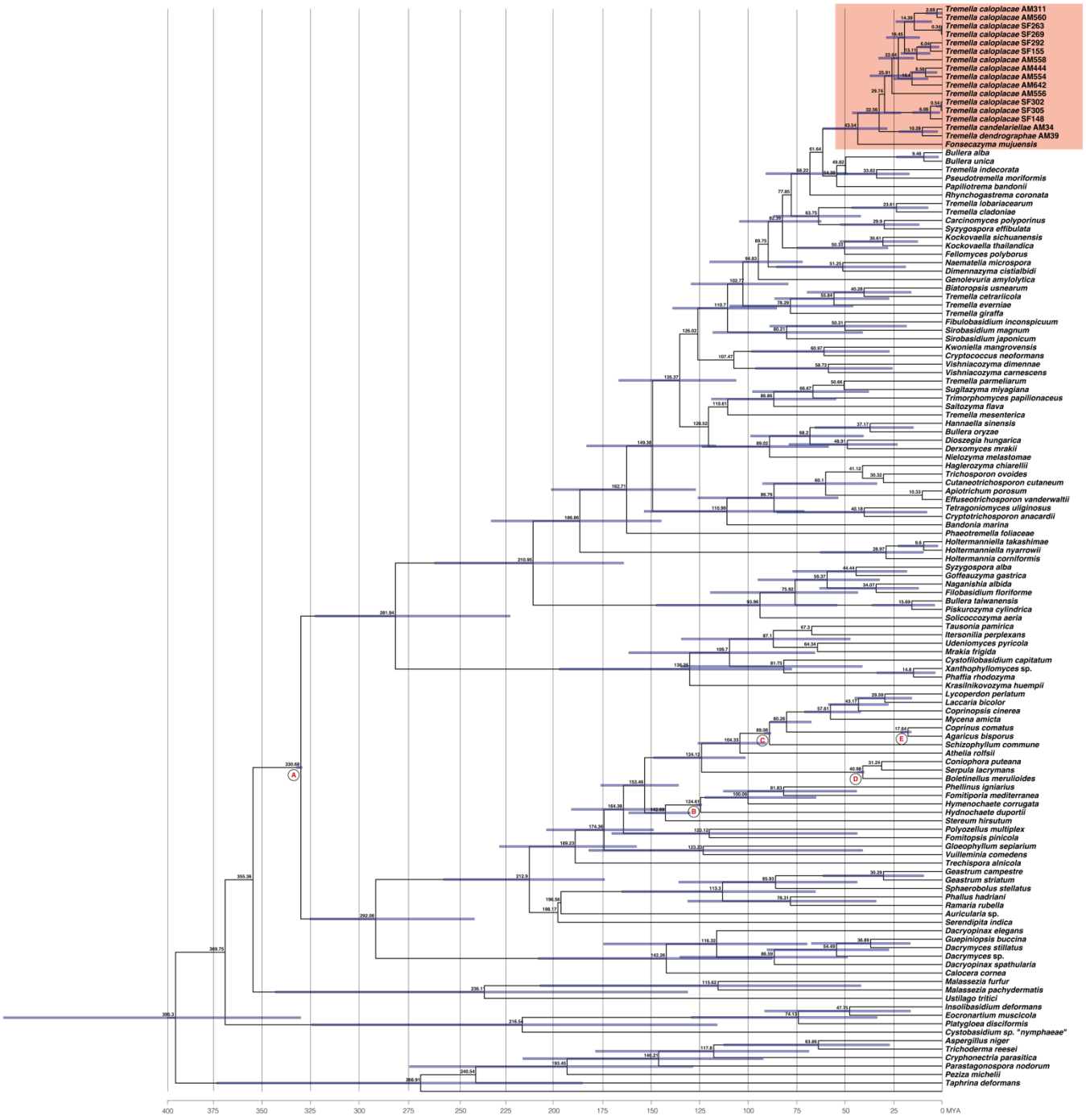

Fig. S1. Divergence times estimations for the 129 taxa representing the main groups of Basidiomycota, based on the five-marker dataset (nSSU, ITS1, 5.8s, ITS2 and nLSU). The most probable divergence time is indicated for each node. Bars correspond to the 95% highest posterior density (HPD). Nodes letters indicate positions of the fossils used for the dating analyses (see Table S4). Region highlighted is shown in Fig. 4.

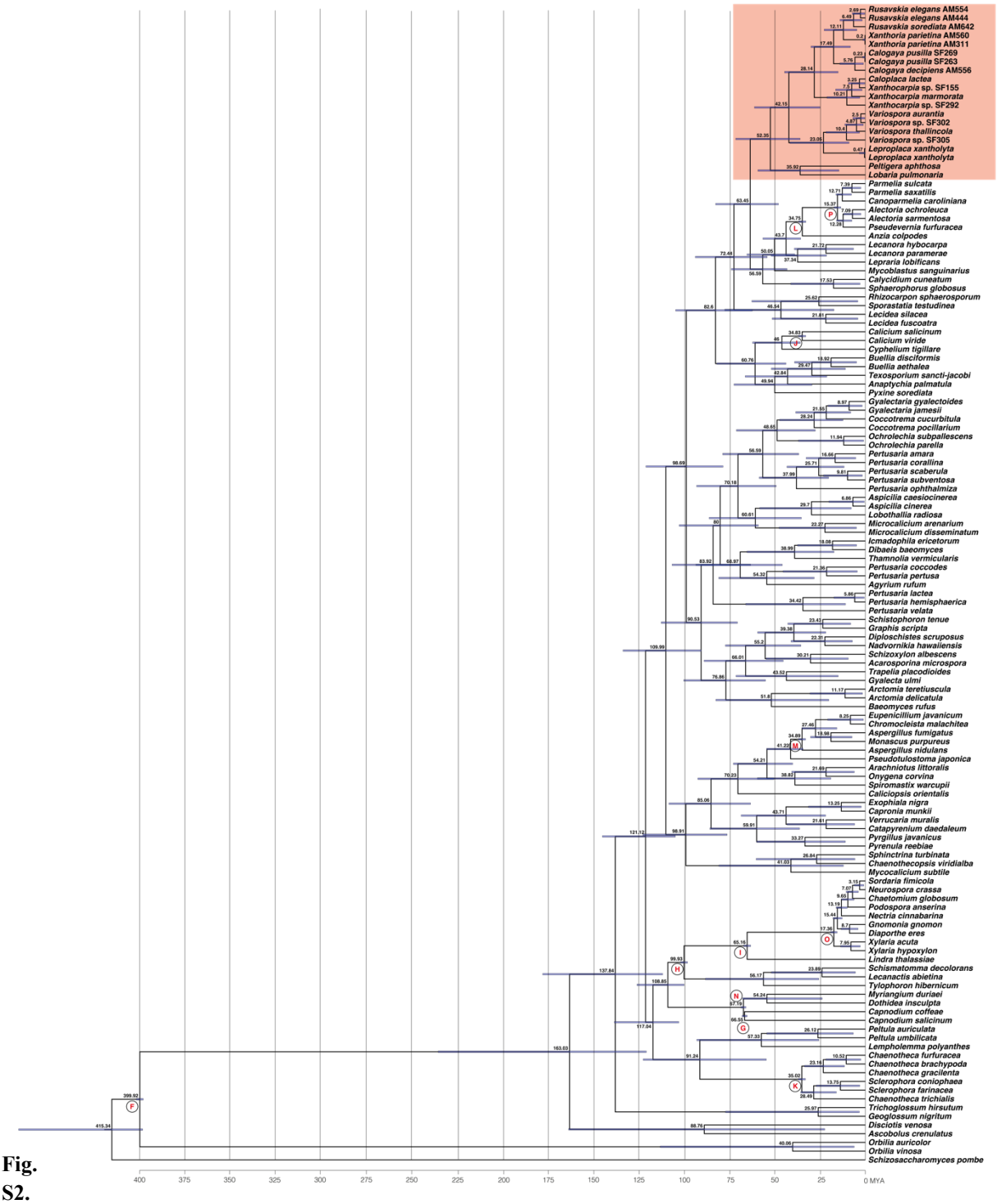

Fig. S2.

Divergence times estimations for the 133 taxa representing the main groups of Ascomycota, based on the five-marker dataset (nSSU, ITS1, 5.8s, ITS2 and nLSU). The most probable divergence time is indicated for each node. Bars correspond to the 95% highest posterior density (HPD). Nodes letters indicate positions of the fossils used for the dating analyses (see Table S4). Region highlighted is shown in Fig. 4.

Table S1. Herbarium material of potential hosts held in S, checked for the presence of galls. We do not have a list of specimens reviewed at UPS, for this reason this information is not included in the table.

Species	Year	Country	Herbarium code (S)
Athallia			
Athallia cerinella	1992	Sweden	F93055
	2004	Estonia	F57285
	2009	Sweden	F140709
Athallia cerinelloides	1991	Austria	F101078
Athallia holocarpa	1992	Sweden	L73064
	1992	United States, Minnesota	F101511
	1992	Sweden	L73051
	1992	United States, Wisconsin	F101506
	1992	Sweden	L73050
	1994	United States, Minnesota	F101509
	1996	Sweden	F172394
	2001	Sweden	F61554
	2002	Sweden	F57725
	2002	United States, California	L47354
	2003	Sweden	L56423
	2004	Sweden	F61539
	2004	Sweden	F57537
	2004	Sweden	F61546
	2004	Sweden	F61659
	2004	Sweden	F57475
	2004	Sweden	F57242
	2004	Sweden	F57531
	2004	Sweden	F61658
	2004	Sweden	F57245
	2005	Sweden	F57356
	2005	Sweden	F52752
	2005	Sweden	F57289
	2005	Sweden	F57288
	2005	United States, Michigan	F52791
	2005	United States, South Dakota	F52785
	2006	Sweden	F61213
	2007	Sweden	F81531
	2008	United States, Pennsylvania	F143905
	2008	United States, Pennsylvania	F143869
	2009	Sweden	F140711
	2009	Sweden	F139384
	2010	Sweden	F178518
	2010	Sweden	F179359
Athallia pyracea	1991	Sweden	L73149
	1992	Sweden	L73059
	1998	United States, North Dakota	F236613
	2007	Sweden	F81532
	2007	Sweden	F87833
	2007	Sweden	F151806
	2009	Sweden	F140722
	2009	Sweden	F140710
	2009	Sweden	F151249
	2010	Sweden	F179358
	2010	Sweden	F178233
	2010	Sweden	F179053
	2010	Sweden	F401161
	2011	Sweden	F207680
	2011	Sweden	F204893

(Continue)

Species	Year	Country	Herbarium code (S)
Athallia pyracea	2015	Sweden	F285250
	2016	Sweden	F299251
	2016	Sweden	F299006
	2016	Sweden	F301124
Athallia saxifragarum	1996	Italy	F106004
Athallia scopularis	1992	Sweden	L52520
	1999	Sweden	L6154
	2009	Sweden	F138192
	2009	Sweden	F283447
Athallia vitellinula	1997	United States, Nebraska	F145075
Blastenia			
Blastenia crenularia	1991	Sweden	L31649
	1992	Sweden	F176538
	2008	Sweden	F122058
	2008	Sweden	F122636
	2008	Sweden	F120202
Blastenia subochracea	1996	Italy	F106941
Bryoplaca			
Bryoplaca jungermanniae	1991	Greenland	F101972
	2002	Greenland	L59900
	2004	Sweden	F61558
	2004	Sweden	F57527
	2008	Sweden	F122435
	2015	Sweden	F277777
	2015	Sweden	F277449
	2015	Sweden	F285591
Bryoplaca sinapisperma	1996	Sweden	L52521
	1998	United States, Wyoming	F144522
	2000	Sweden	L27607
	2000	Sweden	L27006
	2000	Sweden	L27690
	2000	Sweden	L29335
	2000	Sweden	L29337
	2000	Sweden	F61635
	2000	Sweden	F61636
	2004	Sweden	F57393
	2006	Sweden	F61003
	2008	Sweden	F122060
	2008	Canada, Ontario	F143898
	2009	Sweden	F401209
	2011	Sweden	F304745
	2012	Austria	F262464
	2012	Sweden	F403524
	2012	Sweden	F240340
	2013	Sweden	F262765
	2014	Sweden	F259922
	2014	Sweden	F260279
Calogaya			
Calogaya biatorina	1996	Italy	F92038
Calogaya decipiens	1990	Estonia	F94628
	1992	Sweden	F94524
	1992	Sweden	F94526
	1994	Sweden	L52504
	1995	Greenland	F94678
	1996	Italy	F94608
	1998	United States, North Dakota	L5866
	2006	United States, Wyoming	F117283
Calogaya lobulata	1996	Italy	F103398

(Continue)

Species	Year	Country	Herbarium code (S)
Calogaya saxicola	1990	Estonia	F105851
	1990	Canada, Northwest Territories	L45020
	1991	Sweden	F105391
	1991	Antarctica	F158927
	1992	Sweden	F105291
	1992	Sweden	F176571
	1992	Antarctica	F158925
	1992	Antarctica	F158926
	1992	Antarctica	F158928
	1992	Antarctica	F158929
	1996	Italy	F105789
	1996	France	F221084
	1997	United States, Nebraska	F105995
	1998	United States, Montana	L11772
	1999	United States, Arizona	L61972
	2000	Sweden	L29067
	2004	Estonia	L60463
	2007	United States, Wyoming	F144341
	2008	Sweden	F124313
	2009	Sweden	F140711
Caloplaca			
Pyrenodesmia alociza	1996	Italy	F91079
Caloplaca cerina	1990	Sweden	F92376
	1990	Sweden	F92328
	1990	Canada, Northwest Territories	L40729
	1991	Greece	F92485
	1993	United States, Minnesota	L59477
	1993	Sweden	L52502
	1993	Sweden	L52500
	1993	Sweden	F92185
	1993	United States, Minnesota	F93038
	1994	Sweden	L52501
	1994	United States, Arizona	F93037
	1996	Italy	F92483
	1996	Italy	F92481
	1997	Mexico, Baja California	F236648
	1997	United States, Minnesota	L3690
	1998	United States, Minnesota	F117314
	1998	United States, North Dakota	F52595
	1998	United States, North Dakota	F236674
	1998	United States, North Dakota	F117264
	1998	United States, North Dakota	F117285
	1999	United States, Minnesota	F117315
	2004	Sweden	F61658
	2004	Sweden	L64230
	2004	Sweden	F57244
	2005	Sweden	L67812
	2005	United States, Michigan	F52591
	2006	United States, Nebraska	F144676
	2006	United States, Nebraska	F144676
	2007	Sweden	F87835
	2007	Sweden	F77017
	2007	United States, South Dakota	F103668
	2009	Sweden	F138056
	2009	Sweden	F151250
	2009	Sweden	F138042
	2010	Sweden	F179355
	2013	Sweden	F254803
Caloplaca chlorina	1990	Sweden	L52503

(Continue)

Species	Year	Country	Herbarium code (S)
Caloplaca chlorina	1991	Sweden	F93324
	1991	Sweden	F176516
	1991	Sweden	F93346
	1992	Sweden	F93320
	1992	Sweden	F93303
	1992	Sweden	F93299
	1992	Sweden	F93318
	1993	Sweden	F93332
	1995	Sweden	F93347
	2000	Sweden	F360700
	2002	Sweden	F209511
	2015	Sweden	F278019
Caloplaca hanneshertelii	2004	Australia	F65750
Caloplaca teicholyta	2012	Spain	F268486
	2013	Spain	F315382
	2013	Spain	F283438
Cerothalia			
Cerothalia luteoalba	1993	Sweden	L52514
	1993	Sweden	L52517
	1993	Sweden	L52515
	1994	Sweden	L52516
	1996	Italy	F103622
Dufourea			
Dufourea capensis	1996	South Africa	L2915
Dufourea elixii	2004	Australia	F65747
	2004	Australia	F65753
	2004	Australia	F65750
Dufourea ligulata	1997	Australia	L43890
	1997	Australia	L43891
	2010	New Zealand	F180782
Flavoplaca			
Flavoplaca arcis	1990	Austria	L2613
Flavoplaca citrina	1991	Antarctica	F158854
	1991	Antarctica	F158923
	1991	Antarctica	F158558
	1991	Antarctica	F158556
	1992	Sweden	L46435
	1992	Sweden	L46437
	1992	Antarctica	F158867
	1992	Sweden	F176562
	1992	Sweden	L46436
	1992	Sweden	L46458
	1992	Antarctica	F158856
	1992	Antarctica	F158924
	1992	Antarctica	F158855
	1992	Antarctica	F158857
	1992	Antarctica	F158858
	1992	Antarctica	F158868
	1992	Antarctica	F158860
	1993	Antarctica	F94071
	1993	Sweden	F80759
	1994	Antarctica	F94070
	1994	United States, California	L44847
	1996	United States, Iowa	L11850
	1997	United States, Minnesota	L3677
	1998	United States, North Dakota	L11833
	2002	China	F52605
	2004	Sweden	F57217
	2004	Sweden	L64461

(Continue)

Species	Year	Country	Herbarium code (S)
Flavoplaca citrina	2005	Sweden	L67834
	2010	Sweden	F178459
	2014	Sweden	F260247
	2014	Sweden	F260000
Flavoplaca coronata	1991	Sweden	L31822
	1996	Italy	F94140
	2000	Poland	L45012
Flavoplaca cranfieldii	2004	Australia	F65746
	2004	Australia	F65748
Flavoplaca dichroa	1999	Sweden	L6158
	2008	Sweden	F122445
	2009	Sweden	F138198
Flavoplaca granulosa	1996	Italy	F100673
Flavoplaca marina	2009	Sweden	F138191
	2009	Sweden	F283446
	2013	Sweden	F254702
Flavoplaca microthallina	2000	Sweden	F61649
	2009	Sweden	F138189
Flavoplaca oasis	1992	Sweden	L73065
	1992	Sweden	L73063
Flavoplaca polycarpa	1996	Sweden	L46234
	2010	Italy	F179433
Gyalolechia			
Gyalolechia arizonica	1994	United States, Arizona	F91712
	2003	United States, Arizona	F58858
Gyalolechia bassiae	2000	Seychelles	L30236
Gyalolechia bracteata	1990	Sweden	L53564
	1991	Sweden	L53562
	1991	Sweden	L53561
	1991	Greenland	F107773
	1993	Sweden	F171860
	1993	Sweden	F207660
	1993	Sweden	L53563
	1993	Sweden	F207659
	1993	Sweden	F207661
	1995	Greenland	F289482
	1996	Sweden	L1115
	1997	United States, Nebraska	L45343
	1998	Greenland	L7593
	1998	Greenland	L7621
	1998	Greenland	L7601
	1999	Greenland	L45554
	1999	Greenland	L45546
	1999	Greenland	L45557
	1999	Greenland	L45572
	1999	Greenland	L45549
	1999	Greenland	L45553
	1999	Greenland	L45560
	1999	Greenland	L45564
	1999	Greenland	L45611
	1999	Greenland	F27298
	1999	Greenland	L45550
	1999	Greenland	L45558
	1999	Greenland	L45563
	2000	Sweden	L17292
	2000	Sweden	L24475
	2000	Sweden	L15059
	2002	Sweden	L42371
	2004	Sweden	L62172

(Continue)

Species	Year	Country	Herbarium code (S)
Gyalolechia bracteata	2004	Sweden	L62176
	2004	Sweden	L62193
	2004	Sweden	F61562
	2006	Sweden	F61126
	2008	Sweden	F122443
	2009	Sweden	F140789
	2010	Spain	F177726
	2011	Sweden	F314357
	2011	Sweden	F314266
	2012	Austria	F262217
	2012	Austria	F262466
	2012	Sweden	F315028
	2012	Austria	F262451
	2012	Sweden	F256990
	2012	Austria	F262491
	2012	Sweden	F315239
	2013	Sweden	F312476
	2013	Sweden	F315275
	2014	Sweden	F259534
	2014	Sweden	F260080
	2014	Sweden	F267981
	2014	Sweden	F259905
	2014	Sweden	F259787
	2014	Sweden	F260917
	2014	Sweden	F260952
	2014	Sweden	F259737
	2014	Sweden	F260495
	2014	Sweden	F260538
	2014	Sweden	F259738
	2015	Sweden	F277703
	2015	Sweden	F277967
	2015	Sweden	F285665
	2015	Sweden	F285799
	2015	Sweden	F285998
Gyalolechia desertorum	1995	Greenland	F149465
	1998	United States, North Dakota	L45340
	2007	United States, Wyoming	F145101
	2008	Norway	F315175
Gyalolechia flavorubescens	1990	Sweden	L52510
	1990	Sweden	F98167
	1991	Sweden	F98013
	1991	Sweden	L52509
	1991	Sweden	F97999
	1991	Sweden	F98069
	1992	United States, Minnesota	F99458
	1992	Sweden	F97820
	1993	Sweden	F360712
	1993	Sweden	L52508
	1993	Sweden	F317706
	1994	Sweden	F172319
	1994	Sweden	F172318
	1994	United States, Minnesota	F99457
	1996	United States, Minnesota	F52760
	1996	Italy	F98217
	1997	Sweden	L13108
	1998	Sweden	F57826
	1998	Sweden	L29054
	1998	Sweden	F404374
	1999	Sweden	L6159

(Continue)

Species	Year	Country	Herbarium code (S)
Gyalolechia flavorubescens	2000	Sweden	F360755
	2000	Sweden	F365789
	2002	Sweden	L49998
	2002	Sweden	L38828
	2002	Sweden	L46374
	2003	Sweden	F59807
	2003	Sweden	L50020
	2004	Sweden	L66118
	2004	Estonia	L60467
	2004	Estonia	L60468
	2004	Sweden	L60551
	2004	Sweden	L62888
	2004	Sweden	F57245
	2004	Sweden	L62760
	2004	Sweden	F57242
	2005	Sweden	L70976
	2005	Sweden	L71197
	2007	Sweden	F91056
	2007	Sweden	F87834
	2007	Sweden	F81525
	2007	Sweden	F76587
	2009	Sweden	F138005
	2010	Sweden	F180087
	2010	Sweden	F178229
	2011	Sweden	F207687
	2013	Sweden	F401208
	2013	Sweden	F254882
	2013	Japan	F255891
	2016	Sweden	F299083
Gyalolechia flavovirescens	1990	Sweden	F99738
	1991	United States, Minnesota	F100316
	1992	Sweden	F176563
	1992	Mexico	L59284
	1994	United States, Arizona	L60986
	1996	Italy	F99835
	1997	United States, Minnesota	L3676
	2004	Estonia	F192837
	2004	United States, Wisconsin	F52773
Gyalolechia persimilis	1993	Mexico	L69868
	1998	Mexico	L59883
	2002	United States, Texas	L59880
	2002	United States, Texas	L59882
	2002	United States, Texas	L59881
	2010	United States, New Mexico	F236670
Gyalolechia stantonii	1994	United States, California	L59427
	1998	United States, California	F236655
	1998	United States, California	L10052
Gyalolechia stipitata	1990	Mexico	L10053
	1991	Mexico	L45341
	1992	Mexico	L11784
	1993	Mexico	L5891
	1994	United States, California	L45217
	1998	United States, California	L45219
Gyalolechia subbracteata	1995	Italy	F157423
	2012	Spain	F315284
	2013	Spain	F315376
	2013	Spain	F315220
	2013	Spain	F315240
Gyalolechia xanthostigmoidea	1997	United States, Arizona	L10035

(Continue)

Species	Year	Country	Herbarium code (S)
Olegblumia			
Olegblumia demissa	1994	Mexico	F273717
Pachypeltis			
Pachypeltis castellana	1996	Greenland	F90643
	1998	Greenland	L7623
	1998	Greenland	L7646
	1999	Greenland	L45569
Pachypeltis cladodes	1994	United States, Arizona	F74364
	1998	United States, Wyoming	L11759
	2007	United States, Wyoming	F144238
Parvoplaca			
Parvoplaca tiroliensis	1991	Sweden	F107621
	2004	Sweden	F244996
	2015	Sweden	F277933
	2015	Sweden	F277934
	2015	Sweden	F277936
	2015	Sweden	F277935
	2015	Sweden	F277937
Polycauliona			
Polycauliona bolacina	1993	Mexico	F52587
	1994	United States, California	L58804
	1995	Mexico	L59270
	1995	Mexico	L761
	1998	Mexico	L59267
	1998	United States, California	L10030
	1998	Mexico	L10029
Polycauliona candelaria	1990	Sweden	L54368
	1991	Sweden	L54366
	1991	Canada	F68150
	1991	Sweden	F176363
	1991	Canada	F68151
	1991	Canada	F68152
	1991	Sweden	F176361
	1992	Sweden	L54367
	1992	Greenland	F234445
	1992	Sweden	L66087
	1998	United States, Wyoming	L11143
	1998	United States, Wyoming	L11268
	1999	Sweden	L6153
	2000	Sweden	L62769
	2000	Sweden	L1488
	2003	Sweden	L43629
	2004	Sweden	L62494
	2004	Greenland	L65978
	2004	Sweden	L64449
	2004	Sweden	L64173
	2006	Sweden	F60932
	2008	Sweden	F122937
	2008	Sweden	F126615
	2009	Greenland	F169173
	2010	Sweden	F178492
	2012	Sweden	F240339
	2013	Sweden	F255042
	2014	Sweden	F260359
	2015	Sweden	F285212
Polycauliona coralloides	1995	Mexico	F94131
	1995	United States, California	L1389
	1997	Mexico	L10033
	1997	Mexico	L5863

(Continue)

Species	Year	Country	Herbarium code (S)
Polycauliona ignea	1993	United States, California	L44415
	1995	Mexico	L772
	1996	Mexico	F101614
	1996	United States, California	F101615
	1998	Mexico	F101610
	1998	Mexico	L5867
Polycauliona impolita	1993	Mexico	L44927
	1994	United States, California	F101632
Polycauliona ludificans	1994	United States, California	F52798
	1994	United States, California	F145068
	1994	United States, California	L59454
	1994	United States, California	L59455
Polycauliona luteominia	1996	Mexico	L59292
	2003	United States, California	F145061
Polycauliona nashii	1998	Mexico	F53138
Polycauliona phlogina	2009	Sweden	F138040
Polycauliona pollinarioides	2008	United States, California	F175864
Polycauliona polycarpa	1990	Sweden	L54381
	1991	Sweden	L54379
	1992	Sweden	L66078
	1993	Sweden	F172289
	1994	United States, California	F232365
	1994	United States, California	F232003
	1995	Sweden	L54382
	1997	Sweden	L13195
	1998	Sweden	L54380
	1998	United States, Minnesota	L5884
	1999	Sweden	L29239
	2000	Sweden	L14867
	2000	Sweden	L14911
	2001	Sweden	L24418
	2002	Sweden	L38745
	2002	Sweden	L38826
	2002	Lithuania	F236637
	2002	Sweden	L38731
	2003	Sweden	L50037
	2004	Sweden	L62198
	2004	Sweden	L60533
	2005	Sweden	L67818
	2005	Sweden	L67899
	2005	Sweden	L67901
	2005	Sweden	L60998
	2006	Sweden	F61217
	2007	Sweden	F76404
	2007	Sweden	F76428
	2007	Sweden	F76419
	2007	Sweden	F76423
	2008	Sweden	F124402
	2008	Sweden	F118034
	2008	Sweden	F124305
	2008	Finland	F119188
	2008	Sweden	F118011
	2009	Sweden	F140908
	2009	Sweden	F150995
	2010	Sweden	F179339
	2010	Sweden	F178493
	2010	Sweden	F178938
	2010	Sweden	F179055
	2010	Sweden	F179406

(Continue)

Species	Year	Country	Herbarium code (S)
Polycauliona polycarpa	2010	Sweden	F178533
	2010	Sweden	F179408
	2010	Sweden	F179433
	2011	Finland	F205554
	2014	Sweden	F260289
	2015	Sweden	F285706
Polycauliona rosei	1993	United States, California	L59429
	1994	United States, California	L59432
	1994	United States, California	L10051
Polycauliona stellata	2000	United States, Minnesota	L46923
Polycauliona tenax	1993	Mexico	F52062
	1997	Mexico	L10415
	1998	Mexico	L5885
	2007	United States, South Dakota	F143801
Polycauliona thamnodes	1993	Mexico	F107605
Polycauliona verruculifera	1991	Sweden	F109654
	1997	United Kingdom	L5872
	2014	Norway	F278247
Pyrenodesmia			
Pyrenodesmia chalybaea	1996	Italy	F93193
	2008	Sweden	F122432
	2009	Sweden	F138177
	2013	Sweden	F315021
	2014	Sweden	F268013
Pyrenodesmia peliophylla	1996	Mexico	F104929
	1996	Mexico	L59295
	1998	Mexico	F104928
Pyrenodesmia soralifera	2005	United States, Michigan	F145062
	2005	United States, South Dakota	F117317
	2007	United States, South Dakota	F117279
	2007	United States, South Dakota	F117286
Pyrenodesmia variabilis	1993	Sweden	L52522
	2008	Sweden	F120231
	2009	Sweden	F138181
	2014	Sweden	F267986
	2014	Sweden	F267993
Rufoplaca			
Rufoplaca oxfordensis	1995	United States, Minnesota	L42776
Rufoplaca scotoplaca	1991	Sweden	F106232
	2009	Sweden	F139387
Rufoplaca subpallida	2009	Sweden	F138249
	2009	Sweden	F138248
	2009	Sweden	F138247
Rusavskia			
Rusavskia elegans	1990	Sweden	L54370
	1990	Canada	L41214
	1991	Sweden	F314396
	1991	Sweden	L54369
	1991	Antarctica	F158510
	1991	Antarctica	F158514
	1991	Antarctica	F158645
	1991	Antarctica	F329108
	1992	Antarctica	F158526
	1992	Antarctica	F158525
	1992	Antarctica	F158513
	1992	Antarctica	F158508
	1992	Antarctica	F158527
	1992	Antarctica	F158528

(Continue)

Species	Year	Country	Herbarium code (S)
Rusavskia elegans	1992	Antarctica	F327588
	1992	Greenland	F225138
	1992	Antarctica	F329105
	1993	China	F401995
	1997	United States, Minnesota	L3686
	1998	United States, North Dakota	L11269
	1998	Greenland	L7646
	1999	Greenland	L45560
	1999	Greenland	L45570
	1999	Greenland	L45585
	1999	Greenland	L45618
	2000	Sweden	L24435
	2003	Sweden	L56428
	2003	Sweden	L54965
	2003	Greenland	L59962
	2004	Sweden	L66033
	2004	Sweden	L65982
	2004	Sweden	L61295
	2004	Sweden	L66028
	2004	Sweden	L62525
	2006	Sweden	F60941
	2006	Sweden	F60935
	2006	United States, Wyoming	F144023
	2007	Greenland	F169175
	2007	Canada	F104071
	2011	Sweden	F314967
	2012	Sweden	F240316
	2013	Sweden	F254648
	2013	Sweden	F254650
	2015	Sweden	F285394
Rusavskia sorediata	1990	Sweden	L54383
	1990	United States, Arizona	L62092
	1998	Sweden	L5011
	1998	Sweden	L5010
	1998	Sweden	L5012
	2000	Sweden	L27613
	2004	Sweden	L61320
	2006	Sweden	F60952
	2007	Canada	F143794
	2010	Svalbard and Jan Mayen	F178311
	2012	Sweden	F240166
	2015	Sweden	F285674
Seiphora			
Seiphora californicus	1993	Mexico	L5874
	1997	Mexico	L5875
	1997	Mexico	L10373
	2001	United States, California	F58853
Shackletonia			
Shackletonia buelliae	1991	Antarctica	F158499
Sirenophila			
Sirenophila eos	1997	Australia	F65757
	2004	Australia	F65748
	2004	Australia	F65756
	2012	Australia	F256726
Squamulea			
Squamulea kiamae	2004	Australia	F65749
Squamulea parviloba	1992	Mexico	L59468
	1998	Mexico	L59470
	2002	United States, Texas	L59878

(Continue)

Species	Year	Country	Herbarium code (S)
Squamulea parviloba	2002	United States, Texas	L59877
Squamulea squamosa	1997	United States, Minnesota	L3703
	1998	Mexico	L59426
Squamulea subsoluta	1991	United States, Arizona	L62037
	1995	United States, Minnesota	F52918
	1998	Mexico	L59398
	2001	United States, Minnesota	L59884
	2002	United States, Texas	L59885
	2002	United States, Maryland	L59886
	2005	United States, South Dakota	F117275
Solitaria			
Solitaria chrysophthalma	1991	Sweden	L15511
	1991	United States, Missouri	F93528
	1991	Sweden	L15515
	1991	Sweden	L15516
	1992	Sweden	L15514
	1992	Sweden	L15519
	1992	Sweden	L15513
	1993	Sweden	L15512
	1994	Sweden	L15518
	1994	Sweden	F93408
	1994	United States, Minnesota	F93520
	1995	Sweden	L15517
	1996	Sweden	F169773
	1996	United States, Minnesota	F93524
	1996	United States, Missouri	L59257
	1998	United States, Minnesota	L59838
	1998	United States, North Dakota	L59272
	2000	United States, Missouri	L59278
	2000	United States, Missouri	F52598
	2000	United States, Missouri	L59837
	2000	United States, Missouri	L59841
	2000	United States, Missouri	L59870
	2011	United States, Minnesota	F265001
Teloschistes			
Teloschistes exilis	1998	Costa Rica	L10375
Teloschistes chrysophthalmus	1993	Mexico	L5876
	1997	Mexico	L5877
	1997	Mexico	L10374
	2006	United States, Nebraska	F144031
Teloschistes flavicans	1992	Hawaii	L10376
	2005	Ecuador, Galapagos	F96051
	2005	Ecuador, Galapagos	F96014
	2005	Ecuador, Galapagos	F238856
Usnochroma			
Usnochroma carphineum	2014	Spain	F268457
	2014	Spain	F268454
Variospora			
Variospora aurantia	1996	Italy	F91751
Variospora dolomiticola	1996	Italy	L73285
	2004	Sweden	F61585
Variospora flavescens	2014	Sweden	F261068
Xanthocarpia			
Xanthocarpia crenulatella	1993	Sweden	L52574
	2009	Sweden	F140707
Xanthocarpia feracissima	1990	United States, Minnesota	F95091
	1994	United States, Minnesota	F95085
	1994	United States, New Jersey	L42714

(Continue)

Species	Year	Country	Herbarium code (S)
Xanthocarpia lactea	1998	United States, Montana	F144370
Xanthocarpia marmorata	1994	United States, California	F52808
	1996	Italy	F93556
Xanthocarpia tominii	1991	Greenland	F107773
	1995	Greenland	F107741
	1998	Greenland	L7577
	1998	Greenland	L7576
	1998	Greenland	L7601
	1998	Greenland	L7621
	2006	United States, Idaho	F117334
Xanthomendoza			
Xanthomendoza borealis	1991	Sweden	L54365
	1992	Antarctica	F158701
	1992	Antarctica	F158769
	1992	Antarctica	F158698
	1992	Antarctica	F158702
	1992	Antarctica	F158699
	1995	Greenland	F191351
	1995	Greenland	F167168
	1996	Greenland	L63705
	1998	Greenland	L7645
	1999	Greenland	L45616
	1999	Greenland	L45544
	1999	Greenland	L45574
	1999	Greenland	L45556
	2001	Greenland	L59938
	2002	Greenland	L59956
	2004	Sweden	L61301
Xanthomendoza fallax	1994	United States, California	F232001
	1997	United States, Minnesota	L3702
	1998	Mexico	L5880
	1998	United States, North Dakota	L45335
	1998	United States, Arizona	L10412
	1998	Mexico	L10413
	1998	United States, North Dakota	L11270
	2005	United States, South Dakota	F117308
	2006	United States, Nebraska	F143958
	2007	United States, South Dakota	F103672
Xanthomendoza fulva	1990	Sweden	L54374
	1990	Sweden	L54371
	1990	Sweden	L54373
	1990	Sweden	L54375
	1991	Germany	L19
	1992	Sweden	L54372
	1994	Sweden	F172081
	1996	Sweden	F362993
	1996	United States, Minnesota	F143962
	1997	Sweden	L41117
	1997	Sweden	L24445
	1997	Sweden	F162581
	1997	United States, Minnesota	L3685
	1998	Sweden	L29061
	1998	Sweden	L29563
	1998	United States, Wyoming	L11144
	2000	Sweden	L14905
	2000	Sweden	L17266
	2001	Sweden	L24416
	2002	Sweden	L59238
	2002	Sweden	L38823

(Continue)

Species	Year	Country	Herbarium code (S)
Xanthomendoza fulva	2003	United States, Wisconsin	F52582
	2003	Greenland	L59963
	2003	United States, New Jersey	L59810
	2003	Sweden	L43629
	2004	Sweden	L62179
	2005	Sweden	L67851
	2005	Sweden	F57544
	2006	Sweden	F61222
	2006	Sweden	F61229
	2006	United States, Oregon	F117310
	2007	Sweden	F76579
	2009	Sweden	F150308
	2010	Sweden	F179340
	2011	Finland	F205555
	2011	Sweden	F206718
	2011	Sweden	F206719
	2011	Sweden	F206717
	2012	Sweden	F235261
	2012	Sweden	F235087
	2015	Sweden	F286114
	2015	Sweden	F286133
Xanthomendoza galericulata	2006	United States, Washington	F144024
	2006	United States, Utah	F117311
Xanthomendoza hasseana	1998	United States, North Dakota	L5881
	2005	United States, South Dakota	F117312
Xanthomendoza mendozae	1997	United States, Arizona	L62132
	1998	United States, Montana	L11142
Xanthomendoza montana	1990	United States, Arizona	L60363
	1998	United States, Montana	L5882
	1998	United States, Montana	L11142
	2005	United States, Arizona	F60760
	2006	United States, Wyoming	F143941
Xanthomendoza poeltii	1995	Sweden	L1492
	1995	Sweden	L1493
Xanthomendoza trachyphylla	1994	United States, Arizona	F107809
	1994	United States, Nebraska	F107817
	1994	United States, Nebraska	F107820
	1997	United States, Nebraska	L5871
	1998	United States, North Dakota	L45338
	2006	United States, Wyoming	F117333
Xanthomendoza ulophyllodes	2003	United States, Wisconsin	L59441
	2004	United States, Wisconsin	F52098
	2005	United States, South Dakota	F117313

Table S2. Sequences used in phylogenetic analyses (sequences newly produced for this study are highlighted in bold font) and GenBank accession numbers. Voucher information is given for newly produced sequences.

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Basidiomycota				
Agaricomycotina				
Agaricomycetes				
Agaricales				
Agaricus bisporus	FJ641895	AM930981	DQ071710	
Coprinopsis cinerea	JN939901	FJ904826	KM272007	
Coprinus comatus	AY665772	AY854066	AY635772	
Laccaria bicolor	JN939989	DQ179123	DQ071702	
Lycoperdon perlatum	KY418911	LN714568	KY418842	
Mycena amicta	DQ457693	JF908394	KC691216	
Schizophyllum comune	MG569497	LC312146	DQ071725	
Atheliales				
Athelia rolfsii	AY665774	DQ484060	AY635773	
Auriculariales				
Auricularia sp.	DQ234542	DQ200918	AY634277	
Boletales				
Boletinellus merulioides	AY662668	DQ200922	AY684153	
Coniophora puteana	AJ488581	AB592334	GU187578	
Serpula lacrymans	GU187649	AF335274	AB733419	
Corticiales				
Vuilleminia comedens	AF518594	DQ398959	AF518666	
Geastrales				
Geastrum campestre	JN940194	KT985474	JN939555	
Geastrum striatum	JN940211	JN845124	JN939557	
Sphaerobolus stellatus	AF026618	KM586165	AF393077	
Gloeophyllales				
Gloeophyllum sepiarium	AJ540308	AJ344141	AJ583432	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Gomphales				
Ramaria rubella	AY707095	AY854078	AY645057	
Hymenochaetales				
Fomitiporia mediterranea	AY662664	AF515586	AY621000	
Hydnochaete duportii	AY662669	DQ404386	AY635770	
Hydnoporia corrugata	AF518579	JN230419	JQ279621	
Phellinus igniarius	AF026614	MF782801	JX484011	
Phallales				
Phallus hadriani	AY771601	DQ404385	AY885165	
Polyporales				
Fomitopsis pinicola	AY705967	AY854083	AY684164	
Russulales				
Stereum hirsitum	AF026588	AF218400	AF393078	
Sebacinales				
Serendipita indica	AY293147	DQ411527	AY293202	
Thelephorales				
Polyozellus multiplex	AY771600	DQ411528	AY634275	
Trechisporales				
Trechispora alnicola	AY657012	DQ411529	AY635768	
Dacrymycetes				
Dacrymycetales				
Calocera cornea	AY771610	AY789083	AY701526	
Dacrymyces stillatus	L22258	MH856306	FJ644516	
Dacrymyces sp.	AY705954	DQ205684	AY691892	
Dacryopinax elegans	AB712513	AB712471	AB712433	
Dacryopinax spathularia	AY771603	AY854070	AY701525	
Guepiniopsis buccina	DQ667157	DQ206986	AY745711	
Tremellomycetes				
Cystofilobasidiales				
Cystofilobasidium capitatum	D12805	F444300	AF075466	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Cystofilobasidiales				
Itersonilia perplexans	DQ667162	DQ667163	DQ667161	
Krasilnikovozyma huempii	AB032636	AF444322	AF189844	
Mrakia frigida	DQ831017	DQ831018	DQ831016	
Phaffia rhodozyma	NG_061157	NR_111043	NG_042361	
Tausonia pamirica	NG_061158	NR_154490	NG_057760	
Udeniomyces pyricola	D31659	AF444402	AF075507	
Xanthophillomyces sp.	D31656	AF444488	AF444721	
Filobasidiales				
Filobasidium floriforme	D13460	AF190007	AF075498	
Goffeauzyma gastrica	AB032633	AF145323	AF137600	
Naganishia albida	AB032616	AF145321	AF075474	
Piskurozyma cylindrica	NG_061150	AF444360	NG_057656	
Piskurozyma taiwanensis	AB072234	KY104664	AB079065	
Solicoccozyma aeria	AB032614	AF145324	AF075486	
Holtermanniales				
Holtermannia corniformis	*AF053718*	*AF410472*	*AF189843*	
Holtermanniella nyarrowii	*KF036643*	*AY006481*	*AY006480*	
Holtermanniella takashimae	*NG_061154*	*NR_137721*	*NG_060626*	
Tremellales				
Apiotrichum porosum	NG_060985	NR_073209	-	
Bandonia marina	NG_060984	NR_144778	NG_058617	
Biatoropsis usnearum	JN043542	JN053488	JN043594	
Bullera alba	-	NR_111083	NG_042387	
Bullera oryzae	D31652	AF314986	AF075511	
Bullera unica	D78330	AF444441	AF075524	
Carcinomyces effibulatus	JN043554	JN053500	JN043606	
Carcinomyces polyporinus	JN043555	JN053501	JN043607	
Cryptococcus neoformans	BR000310	BR000310	-	
Cryptotrichosporon anacardii	DQ242635	AY549986	AY550003	
Cutaneotrichosporon cutaneum	AB001753	AF444325	AF075483	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Tremellales				
Derxomyces mrakii	D78325	AB022932	AB118871	
Dimennazyma cisti-albidi	NG_061149	NR_144841	NG_059152	
Dioszegia hungarica	AB032638	AF444379	AF075503	
Effuseotrichosporon vanderwaltii	-	NR_153975	KY107670	
Fellomyces polyborus	KF036676	NR_073238	NG_057660	
Fibulobasidium inconspicuum	JN043552	JN053498	JN043604	
Fonsecazyma mujuensis	NG_061151	NR_137814	NG_058290	
Genolevuria amylolytica	NG_061148	NR_137810	NG_057728	
Haglerozyma chiarellii	KF036711	GQ338074	EU030272	
Hannaella sinensis	NG_061040	NR_155150	NG_042362	
Kockovaella sichuanensis	AB032662	AF444461	AF189879	
Kockovaella thailandica	NG_061038	NR_077103	NG_057650	
Kwoniella mangrovensis	NG_061155	AF444646	NG_042391	
Naematelia microspora	-	AF042435	AF042253	
Nielozyma melastomae	AB118872	NR_154220	NR_154220	
Papiliotrema bandonii	GU327539	GU327539	AF416642	
Phaeotremella foliacea	NG_062976	AF042415	AF042233	
Pseudotremella moriformis	U00977	AF042426	AF042244	
Rhynchogastrema coronata	-	LN870267	KJ170152	
Saitozyma flava	AB032629	AF444338	AF075497	
Sirobasidium japonicum	LC203421	LC203422	LC016573	
Sirobasidium magnum	JN043551	NR_154446	NG_057646	
Sugitazyma miyagiana	D31651	NR_073237	NG_058409	
Syzygospora alba	JN043563	JN053509	JN043616	
Tetragoniomyces uliginosus	-	-	JN043621	
Tremella caloplacae* AM556 on *Calogaya decipiens	**OQ152058**	**OQ192933**	**OQ176383**	Sweden (Vadstena) Millanes 850 (S-F253110)
Tremella caloplacae* SF234 on *Calogaya pusilla	-	**OQ192934**	**OQ176384**	Sweden (Öland) Freire-Rallo S33 (S)
Tremella caloplacae* SF237 on *Calogaya pusilla	-	**OQ192935**	**OQ176385**	Sweden (Öland) Freire-Rallo S34 (S)
Tremella caloplacae* SF263 on *Calogaya pusilla	**OQ152059**	**OQ192936**	**OQ176386**	Sweden (Öland) Freire-Rallo S44 (S)
Tremella caloplacae* SF269 on *Calogaya pusilla	**OQ152060**	**OQ192937**	**OQ176387**	Sweden (Öland) Freire-Rallo S45 (S)

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Tremellales				
Tremella caloplacae* SF162 on *Leproplaca xantholyta	-	**OQ192938**	-	Poland (Kocieliska Valley) Wetmore 77310 (S-L59446)
Tremella caloplacae* AM315E on *Rusavskia elegans	-	**OQ192939**	**OQ176388**	Sweden (Abisko) Millanes 908 (S-F255314)
Tremella caloplacae* AM349E on *Rusavskia elegans	-	**OQ192940**	**OQ176389**	Norway (Jotunheimen) Millanes 808 (S-F255312)
Tremella caloplacae* AM443 on *Rusavskia elegans	-	**OQ192941**	**OQ176390**	Norway (Varanger) Millanes 1085 (S)
Tremella caloplacae* AM444 on *Rusavskia elegans	-	**OQ192942**	**OQ176391**	Norway (Varanger) Millanes 1113 (S)
Tremella caloplacae* AM554 on *Rusavskia elegans	**OQ152061**	**OQ192943**	**OQ176392**	Sweden (Abisko) Millanes 904 (S-F255313)
Tremella caloplacae* SF161 on *Rusavskia elegans	-	**OQ192944**	-	Sweden (Ångermanland) Odelvik, Hedenäs & Rönblom 14-453 (S-F253739)
Tremella caloplacae* AM32 on *Rusavskia sorediata	-	**OQ192945**	**OQ176393**	Greenland (Qagssiarsuk) Kukwa 4385a (S-F102490)
Tremella caloplacae* AM642 on *Rusavskia sorediata	-	**OQ192946**	**OQ176394**	Canada (British Columbia) Goward 01-608 (UBC)
Tremella caloplacae* SF148 on *Variospora aurantia	**OQ152062**	**OQ192947**	**OQ176395**	Ukraine (Kamianets-Podilskyi) Kukwa 1851 (S-F117262)
Tremella caloplacae* AM780 on *Variospora dolomiticola	-	**OQ192948**	**OQ176396**	Crete Type locality (Greece) Diederich 18575 (herb. Diederich)
Tremella caloplacae* AM782 on *Variospora flavescens	-	**OQ192949**	**OQ176397**	Luxembourg Diederich 18559 (herb. Diederich)
Tremella caloplacae* SF149 on *Variospora flavescens	-	**OQ192950**	**OQ176398**	Sweden (Gotland) Westberg, Košuthová, Prieto GTL24 (S-F268002)
Tremella caloplacae* SF243 on *Variospora flavescens	-	**OQ192951**	**OQ176399**	Sweden (Öland) Freire-Rallo S37 (S)
Tremella caloplacae* SF254 on *Variospora flavescens	-	**OQ192952**	**OQ176400**	Sweden (Öland) Freire-Rallo S41 (S)
Tremella caloplacae* SF257 on *Variospora flavescens	-	**OQ192953**	**OQ176401**	Sweden (Öland) Freire-Rallo S42 (S)
Tremella caloplacae* SF260 on *Variospora flavescens	-	**OQ192954**	**OQ176402**	Sweden (Öland) Freire-Rallo S43 (S)
Tremella caloplacae* SF266 on *Variospora flavescens	-	**OQ192955**	**OQ176403**	Sweden (Öland) Freire-Rallo S45 (S)
Tremella caloplacae* SF272 on *Variospora flavescens	-	**OQ192956**	**OQ176404**	Sweden (Öland) Freire-Rallo S46 (S)
Tremella caloplacae* SF279 on *Variospora flavescens	-	**OQ192957**	**OQ176405**	Sweden (Öland) Freire-Rallo S50 (S)
Tremella caloplacae* SF150 on *Variospora thallincola	-	**OQ192958**	**OQ176406**	UK (England, South Devon) Kärnefelt 970901 (S-L5870)
***Tremella caloplacae* SF302 on *Variospora* sp.**	-	**OQ192959**	**OQ176407**	Spain (Guadalajara) Millanes & Freire-Rallo 1370 (S)
***Tremella caloplacae* SF305 on *Variospora* sp.**	**OQ152063**	**OQ192960**	**OQ176408**	Spain (Guadalajara) Millanes & Freire-Rallo 1371 (S)
Tremella caloplacae* AM558 on *Xanthocarpia lactea	-	**OQ192961**	**OQ176409**	Austria (Mühlgraben) Hafellner & Maurer 6-feb-1990 (GZU)
***Tremella caloplacae* SF155 on *Xanthocarpia* sp.**	-	**OQ192962**	**OQ176410**	Estonia (Harjumaa) Thor 8202 (S-F70137)
***Tremella caloplacae* SF156 on *Xanthocarpia* sp.**	-	**OQ192963**	**OQ176411**	Estonia (Harjumaa) Thor 8202 (S-F70137)
***Tremella caloplacae* SF291 on *Xanthocarpia* sp.**	-	**OQ192964**	**OQ176412**	Spain (Guadalajara) Millanes & Freire-Rallo 1365 (S)
***Tremella caloplacae* SF292 on *Xanthocarpia* sp.**	**OQ152064**	**OQ192965**	**OQ176413**	Spain (Guadalajara) Millanes & Freire-Rallo 1365 (S)
Tremella caloplacae* AM310 on *Xanthoria parietina	-	**OQ192966**	**OQ176414**	Austria (Ramsau) Obermayer 25xi2011 (GZU)

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Tremellales				
Tremella caloplacae* AM311 on *Xanthoria parietina	-	**OQ192967**	**OQ176415**	Austria (Graz) Fleischhacker 11010 (GZU)
Tremella caloplacae* AM352E on *Xanthoria parietina	-	**OQ192968**	**OQ176416**	Spain (Segovia) Zamora 8-v-2010 (S)
Tremella caloplacae* AM353E on *Xanthoria parietina	-	**OQ192969**	**OQ176417**	Spain (Madrid) Zamora 18-xii-2011 (S)
Tremella caloplacae* AM446 on *Xanthoria parietina	-	**OQ192970**	**OQ176418**	Spain (Monfragüe) Millanes 1192 (S)
Tremella caloplacae* AM447 on *Xanthoria parietina	-	**OQ192971**	**OQ176419**	Spain (Monfragüe) Millanes 1197 (S)
Tremella caloplacae* AM553 on *Xanthoria parietina	-	**OQ192972**	**OQ176420**	Sweden (Vadstena) Millanes 849 (S-F253109)
Tremella caloplacae* AM555 on *Xanthoria parietina	-	**OQ192973**	**OQ176421**	Sweden (Vadstena) Millanes 833 (S-F253105)
Tremella caloplacae* AM559 on *Xanthoria parietina	-	**OQ192974**	**OQ176422**	Austria (Ramsau) Obermayer 12148a1 (GZU)
Tremella caloplacae* AM560 on *Xanthoria parietina	**OQ152065**	**OQ192975**	**OQ176423**	Austria (Ramsau) Obermayer 12147 (GZU)
Tremella caloplacae* AM561 on *Xanthoria parietina	-	**OQ192976**	**OQ176424**	Austria (Graz) Hafellner 1-vii-2010 (GZU)
Tremella caloplacae* AM562 on *Xanthoria parietina	-	**OQ192977**	**OQ176425**	Austria (Ramsau) Obermayer 12148a2 (GZU)
Tremella caloplacae* AM563 on *Xanthoria parietina	-	**OQ192978**	**OQ176426**	Austria (Ramsau) Obermayer 12446 (GZU)
Tremella caloplacae* AM564 on *Xanthoria parietina	-	**OQ192979**	**OQ176427**	Austria (Saualpe) Hafellner 25-xii-2010 (GZU)
Tremella caloplacae* AM565 on *Xanthoria parietina	-	**OQ192980**	**OQ176428**	Slovenia (Bovec) (GZU)
Tremella caloplacae* AM638 on *Xanthoria parietina	-	**OQ192981**	**OQ176429**	Luxembourg Diederich 17385 (herb. Diederich)
Tremella caloplacae* AM639 on *Xanthoria parietina	-	**OQ192982**	**OQ176430**	Luxembourg Diederich 17455 (herb. Diederich)
Tremella caloplacae* AM640 on *Xanthoria parietina	-	**OQ192983**	**OQ176431**	Luxembourg Diederich 17473 (herb. Diederich)
Tremella caloplacae* AM641 on *Xanthoria parietina	-	**OQ192984**	**OQ176432**	Luxembourg Diederich 17740 (herb. Diederich)
Tremella candelariellae	JN043526	JN053470	JN043575	
Tremella cetrariicola	JN043544	JN053490	JN043596	
Tremella cladoniae	JN043532	JN053477	JN043583	
Tremella dendrographae	-	JN053471	JN043576	
Tremella everniae	JN043547	JN053493	JN043599	
Tremella giraffa	JN043537	JN053483	JN043589	
Tremella haematommatis	-	JN053510	JN043617	
Tremella indecorata	JN043557	JN053503	JN043610	
Tremella lobariacearum	JN043529	JN053474	JN043580	
Tremella mesenterica	JN043520	JN053463	JN043568	
Tremella parmeliarum	JN043565	JN053511	JN043618	
Trichosporon ovoides	AB001765	AF444439	AF075523	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Tremellales				
Trimorphomyces papilonaceus	-	AF444483	AF075491	
Vishniacozyma carnescens	NG_060988	NR_130695	NG_058430	
Vishniacozyma dimennae	AB032627	EU266559	AF075489	
Ustilaginomycotina				
Malasseziomycetes				
Malasseziales				
Malassezia furfur	KF706457	NR_149347	NG_057730	
Malassezia pachydermatis	DQ457640	DQ411532	AY745724	
Ustilaginomycetes				
Ustilaginales				
Ustilago tritici	DQ846895	DQ846894	DQ094784	
Pucciniomycotina				
Pucciniomycetes				
Platygloeales				
Eocronartium muscicola	AY123323	-	AF014825	
Insolibasidium deformans	AY123292	MH790966	AF522169	
Platygloea disciformis	DQ234563	DQ234556	AY629314	
Cystobasidiomycetes				
Cystobasidiales				
Cystobasidium sp. "nymphaeae"	AB055189	AB055198	AB055194	
Ascomycota				
Arthoniomycetes				
Arthoniales				
Dendrographa decolorans	AY548809	AY548808	NG_027622	
Lecanactis abietina	AY548805	AY548804	AY548812	
Tylophoron hibernicum	-	-	JF295084	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Coniocybomycetes				
Coniocybales				
Chaenotheca brachypoda	-	AF297963	JX000086	
Chaenotheca furfuracea	JX000068	JX000101	JX000087	
Chaenotheca gracilenta	JX000067	JX000100	JX000084	
Chaenotheca trichialis	JX000069	JX000102	JX000085	
Sclerophora coniophaea	JX000079	-	JX000094	
Sclerophora farinacea	JX000078	JX000113	JX000095	
Dothideomycetes				
Capnodiales				
Capnodium coffeae	DQ247808	DQ491515	GU214400	
Capnodium salicinum	DQ677997	MH855469	MH866941	
Dothideales				
Dothidea insculpta	DQ247810	AF027764	DQ247802	
Myriangiales				
Myriangium duriaei	AF242266	MH855793	AY016365	
Pleosporales				
Parastagonospora nodorum	KY090706	KF251185	KF251688	
Eurotiomycetes				
Chaetothyriales				
Capronia munkii	EF413603	AF050250	EF413604	
Exophiala nigra	FJ358312	EF551550	FJ358244	
Coryneliales				
Caliciopsis orientalis	DQ471039	KP881690	DQ470987	
Elaphomycetales				
Pseudotulostoma japonica	AB161196	-	AB161194	
Eurotiales				
Aspergillus fumigatus	KM103660	MN638754	MH868658	
Aspergillus nidulans	NG_064803	AF455505	KP172159	
Aspergillus niger	NG_065763	NR_111348	NG_055744	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Eurotiales				
Chromocleista malachitea	FJ358346	KC773838	FJ358281	
Eupenicillium javanicum	EF413620	GU981614	EF413621	
Monascus purpureus	DQ782881	DQ782847	DQ782908	
Mycocaliciales				
Chaenothecopsis viridialba	-	JX000103	AY853365	
Mycocalicium subtile	JX000072	AF225445	AY853379	
Sphinctrina turbinata	EF413631	AY795877	EF413632	
Onygenales				
Arachniotus littoralis	FJ358340	AB566293	FJ358272	
Onygena corvina	FJ358352	MH857958	FJ358287	
Spiromastix warcupii	DQ782882	DQ782848	DQ782909	
Pyrenulales				
Pyrenula reebiae	AY641001	DQ782845	AY640962	
Pyrgillus javanicus	DQ823110	DQ826741	DQ823103	
Verrucariales				
Catapyrenium daedaleum	EF689830	JX000099	EF643748	
Verrucaria muralis	EF689878	EU010261	EF689878	
Geoglossomycetes				
Geoglossales				
Geoglossum nigritum	AY544694	DQ491490	AY544650	
Trichoglossum hirsutum	AY544697	DQ491494	AY544653	
Lecanoromycetes				
Agyriales				
Agyrium rufum	KR017244	JX000097	EF581826	
Arctomiales				
Arctomia delicatula	KR017255	-	AY853355	
Arctomia teretiuscula	-	-	DQ007346	
Baeomycetales				
Baeomyces rufus	AF113718	AF448457	JX000080	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Caliciales				
Anaptychia palmatula	DQ883792	HQ650702	DQ883801	
Buellia aethalea	AJ549801	JX000098	JX000081	
Buellia disciformis	AF241543	AY143392	JX000082	
Calicium salicinum	KF157970	KX512919	KX512861	
Calicium tigillare	AF241545	JX000104	JX000088	
Calicium viride	-	HQ650703	AY340538	
Pyxine sorediata	DQ973012	JX000111	JX000093	
Texosporium sancti-jacobi	AF085472	KX512941	JX000096	
Lecanorales				
Alectoria ochroleuca	DQ983483	HQ650597	DQ986801	
Alectoria sarmentosa	AF140233	DQ979998	DQ899290	
Anzia colpodes	DQ899293	DQ980000	DQ923651	
Calycidium cuneatum	-	JX000114	JX000083	
Canoparmelia caroliniana	AY584658	DQ782833	AY584634	
Lecanora hybocarpa	DQ782883	DQ782849	DQ782910	
Lecanora paramerae	-	EF105413	EF105422	
Lepraria lobificans	DQ986733	HQ650623	DQ986768	
Mycoblastus sanguinarius	DQ782879	MK811665	KJ766602	
Parmelia saxatilis	AF117985	AF058037	AY300849	
Parmelia sulcata	-	GU994574	GU994621	
Pseudevernia furfuracea	AY548817	AY611112	AY607826	
Sphaerophorus globosus	AF117983	HQ650622	DQ986767	
Lecideales				
Lecidea fuscoatra	DQ912310	HQ650707	DQ912332	
Lecidea silaceae	DQ986723	HQ650629	AY756340	
Ostropales				
Diploschistes scruposus	AF279388	HQ650716	AF279389	
Graphis scripta	AF038878	AF229195	AY853370	
Gyalecta ulmi	AF088237	HQ650713	AF465463	
Nadvornikia hawaiiensis	-	-	HQ639659	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Ostropales				
Schistophoron tenue	-	JX000112	EU544932	
Schizoxylon albescens	JX000077	HQ287353	DQ401144	
Ostroporales				
Acarosporina microspora	AY584667	DQ782834	AY584643	
Peltigerales				
Lobaria pulmonaria	MH887509	HM448799	AY340548	
Peltigera aphthosa	AY424225	AY424225	AF286759	
Pertusariales				
Aspicilia caesiocinerea	DQ986736	HQ650636	DQ780303	
Aspicilia cinerea	DQ986735	HQ650637	DQ780304	
Coccotrema cucurbitula	AF274114	AF329162	AF274092	
Coccotrema pocillarium	AF274113	AF329167	AF274093	
Dibaeis baeomyces	AF113713	DQ782844	AF279385	
Gyalectaria gyalectoides	-	-	GU980983	
Gyalectaria jamesii	-	-	GU980984	
Icmadophila ericetorum	DQ883704	MK812230	DQ883694	
Lepra amara	AF356682	HQ650677	AF274101	
Lepra corallina	JX000074	FR799261	AY300850	
Lepra ophthalmiza	JX000076	KU894635	AY568006	
Lepra scaberula	AF274105	-	AF274099	
Lepra subventosa	-	-	AY300854	
Lobothallia radiosa	-	JF703124	DQ780306	
Microcalicium ahlneri	JX000070	JX000108	-	
Microcalicium arenarium	-	JX000107	JX000091	
Microcalicium disseminatum	JX000071	-	JX000092	
Ochrolechia parella	AF274109	AF332123	AF274097	
Ochrolechia subpallescens	-	-	GU980985	
Pertusaria coccodes	-	MN387089	AF279295	
Pertusaria pertusa	AY779282	AF332127	AF279300	
Thamnolia vermicularis	AF085472	MF149097	AY853395	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Pertusariales				
Varicellaria hemisphaerica	DQ902340	HQ650676	AF381556	
Varicellaria lactea	-	-	AF381557	
Varicellaria velata	JX000075	JX000109	AY300855	
Rhizocarpales				
Rhizocarpon sphaerosporum	-	-	AY853390	
Sporastatia testudinea	AY641009	-	AY640969	
Teloschistales				
***Calogaya decipiens* AM556**	-	**OQ249812**	**OQ192200**	Sweden (Vadstena) Millanes 850 (S-F253110)
Calogaya decipiens	AJ535280	-	-	
***Calogaya pusilla* SF234**	-	**OQ249813**	**OQ192201**	Sweden (Öland) Freire-Rallo S33 (S)
***Calogaya pusilla* SF237**	-	**OQ249814**	**OQ192202**	Sweden (Öland) Freire-Rallo S34 (S)
***Calogaya pusilla* SF263**	-	**OQ249815**	**OQ192203**	Sweden (Öland) Freire-Rallo S44 (S)
***Calogaya pusilla* SF269**	-	-	**OQ192204**	Sweden (Öland) Freire-Rallo S45 (S)
Leproplaca xantholyta	-	JQ301670	-	
***Leproplaca xantholyta* SF162**	-	**OQ249816**	**OQ192205**	Poland (Kocieliska Valley) Wetmore 77310 (S-L59446)
***Rusavskia elegans* AM315E**	-	**OQ249817**	**OQ192206**	Sweden (Abisko) Millanes 908 (S-F255314)
***Rusavskia elegans* AM349E**	-	**OQ249818**	**OQ192207**	Norway (Jotunheimen) Millanes 808 (S-F255312)
***Rusavskia elegans* AM443**	-	**OQ249819**	**OQ192208**	Norway (Varanger) Millanes 1085 (S)
***Rusavskia elegans* AM444**	-	**OQ249820**	**OQ192209**	Norway (Varanger) Millanes 1113 (S)
***Rusavskia elegans* AM554**	-	**OQ249821**	**OQ192210**	Sweden (Abisko) Millanes 904 (S-F255313)
Rusavskia elegans	JQ301640	-	-	
***Rusavskia elegans* SF161**	-	**OQ249822**	**OQ192211**	Sweden (Ångermanland) Odelvik, Hedenäs & Rönblom 14-453 (S-F253739)
***Rusavskia sorediata* AM32**	-	**OQ249823**	**OQ192212**	Greenland (Qagssiarsuk) Kukwa 4385a (S-F102490)
***Rusavskia sorediata* AM642**	-	**OQ249824**	**OQ192213**	Canada (British Columbia) Goward 01-608 (UBC)
Rusavskia sorediata	AM495020	-	-	
Variospora aurantia	-	KC179470	KC179261	
***Variospora flavescens* SF149**	-	**OQ249825**	-	Sweden (Gotland) Westberg, Košuthová, Prieto GTL24 (S-F268002)
***Variospora flavescens* SF243**	-	**OQ249826**	-	Sweden (Öland) Freire-Rallo S37 (S)
***Variospora flavescens* SF254**	-	**OQ249827**	**OQ192214**	Sweden (Öland) Freire-Rallo S41 (S)

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Teloschistales				
***Variospora flavescens* SF257**	-	**OQ249828**	**OQ192215**	Sweden (Öland) Freire-Rallo S42 (S)
***Variospora flavescens* SF260**	-	**OQ249829**	**OQ192216**	Sweden (Öland) Freire-Rallo S43 (S)
***Variospora flavescens* SF266**	-	**OQ249830**	**OQ192217**	Sweden (Öland) Freire-Rallo S45 (S)
***Variospora flavescens* SF272**	-	**OQ249831**	**OQ192218**	Sweden (Öland) Freire-Rallo S46 (S)
***Variospora flavescens* SF279**	-	**OQ249832**	**OQ192219**	Sweden (Öland) Freire-Rallo S50 (S)
Variospora thallincola	JQ301620	JQ301667	JQ301563	
***Variospora* sp. SF302**	-	**OQ249833**	**OQ192220**	Spain (Guadalajara) Millanes & Freire-Rallo 1370 (S)
***Variospora* sp. SF305**	-	**OQ249834**	**OQ192221**	Spain (Guadalajara) Millanes & Freire-Rallo 1371 (S)
Xanthocarpia lactea	-	HQ699644	-	
***Xanthocarpia lactea* AM558**	-	**OQ249835**	-	Austria (Mühlgraben) Hafellner & Maurer 6-feb-1990 (GZU)
Xanthocarpia marmorata	-	MN512254	KT291553	
***Xanthocarpia* sp. SF155**	-	**OQ249836**	**OQ192222**	Estonia (Harjumaa) Thor 8202 (S-F70137)
***Xanthocarpia* sp. SF291**	-	**OQ249837**	**OQ192223**	Spain (Guadalajara) Millanes & Freire-Rallo 1365 (S)
***Xanthocarpia* sp. SF292**	-	**OQ249838**	**OQ192224**	Spain (Guadalajara) Millanes & Freire-Rallo 1365 (S)
***Xanthoria parietina* AM310**	-	**OQ249839**	**OQ192225**	Austria (Ramsau) Obermayer 25xi2011 (GZU)
***Xanthoria parietina* AM311**	-	**OQ249840**	**OQ192226**	Austria (Graz) Fleischhacker 11010 (GZU)
Xanthoria parietina	JQ301641	-	-	
***Xanthoria parietina* AM352E**	-	**OQ249841**	**OQ192227**	Spain (Segovia) Zamora 8-v-2010 (S)
***Xanthoria parietina* AM353E**	-	**OQ249842**	**OQ192228**	Spain (Madrid) Zamora 18-xii-2011 (S)
***Xanthoria parietina* AM446**	-	**OQ249843**	**OQ192229**	Spain (Monfragüe) Millanes 1192 (S)
***Xanthoria parietina* AM447**	-	**OQ249844**	**OQ192230**	Spain (Monfragüe) Millanes 1197 (S)
***Xanthoria parietina* AM553**	-	**OQ249845**	**OQ192231**	Sweden (Vadstena) Millanes 849 (S-F253109)
***Xanthoria parietina* AM555**	-	**OQ249846**	**OQ192232**	Sweden (Vadstena) Millanes 833 (S-F253105)
***Xanthoria parietina* AM559**	-	**OQ249847**	**OQ192233**	Austria (Ramsau) Obermayer 12148a1 (GZU)
***Xanthoria parietina* AM560**	-	**OQ249848**	**OQ192234**	Austria (Ramsau) Obermayer 12147 (GZU)
Xanthoria parietina	JQ301641	-	-	
***Xanthoria parietina* AM561**		**OQ249849**	**OQ192235**	Austria (Graz) Hafellner 1-vii-2010 (GZU)
***Xanthoria parietina* AM562**	-	**OQ249850**	**OQ192236**	Austria (Ramsau) Obermayer 12148a2 (GZU)
***Xanthoria parietina* AM563**	-	**OQ249851**	**OQ192237**	Austria (Ramsau) Obermayer 12446 (GZU)
***Xanthoria parietina* AM564**	-	**OQ249852**	**OQ192238**	Austria (Saualpe) Hafellner 25-xii-2010 (GZU)

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Teloschistales				
***Xanthoria parietina* AM565**	-	**OQ249853**	**OQ192239**	Slovenia (Bovec) (GZU)
***Xanthoria parietina* AM638**	-	**OQ249854**	**OQ192240**	Luxembourg Diederich 17385 (herb. Diederich)
***Xanthoria parietina* AM639**	-	**OQ249855**	**OQ192241**	Luxembourg Diederich 17455 (herb. Diederich)
***Xanthoria parietina* AM640**	-	**OQ249856**	**OQ192242**	Luxembourg Diederich 17473 (herb. Diederich)
***Xanthoria parietina* AM641**	-	**OQ249857**	**OQ192243**	Luxembourg Diederich 17740 (herb. Diederich)
Trapeliales				
Trapelia placodioides	AF119500	AF274081	AF274103	
Lichinomycetes				
Lichinales				
Lempholemma polyanthes	AF356690	JX000106	JX000090	
Peltula auriculata	DQ832332	DQ832329	DQ832330	
Peltula umbilicata	DQ782887	DQ832333	DQ832334	
Orbiliomycetes				
Orbiliales				
Orbilia auricolor	DQ471001	DQ491512	DQ470953	
Orbilia vinosa	DQ471000	DQ491511	DQ470952	
Pezizomycetes				
Pezizales				
Ascobolus crenulatus	AY544721	DQ491504	AY544678	
Peziza michelii	DQ646545	DQ200839	AY500549	
Disciotis venosa	AY544711	DQ491503	AY544667	
Pyricularia grisea	AF277123	MH864859	MH877665	
Sordariomycetes				
Diaphorthales				
Cryphonectria parasitica	L42441	KC851951	NG_027589	
Diaporthe eres	DQ471015	EU805539	AF408350	
Gnomonia gnomon	DQ471019	EU254780	EU255092	
Hypocreales				
Trichoderma reesei	KY100257	KY792626	KU729195	

(Continue)

Taxon names	nuSSU	ITS	nuLSU	Specimen data
Lulworthiales				
Nectria cinnabarina	JX000073	HM484710	AF193237	
Lindra thalassiae	DQ470994	DQ491508	DQ470947	
Sordariales				
Chaetomium globosum	KM096357	DQ336707	AY346272	
Neurospora crassa	XR_898133	AF388914	XR_898136	
Podospora anserina	MG708375	AF388930	FR774293	
Sordaria fimicola	X69851	MH856730	MH869927	
Xylariales				
Xylaria acuta	JQ419764	JQ862676	JQ862609	
Xylaria hypoxylon	AY544692	DQ491487	AY544648	
Saccharomycotina				
Saccharomycetes				
Saccharomycetales				
Scheffersomyces stipitis	NG_063362	NR_111693	NG_042637	
Taphrinomycotina				
Schizosaccharomycetes				
Schizosaccharomycetales				
Schizosaccharomyces pombe	X54866	MH595429	Z19136	
Taphrinomycetes				
Taphrinales				
Taphrina deformans	DQ471024	AF492093	DQ470973	

Table S3. Primers newly designed for this study.

Primer name	Nucleotide sequence
nSSU	
Tc2SSU1-3_XpXcsp	5' TTCATAGACCCGGAGGTCGAAATC 3'
Tc2SSU1-3_Cgp	5' TTTGTAGACCCAGAGGTCAAAATC 3'
Tc2SSU1-3_Vsp1	5' TTTATAGACCGAGAGGTCAAAATC 3'
Tc2SSU1-3_Vsp2	5' TTTATAGACCCAGAGGTCAAAATC 3'
Tc3SSU1-3_Cgp	5' ATGTTTGTCATGAGGTAGACCCG 3'
Tc3SSU1-3_Vsp	5' ATGTTTGTTATAGGGTAGACCCG 3'
ITS	
TRMcal_R2	5' ATGCAAGTGCTATTCCCAC 3'
nLSU	
TelLSU1-5	5' TAGCGCACAAGTAGAGTGATC 3'
TRMLSU_1F	5' ACAAACATCAGTGTAATGAACGTC 3'

Table S4. Information on fossils and settings used for the molecular clock analyses. Letters identify the node position of each fossil in the dated trees of the Basidiomycota and the Ascomycota (Fig. S1 and Fig. S2 respectively). The minimum age of fossils B, C and E were set following Lutzoni et al., 2018. † Fossils used to calibrate the dating analyses of the Basidiomycota; ‡ Fossil used to calibrate both Ascomycota analyses: the one to construct the starting tree and the final dating analyses; § Fossils used to calibrate only the final dating analyses of the Ascomycota.

	Fossil	Refference	Node	Geological age, chart	Prior distribution	Offset	Mean	Standard deviation
A	Fossil septate hyphae with clamp connections	Krings et al., 2011	Base of Basidiomycota	Late Visean, Mississippian	Exponential	330.00	-	-
B	*Quatsinoporites cranhamii* †	Smith et al., 2004	Base of Hymenochaetaceae	Early Cretaceous, Barremian	Exponential	124.00	-	-
C	*Archaeomarasmius lagetti* †	Hibbett et al., 1997	Base of Agaricales	Mid-Cretaceous, New Jersey amber	Exponential	88.30	-	-
D	Fossil ectomycorrhizal fungus †	LePage et al., 1997	Base of Boletales	Middle-Eocene Princeton chert, British Columbia	Exponential	40.20	-	-
E	*Coprinites dominicana* †	Poinar and Singer 1990	Divergence Coprinaceae-Agaricaceae	Lower Oligocene to upper Eocene, Dominican amber	Exponential	15.97	-	-
F	*Palaeopyrenomycites devonicus* ‡	Taylor et al., 2005	Base of Pezizomycetes	low Devonian	Normal	-	400.00	1.00
G	Fossil Metacapnodiaceae sooty molds §	Schmidt et al., 2014	Base of Capnodiales	Early Cretaceous, Charentes amber	Normal	-	100.00	1.00
H	Sooty mold fossil similar to *Curvularia* spores §	Schmidt et al., 2010	Dotideomycetes-Artoniomycetes	Upper Cretaceous, Cenomanian	Normal	-	100.00	1.00
I	*Colletotrichum* sp. §	Kar et al., 2004	Base of Sordariomycetes	Upper Cretaceous	Normal	-	65.20	1.00
J	*Calicium* sp. §	Rikkinen 2003	Base of *Calicium*	Eocene Baltic amber	Normal	-	35.00	1.00
K	*Chaenotheca* sp. §	Rikkinen 2003	Base of Coniocybomycetes	Eocene Baltic amber	Normal	-	35.00	1.00
L	*Anzia electra* §	Rikkinen and Poinar 2002	Base of Parmeliaceae	Eocene Baltic amber	Normal	-	35.00	1.00
M	*Aspergillus collembolorum* §	Dörfelt and Schmidt 2005	Base of *Aspergillus*	Eocene Baltic amber	Normal	-	35.00	1.00
N	*Metacapnodium succinum* §	Rikkinen et al., 2003	Capnodiales-Dothideales	Eocene	Normal	-	33.80	1.00
O	*Xylaria* sp. §	Poinar and Poinar 1999	Base of Xylariales	Eocene-Miocene, Dominican amber	Normal	-	15.97	1.00
P	*Parmelia ambra* §	Poinar et al., 2000	Base of *Parmelia*	Miocene, Dominican amber	Normal	-	15.10	1.00

Table S5. Results of Jane v.4.0 cophylogenetic analyses. Each cost combination was tested for each species delimitation hypotheses presented in Fig. 3. *P*-values of the tests were <0.05.

	Cospeciation	Duplication	Duplication & Host switching	Loss	Failure to diverge	Total
Combination 1 GMYCs	4	0	1	3	0	5
Combination 1 GMYCm	5	0	1	3	0	5
Combination 1 PTP	11	1	4	1	0	10
Combination 1 ABGD	7	0	3	0	0	6
Combination 1 PCI	3	0	1	1	0	3
Combination 1 Adopted consensus	5	0	2	2	0	6
Combination 2 GMYCs	2	0	3	0	0	3
Combination 2 GMYCm	3	0	3	0	0	3
Combination 2 PTP	10	1	5	0	0	6
Combination 2 ABGD	7	0	3	0	0	3
Combination 2 PCI	2	0	2	0	0	2
Combination 2 Adopted consensus	3	0	4	0	0	4
Combination 3 GMYCs	0	0	5	0	0	5
Combination 3 GMYCm	0	0	6	0	0	6
Combination 3 PTP	0	1	15	0	0	16
Combination 3 ABGD	0	0	10	0	0	10
Combination 3 PCI	0	0	4	0	0	4
Combination 3 Adopted consensus	0	0	7	0	0	7
Combination 4 GMYCs	0	0	5	0	0	0
Combination 4 GMYCm	0	0	6	0	0	0
Combination 4 PTP	0	1	15	0	0	16
Combination 4 ABGD	0	0	10	0	0	0
Combination 4 PCI	0	0	4	0	0	0
Combination 4 Adopted consensus	0	0	7	0	0	0
Combination 5 GMYCs	0	0	5	0	0	5
Combination 5 GMYCm	0	0	6	0	0	6
Combination 5 PTP	0	1	15	0	0	16
Combination 5 ABGD	0	0	10	0	0	10
Combination 5 PCI	0	0	4	0	0	4
Combination 5 Adopted consensus	0	0	7	0	0	7
Combination 6 GMYCs	0	0	5	0	0	5
Combination 6 GMYCm	0	0	6	0	0	6
Combination 6 PTP	0	1	15	0	0	0
Combination 6 ABGD	0	0	10	0	0	10
Combination 6 PCI	0	0	4	0	0	4
Combination 6 Adopted consensus	0	0	7	0	0	7
Combination 7 GMYCs	0	0	5	0	0	5
Combination 7 GMYCm	0	0	6	0	0	6
Combination 7 PTP	0	1	15	0	0	16
Combination 7 ABGD	0	0	10	0	0	10
Combination 7 PCI	0	0	4	0	0	4
Combination 7 Adopted consensus	0	0	7	0	0	7
Combination 8 GMYCs	2	0	3	0	0	3
Combination 8 GMYCm	3	0	3	0	0	3
Combination 8 PTP	10	1	5	0	0	5
Combination 8 ABGD	7	0	3	0	0	3
Combination 8 PCI	0	0	2	0	0	2
Combination 8 Adopted consensus	3	0	4	0	0	4
Combination 9 GMYCs	2	0	3	0	0	8
Combination 9 GMYCm	3	0	3	0	0	9
Combination 9 PTP	10	1	5	0	0	21
Combination 9 ABGD	7	0	3	0	0	13
Combination 9 PCI	2	0	2	0	0	6
Combination 9 Adopted consensus	3	0	4	0	0	11

Chapter 2

Five new species in the *Tremella caloplacae* complex

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Abstract

Tremella caloplacae (Zahlbr.) Diederich is a species complex known to include at least nine different species. Here, we formally describe the new species *Tremella elegantis*, *T. nimisiana*, *T. parietinae*, *T. pusillae* and *T. sorediatae*. *Tremella elegantis* induces galls in the hymenium of *Rusavskia elegans* and forms 2-celled basidia, where cells rarely elongate and sometimes give the appearance of two immature, independent basidia. *Tremella nimisiana* has small basidiomata (less than 1 mm diam.), narrowly ellipsoid to pyriform 2-celled, occasionally clavate to subcylindrical 3-celled basidia, and grows in the hymenium of *Xanthocarpia* species. *Tremella parietinae* is characterized by the exclusive growth in the hymenium of *Xanthoria parietina*, the broadly fusiform to ellipsoid probasidia, and the subspherical, pyriform or ellipsoid 2(–3)-celled basidia. *Tremella pusillae* has ellipsoidal probasidia, 2(–3)-celled pyriform or ellipsoidal basidia that sometimes are constricted at the septum, and grows only on *Calogaya pusilla*. *Tremella sorediatae* is characterized by inducing galls on the thallus of *Rusavskia sorediata* and by pyriform to ellipsoid basidia that sometimes are constricted at the septum. Three species are not formally described and are left unnamed as *Tremella* sp. 13 on *Calogaya biatorina*, *Tremella* sp. 14 on *Calogaya decipiens* and *Tremella* sp. 15 on *Polycauliona* sp. *Tremella caloplacae* in the strict sense is re-circumscribed as a species confined to *Variospora* species.

Keywords: basidiomycetes; lichenicolous fungi; molecular phylogeny; species complex; taxonomy; *Tremellales*

Introduction

Tremella Pers. (*Tremellales*, *Agaricomycotina*) is a widespread genus with more than 300 species that grow associated with other fungi, including lichenized fungi. Lichenicolous *Tremella* species were neglected and poorly studied until the publication of the first monograph on lichenicolous heterobasidiomycetes by Diederich (1996). Since then, the number of formally described lichenicolous species has increased to 117, and we expect this number to continue increasing in the coming years (Diederich *et al*. 2022). Species delimitation is often complicated in this group due to the scarcity of morphological characters (Diederich 1996). Still, lichenicolous *Tremella* species are highly specific towards their hosts, usually growing on a single species or genus (Diederich *et al*. 2018), which has been useful for species circumscription (Diederich 1996; Millanes *et al*. 2015; Zamora *et al*. 2016; Diederich *et al*. 2022). Evidence suggests that speciation is driven by host selection rather than by geographical isolation (Werth *et al*. 2013; Millanes *et al.* 2014, 2015, 2016; Diederich & Ertz 2020; Diederich *et al*. 2022).

A large amount of still overlooked diversity in lichenicolous tremellalean fungi is hidden in several species complexes (Diederich *et al*. 2022). *Tremella caloplacae* (Zahlbr.) Diederich was first described as a hyphomycete with large, 1-septate conidia (as *Lindauopsis caloplacae* Zahlbr.; Zahlbruckner 1906). The description was based on a specimen growing in the hymenium of *Caloplaca callopisma* (Ach.) Th. Fr. (currently *Variospora aurantia* (Pers.) Arup *et al.*), collected in Crete. In his monograph of lichenicolous heterobasidiomycetes, Diederich (1996) described a species of lichenicolous *Tremella* growing in the hymenium of specimens of *Caloplaca* collected in Austria and Great Britain, but he left the species unnamed as *Tremella* sp. 1 due to the lack of taxonomically useful morphological characters of the specimens and the

impossibility of finding differences between this species and *Tremella rinodinae* Diederich & M.S. Christ. The species was not formally described until 2003 (Sérusiaux *et al*. 2003), when enough material from new localities had been collected and studied. Sérusiaux *et al.* (2003) studied the type of *Lindauopsis caloplacae* Zahlbr. and concluded that the structures that Zahlbruckner (1906) illustrated in great detail (and interpreted as conidia) in reality correspond to tremelloid basidia with one transverse primary septum. The species corresponds to '*Tremella* sp. 1' (*sensu* Diederich 1996) and was combined in *Tremella* and reported on several species of *Caloplaca* s. lat. by Sérusiaux *et al.* (2003). Later, Diederich (2007) reported the species as growing on *Xanthoria sorediata* (Vain.) Poelt from Canada and Greenland. The two collections on *X. sorediata* could not be distinguished microscopically from the material on *Caloplaca* s. lat., although they were macroscopically very distinct. That led Diederich (2007) to suggest that *Tremella caloplacae* could represent a species complex needing further study, also considering the polyphyly of both *Caloplaca* and *Xanthoria*, which was already acknowledged at that time by Gaya *et al*. (2003). The first molecular data obtained from *Tremella caloplacae* also supported this hypothesis (Millanes *et al.* 2011), although this was not discussed by those authors.

Freire-Rallo *et al*. (2023) studied the phylogenetic relationships and species boundaries of 52 specimens of *Tremella caloplacae* s. lat. growing on different hosts of the *Teloschistaceae*. That study showed that *Tremella caloplacae* s. lat. is indeed a species complex including at least six putative new species, each of them restricted to grow and develop on a single host species or genus. The specimens analyzed in Freire-Rallo *et al.* (2023), to which we refer here for practical reasons as the *Tremella caloplacae* species complex, are studied in this work at a morphological level. In addition, we have

sequenced two specimens of *Tremella* growing on *Calogaya biatorina* (A. Massal.) Arup *et al.* and on *Polycauliona* sp., and these are also included in this study to test their phylogenetic position.

The aim of this work is to formally describe five species and to tentatively describe three other species within the *Tremella caloplacae* complex, combining morphological, ecological and molecular data, and studying the phylogenetic relationships within the group.

Materials and Methods

Morphological studies

Specimens studied are housed in the ANGUC, BR, GZU, MARSSJ, S and UBC herbaria (abbreviations according to Index Herbariorum http://sweetgum.nybg.org/science/ih/) or the private collection of Javier Etayo (Table 1). Macroscopic characters were observed using an Olympus SZX16 or a Leica MZ 7.5 dissecting microscope. Images of macroscopic traits were captured with either a Sony Alpha A6000 camera on an Olympus SZX16 dissecting microscope or a Canon 6D camera with Nikon BD Plan 5 or 10 microscope objectives, using StackShot (Cognisys) and Helicon Focus (HeliconSoft) for increasing the depth of field. The samples were first moistened with distilled water and then the surface of the gall cut with a razor blade to maximize the amount of tremellalean hyphae taken. Sections were pretreated with a solution of KOH (5%) and stained with phloxine B (1% in water), following the methods used by Diederich (1996). Microscopic photographs were captured with a Sony Alpha A6000 or an Olympus DP23 camera on an Olympus BX51 microscope, or a Leica EC3 camera on a Leica DMLB microscope, without or with DIC optics. Values of the measured structures were rounded to the nearest

0.5 µm. The basidiospore apiculus is not included in the measurements. Microscopic images were adjusted with Helicon Focus to increase the depth of field. Measurements are presented with a range representing the 69% probability interval; within parentheses the smallest and largest measurements are specified followed by the mean ($\bar{x}$) minus and plus the standard deviation (SD) as follows: (min–) ($\bar{x}$ − SD) – ($\bar{x}$ + SD) (–max). Basidiospore length/width ratio is expressed as Q. The total number of measurements (*n*) is given within parentheses. Mycological terminology used follows Diederich (1996), Kirk *et al*. (2008) and Diederich *et al.* (2022).

Molecular studies

DNA extraction, amplification and sequencing. DNA was extracted directly from the specimens studied (Table 1), either freshly collected or herbarium material. Total DNA extraction was performed using the Qiagen DNeasy Plant Mini Kit, following the manufacturer's instructions but repeating the final elution of 50 µl with water twice.

For PCR amplification, we combined general fungal primers with others specifically designed to amplify DNA from the tremellalean fungi (Millanes *et al*. 2011; Freire-Rallo *et al.* 2023). To perform the PCRs, we used the primers ITS1F (Gardes & Bruns 1993), BasidLSU3-3 (Millanes *et al.* 2011) and TRMcal_R2 (Freire-Rallo *et al.* 2023) to amplify the internal transcribed spacer I, the 5.8S rDNA gene and the internal transcribed spacer II gene. PCR reactions were carried out using Illustra^{TM} Hot Star PCR beads, according to the manufacturer's instructions. For ITS1F/BasidLSU3-3 and ITS1F/TRMcal_R2, we

Table 1. *Tremella* sequences newly generated in this study (bold), or downloaded from GenBank, with specimen data. Type specimens are indicated by (T).

Species names (DNA extraction number)	Host	ITS	LSU	Specimen data
Tremella caloplacae (SF148)	*Variospora aurantia*	OQ192947	OQ176395	Ukraine. *Kukwa* 1851 (S-F117262)
T. caloplacae (SF302)	*Variospora aurantia*	OQ192959	OQ176407	Spain. *Millanes* 1370 & *Freire-Rallo* (S)
T. caloplacae (AM780)	*Variospora dolomiticola*	OQ192948	OQ176396	Greece. *Diederich* 18575 (BR)
T. caloplacae (SF305)	*Variospora dolomiticola*	OQ192960	OQ176408	Spain. *Millanes* 1371 & *Freire-Rallo* (S)
T. caloplacae (AM782)	*Variospora flavescens*	OQ192949	OQ176397	Luxembourg. *Diederich* 18559 (BR)
T. caloplacae (SF149)	*Variospora flavescens*	OQ192950	OQ176398	Sweden. *Westberg, Košuthová, Prieto* GTL24 (S-F268002)
T. caloplacae (SF243)	*Variospora flavescens*	OQ192951	OQ176399	Sweden. *Freire-Rallo* S37 (S)
T. caloplacae (SF254)	*Variospora flavescens*	OQ192952	OQ176400	Sweden. *Freire-Rallo* S41 (S)
T. caloplacae (SF257)	*Variospora flavescens*	OQ192953	OQ176401	Sweden. *Freire-Rallo* S42 (S)
T. caloplacae (SF260)	*Variospora flavescens*	OQ192954	OQ176402	Sweden. *Freire-Rallo* S43 (S)
T. caloplacae (SF266)	*Variospora flavescens*	OQ192955	OQ176403	Sweden. *Freire-Rallo* S45 (S)
T. caloplacae (SF272)	*Variospora flavescens*	OQ192956	OQ176404	Sweden. *Freire-Rallo* S46 (S)
T. caloplacae (SF279)	*Variospora flavescens*	OQ192957	OQ176405	Sweden. *Freire-Rallo* S50 (S)
T. caloplacae (SF150)	*Variospora thallincola*	OQ192958	OQ176406	United Kingdom. *Kärnefelt* 970901 (S-L5870)
T. elegantis (AM315E)	*Rusavskia elegans*	OQ192939	OQ176388	Sweden. *Millanes* 908 (S-F255314)
T. elegantis (AM349E)	*Rusavskia elegans*	OQ192940	OQ176389	Norway. *Millanes* 808 (S-F255312)
T. elegantis (AM443)	*Rusavskia elegans*	OQ192941	OQ176390	Norway. *Millanes* 1085 (S)
T. elegantis (AM444) (T)	*Rusavskia elegans*	OQ192942	OQ176391	Norway. *A. Millanes* 1113 (S)
T. elegantis (AM554)	*Rusavskia elegans*	OQ192943	OQ176392	Sweden. *Millanes* 904 (S-F255313)
T. elegantis (SF161)	*Rusavskia elegans*	OQ192944	-	Sweden. *Odelvik, Hedenäs* & *Rönblom* 14-453 (S-F253739)
T. nimisiana (SF155)	*Xanthocarpia ferrarii*	OQ192962	OQ176410	Estonia. *Thor* 8202 (S-F70137)
T. nimisiana (AM558)	*Xanthocarpia lactea*	OQ192961	OQ176409	Austria. *Hafellner* 24839 (GZU)
T. nimisiana (SF291) (T)	*Xanthocarpia marmorata*	OQ192964	OQ176412	Spain. *Millanes* 1365 & *Freire-Rallo* (S)

(Continue)

| Species names (DNA extraction number) | Host | ITS | LSU | Specimen data |

T. nimisiana (SF156)	*Xanthocarpia* sp.	OQ192963	OQ176411	Estonia. *Thor* 8202 (S-F70137)
T. nimisiana (SF292)	*Xanthocarpia* sp.	OQ192965	OQ176413	Spain. *Millanes* 1356 & *Freire-Rallo* (S)
***T. nimisiana* (SF414)**	*Xanthocarpia* sp.	**OQ418449**	-	France. *B. de Lesdain* 1906 (ANGUC)
T. parietinae (AM310)	*Xanthoria parietina*	OQ192966	OQ176414	Austria. *Obermayer* 12148a (GZU)
T. parietinae (AM311)	*Xanthoria parietina*	OQ192967	OQ176415	Austria. *Fleischhacker* 11010 (GZU)
T. parietinae (AM352E)	*Xanthoria parietina*	OQ192968	OQ176416	Spain. *Zamora, Zamora* & *Señoret* 8-v-2010 (G)
T. parietinae (AM353E)	*Xanthoria parietina*	OQ192969	OQ176417	Spain. *Vivas, Zamora* & *Zamora* 18-xii-2011 (G)
T. parietinae (AM446)	*Xanthoria parietina*	OQ192970	OQ176418	Spain. *Millanes* 1192 (S)
T. parietinae (AM447)	*Xanthoria parietina*	OQ192971	OQ176419	Spain. *Millanes* 1197 (S)
T. parietinae (AM553)	*Xanthoria parietina*	OQ192972	OQ176420	Sweden. *Millanes* 849 (S)
T. parietinae (AM555)	*Xanthoria parietina*	OQ192973	OQ176421	Sweden. *Millanes* 833 (S)
T. parietinae (AM559)	*Xanthoria parietina*	OQ192974	OQ176422	Austria. *Obermayer* 12148a1 (GZU)
T. parietinae (AM561)	*Xanthoria parietina*	OQ192976	OQ176424	Austria. *Hafellner* 77075 (GZU)
T. parietinae (AM562)	*Xanthoria parietina*	OQ192977	OQ176425	Austria. *Obermayer* 12148a1 (GZU)
T. parietinae (AM563)	*Xanthoria parietina*	OQ192978	OQ176426	Austria. *Obermayer* 12446 (GZU)
T. parietinae (AM564)	*Xanthoria parietina*	OQ192979	OQ176427	Austria. *Hafellner* 77065 (GZU)
T. parietinae (AM565)	*Xanthoria parietina*	OQ192980	OQ176428	Slovenia. *Hafellner* 77507 (GZU)
T. parietinae (AM638)	*Xanthoria parietina*	OQ192981	OQ176429	Luxembourg. *Diederich* 17385 (BR)
T. parietinae (AM639)	*Xanthoria parietina*	OQ192982	OQ176430	Luxembourg. *Diederich* 17455 (BR)
T. parietinae (AM640)	*Xanthoria parietina*	OQ192983	OQ176431	Luxembourg. *Diederich* 17473 (BR)
T. parietinae (AM641)	*Xanthoria parietina*	OQ192984	OQ176432	Luxembourg. *Diederich* 17740 (BR)
***T. parietinae* (SF390) (T)**	*Xanthoria parietina*	**OQ418450**	-	Spain. *Millanes* 1328 (S)
***T. parietinae* (SF391)**	*Xanthoria parietina*	**OQ418451**	-	Spain. *Millanes* 1364 (S)
***T. parietinae* (SF392)**	*Xanthoria parietina*	**OQ418452**	-	Spain. *Millanes* 1304 (S)
***T. parietinae* (SF394)**	*Xanthoria parietina*	**OQ842302**	-	Spain. *Freire Rallo* S128 (S)

(Continue)

Species names (DNA extraction number)	**Host**	**ITS**	**LSU**	**Specimen data**

***T. parietinae* (SF397)**	*Xanthoria parietina*	**OQ842303**	-	Spain. *Etayo* 31851 (hb. Etayo)
T. pusillae (SF234) (T)	*Calogaya pusilla*	OQ192934	OQ176384	Sweden. *Freire-Rallo* S33 (S)
T. pusillae (SF237)	*Calogaya pusilla*	OQ192935	OQ176385	Sweden. *Freire-Rallo* S34 (S)
T. pusillae (SF263)	*Calogaya pusilla*	OQ192936	OQ176386	Sweden. *Freire-Rallo* S44 (S)
T. pusillae (SF269)	*Calogaya pusilla*	OQ192937	OQ176387	Sweden. *Freire-Rallo* S45 (S)
T. sorediatae (AM32) (T)	*Rusavskia sorediata*	OQ192945	OQ176393	Greenland. *Kukwa* 4385a (UGDA)
T. sorediatae (AM642)	*Rusavskia sorediata*	OQ192946	OQ176394	Canada. *Goward* 01-608 (UCB)
***Tremella* sp. 13 (SF401)**	*Calogaya biatorina*	**OQ418453**	-	Spain. *Etayo* 20762 (hb. Etayo)
Tremella sp. 14 (AM556)	*Calogaya decipiens*	OQ192933	OQ176383	Sweden. *Millanes* 850 (S-F253110)
***Tremella* sp. 15 (SF399)**	*Polycauliona* sp.	**OQ418454**	-	Spain. *Etayo* 19125 (hb. Etayo)
***T. candelariellae* (AM384)**	*Candelaria concolor*	**OQ418455**	**OQ410474**	Spain. *Zamora* 13-iii-2010 (G)

ran an initial denaturing at 95 °C for 5 min; four cycles of 95 °C for 40 s, 53 °C for 40 s and 72 °C for 90 s; four cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; 32 cycles of 95 °C for 30 s, 47 °C for 30 s and 72 °C for 90 s; a final extension step of 72 °C for 10 min. For PCR samples we added 2 µl of DNA extraction and 0.5 µl of each primer (primer concentration 10 µM). Before sequencing, we purified PCR products using 5 µl of Exo-sap-IT© (USB Corporation) added to 22 µl volume of amplification product. Sequencing was performed at the Molecular Systematic Laboratory of the Swedish Museum of Natural History, at the Genomic Unit in Rey Juan Carlos University, or by Macrogen Europe (Amsterdam, the Netherlands) or Macrogen Spain (Madrid, Spain).

Sequence alignment and phylogenetic analysis. Newly produced sequences were assembled and edited with Geneious Prime® v. 2021.0.3. (https://www.geneious.com). A data matrix was produced for subsequent phylogenetic analyses using sequences of the ITS1, 5.8S, ITS2 and LSU nuclear rDNA produced by us or retrieved from GenBank (Table 1). *Tremella candelariellae* was used as outgroup (Table 1) based on previous literature (Millanes *et al*. 2011; Liu *et al*. 2015) and from preliminary trees. Sequences were aligned using the Q-INS-I algorithm as implemented in MAFFT v. 7 (Katoh *et al*. 2019). Misaligned positions, major insertions and ambiguous and/or divergent regions were identified and deleted with Gblocks v. 0.91b (Castresana 2000), following the relaxed conditions described by Talavera & Castresana (2007). Alignments were then checked with Mesquite (Maddison & Maddison 2021) and terminal gaps were converted to missing data.

We considered four independent partitions, ITS1, 5.8S, ITS2 and nuLSU, in all analyses. Each partition was analyzed individually by maximum likelihood ultrafast bootstrap (UF-

BS) in IQ-TREE to assess for conflicts. Strongly supported clades (IQ-TREE UF-BS > 95%) in disagreement were considered to be an indication of significant conflict (Mason-Gamer & Kellogg 1996; Hoang *et al.* 2018). Since no conflict was detected in our data sets, they were combined and then analyzed using maximum likelihood (ML) and Bayesian approaches. Maximum likelihood analyses were carried out in IQ-TREE (Nguyen *et al*. 2015). Model selection for each partition was achieved using ModelFinder in IQ-TREE (Kalyaanamoorthy *et al*. 2017), with the corrected Akaike information criterion (AICc). Following this scheme, the TIM2e + I model was selected forITS1 and 5.8S, TIM2e + Γ4 for ITS2, and TIM3 + F + Γ4 for nuLSU. We assessed node support by standard bootstrap using 1000 bootstrap pseudoreplicates. Bayesian analyses were performed by Markov chain Monte Carlo (MCMC) sampling as implemented in the software MrBayes v. 3.2.7a (Ronquist *et al.* 2012). Since not all models tested by ModelFinder in IQ-TREE can be directly implemented in MrBayes, for the Bayesian analyses we selected from a subsample of substitution models using the corrected Akaike information criterion (AICc) as implemented in jModelTest 2 (Darriba *et al.* 2012), allowing only three substitution schemes, using full likelihood optimization and four discrete gamma categories. In this case, JC was selected for ITS1 and 5.8S, and SYM + Γ for ITS2 and nuLSU rDNA. The combined analyses treated the different regions as separate partitions with topology linked across partitions but separate model parameter values and proportional rates across partitions. For each combined data set, two parallel runs were performed, each with four chains, three of which were incrementally heated with a temperature of 0.15. The analyses were diagnosed for convergence every 100 000 generations and were set to halt automatically when the average standard deviation of splits across runs in the last half of the analysis descended below 0.01. Every 100th tree

was saved. The first 50% of each run was discarded as burn-in. Both ML and Bayesian analyses were performed on the CIPRES Web Portal (Miller *et al*. 2015).

Results and Discussion

Eight sequences of the ITS region were newly produced for this study. The alignment included these newly generated sequences, combined with sequences of ITS and nuLSU sequenced by Freire-Rallo *et al.* (2023) or retrieved from GenBank (Table 1). A data matrix corresponding to the ITS and nuLSU regions was generated with a total of 1360 characters (ITS1, 1–122; 5.8S, 123–276; ITS2, 277–595; nuLSU, 596–1360). The best tree obtained from the ML analysis had a ln-likelihood value of −2998.5609. The Bayesian analysis halted after 900 000 generations, at which time the average standard deviation of split frequencies across runs was 0.0098, indicating that the three runs had converged (< 0.01). A majority-rule consensus tree was constructed from the 9000 trees of the stationary tree sample. No incongruences were found among the topologies of the trees obtained with ML and Bayesian inference and therefore only the best tree from the ML analysis is shown in Figure 1.

The phylogenetic analyses showed at least nine distinct lineages. Most of them coincide with the species delimitation analyses performed by Freire-Rallo *et al*. (2023), who discussed at least nine different species growing on different hosts: 1) on *Xanthoria parietina* (L.) Th. Fr., 2) on *Xanthocarpia lactea* (A. Massal.) A. Massal., 3) on *Xanthocarpia* sp., 4) on *Calogaya decipiens* (Arnold) Arup *et al.*, 5) on *Calogaya pusilla* (A. Massal.) Arup *et al.*, 6) on *Rusavskia elegans* (Link) S.Y. Kondr. & Kärnefelt, 7) on *Rusavskia sorediata* (Vain.) S.Y. Kondr. & Kärnefelt, 8) on *Variospora* sp., and 9) on *Leproplaca xantholyta* (Nyl.) Nyl. Not all the species delimited in Freire-Rallo *et al.*

(2023) will be formally described in this work. The specimen of *T. caloplacae* s. lat. on *Leproplaca xantholyta* is in a very poor condition so it has not been possible to obtain sufficient morphological data for a full description. Although *T. caloplacae* s. lat. on *Calogaya decipiens* is one of the species delimited in Freire-Rallo *et al.* (2023), we refrain from describing a species based on a single specimen and instead leave it unnamed as *Tremella* sp. 14, until more specimens are available. The same applies to *Tremella* sp. 13 on *Calogaya biatorina* and *Tremella* sp. 15 on *Polycauliona* sp. The latter two samples were not included in Freire-Rallo *et al.* (2023) and were sequenced for the first time in this study. The specimens growing on *Xanthocarpia* species are all included in the newly described *Tremella nimisiana,* although our current and previous phylogenetic results (Freire-Rallo *et al.* 2023) clearly show that *Tremella* material growing on *Xanthocarpia* represents a species complex. This conservative decision was made owing to the low number of specimens available growing on each *Xanthocarpia* host, and to the scarcity of diagnostic characters, which makes a morphological characterization based on a small number of samples challenging.

There are also other lichenicolous *Tremella* species known to grow on *Teloschistaceae* hosts (Diederich *et al*. 2022): *Tremella teloschistis* Diederich *et al*., which is phylogenetically not close to *Tremella caloplacae* s. lat., on *Teloschistes exilis*; *T. occultixanthoriae* Diederich *et al*. on *Xanthoria parietina*; *T. pisutiellae* Diederich & W. R. Buck on *Pisutiella conversa*; *T. xanthomendozae* Diederich *et al*. on *Xanthomendoza weberi* (S.Y. Kondr. & Kärnefelt) L. Lindblom; and *Tremella* sp. 11 on *Pyrenodesmia chalybaea* (Fr.) A. Massal., from which there are no sequences and which is therefore not included in this work. These species are, however, included in our key to facilitate the

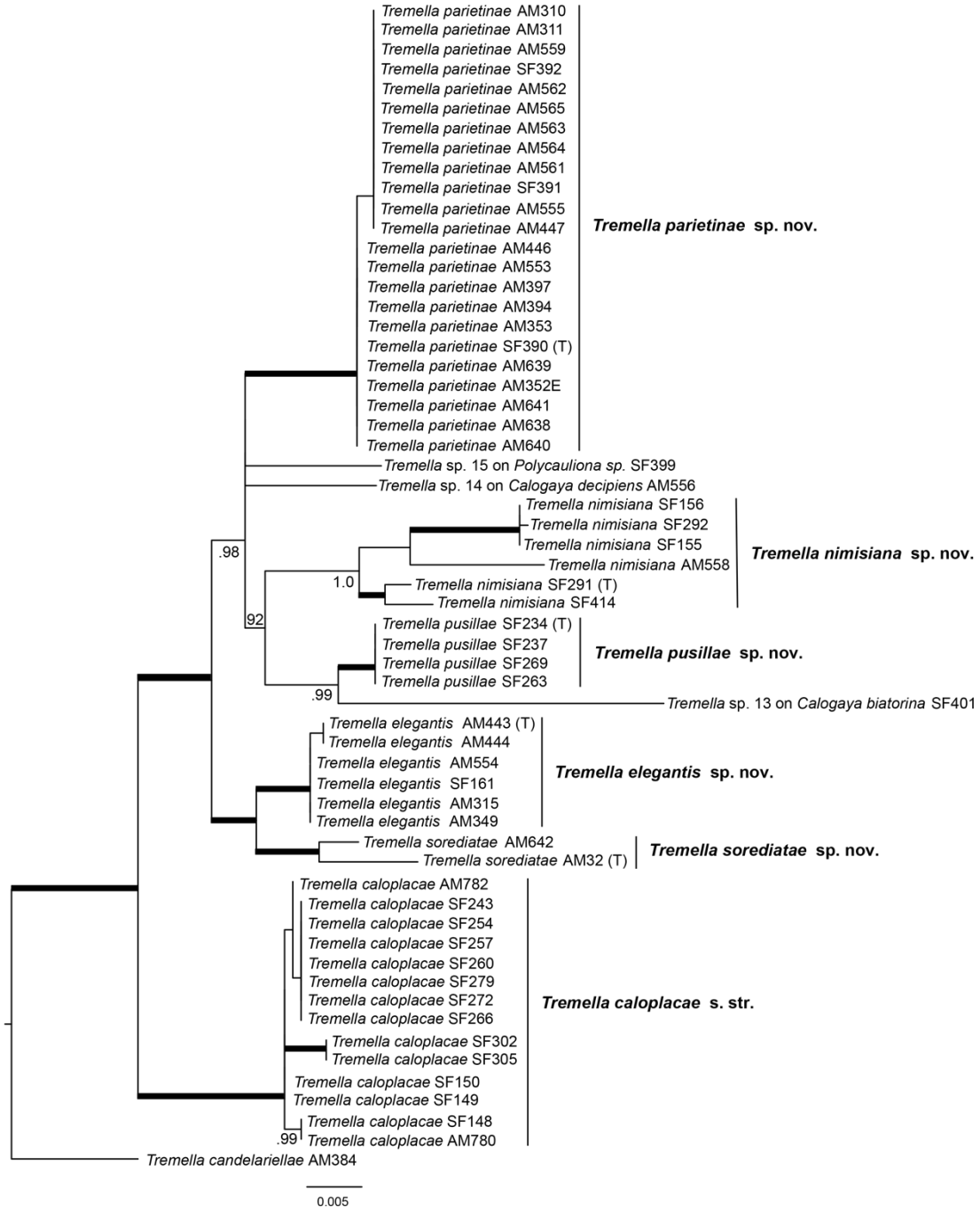

Fig. 1. Phylogram based on ITS and nuLSU sequences of *Tremella* species, corresponding to the best tree recovered in the maximum likelihood analysis (ML), with information on the Bayesian posterior probability (PP) values added. Branches in bold indicate nodes supported by both PP≥ 0.95 and ML bootstrap (MLBS) values ≥ 70. When nodes received support only from one of the two methods, ML-BS values ≥ 70 are indicated above branches and PP values ≥ 0.95, below branches. Species names are indicated in the right margin. Types are indicated with '(T)'. Branch lengths are scaled to the expected number of substitutions per site.

identification of the newly described species, and to complement recently published keys (Diederich *et al*. 2022).

Key to species

1 Basidia devoid of epibasidia; on the lower thallus surface of *Xanthoria parietina* ……..……**T. occultixanthoriae**

Basidia with epibasidia……..……2

2(1) Basidiomatal galls on the host thallus……..…….……..3

Basidia immersed in the hymenium of the host……..5

3(2) Basidiomatal galls elongated; on *Teloschistes exilis* ……....**T. teloschistis**

Basidiomatal galls subspherical; on other hosts……..4

4(3) Basidia transversely or obliquely septate, 5.5–9 µm wide; basidiospores 5–7.5 × 4.5–6 µm; on *Xanthomendoza weberi* ……...**T. xanthomendozae**

Basidia transversely or obliquely septate, 8.7–13 µm wide or longitudinally septate, 13.2–16.2 µm wide; basidiospores slightly smaller; on *Rusavskia sorediata* ……...……**T. sorediatae**

5(2) Infected apothecia without visible symptoms or only slightly swollen; basidia 2-celled, transversely septate……6

Infected apothecia strongly swollen at maturity, with convex basidiomatal galls; basidia 2-celled or rarely 3-celled……7

6(5) Basidia 17–24 × 7–10 µm; on *Pisutiella conversa* ……**T. pisutiellae**

Basidia shorter, 12–18 × 7–9 µm; on *Pyrenodesmia chalybaea*……..…

……..**Tremella** sp. 11

7(5) Basidiomata often > 1 mm diam…….……8

Basidiomata < 1 mm diam…….……11

8(7) Clamped hyphae not or rarely observed; probasidia subspherical to ellipsoidal, not fusiform……...……..…...9

Clamped hyphae present; probasidia ellipsoidal to broadly fusiform……10

9(8) Basidia 2-celled; basidiospores 3.8–9.6 × 3.8–7.9 µm; on *Variospora* spp…… ……..…….……**T. caloplacae**

Basidia 2-celled or rarely 3-celled; basidiospores 7.6–9.2 × 6.7–10.2 µm; on *Calogaya pusilla*……..……..**T. pusillae**

10(8) Basidia 2-celled or rarely 3-celled, not elongating separately; basidiospores 6.5–11.9 × 5.7–11.9 µm; on *Xanthoria parietina*……**T. parietinae**

Basidia 2-celled, when longitudinally septate, cells occasionally elongating and growing separately; basidiospores smaller (one measurement of 5.5 × 6.0 µm); on *Rusavskia elegans*…….……..……**T. elegantis**

11(7) Clamped hyphae not observed; basidia with transverse septa often constricted at the septum…….……..12

Clamped hyphae present; basidia with transverse septa rarely constricted at the septum……..…..….13

12(11) Basidia 2-celled or rarely 3-celled; basidiospores 8.7–10.2 × 8.2–10.3 µm; on *Calogaya biatorina*……...…….**Tremella** sp. 13

Basidia 2-celled, when transversely septate sometimes upper part subglobose and wider than the lower part; basidiospores smaller (one measurement of 5.5 × 4.5 µm); on *Calogaya decipiens*…….……..**Tremella** sp. 14

13(11) Basidia 2-celled and ellipsoid to pyriform, rarely 3-celled and clavate to subcylindrical; basidiospores 7.5–10.3 × 6.9–10.1 µm; on *Xanthocarpia* spp. ……..…….**T. nimisiana**

Basidia 2-celled, elongate, very narrowly ellipsoid to ellipsoid; basidiospores

smaller (one measurement of 6.5 × 7.1 µm); on *Polycauliona* sp……

…….**Tremella** sp. 15

Taxonomy

Tremella caloplacae (Zahlbr.) Diederich

In Sérusiaux *et al*., *Lejeunia n. s*. **173**, 31 (2003).—*Lindauopsis caloplacae* Zahlbr., *Ber. Deutsch. Bot. Ges.* **24**, 145 (1906); type: Crete, 'an Kalkfelsen bei Kristallenia', 1904, *R. Sturany* (W 11196, lichenicolous fungus in apothecia of *Variospora aurantia*: lectotype, designated by Sérusiaux *et al.* (2003)).

= *Tremella* sp. 1, in Diederich, *Bibl. Lichenol*. **61**, 167 (1996).

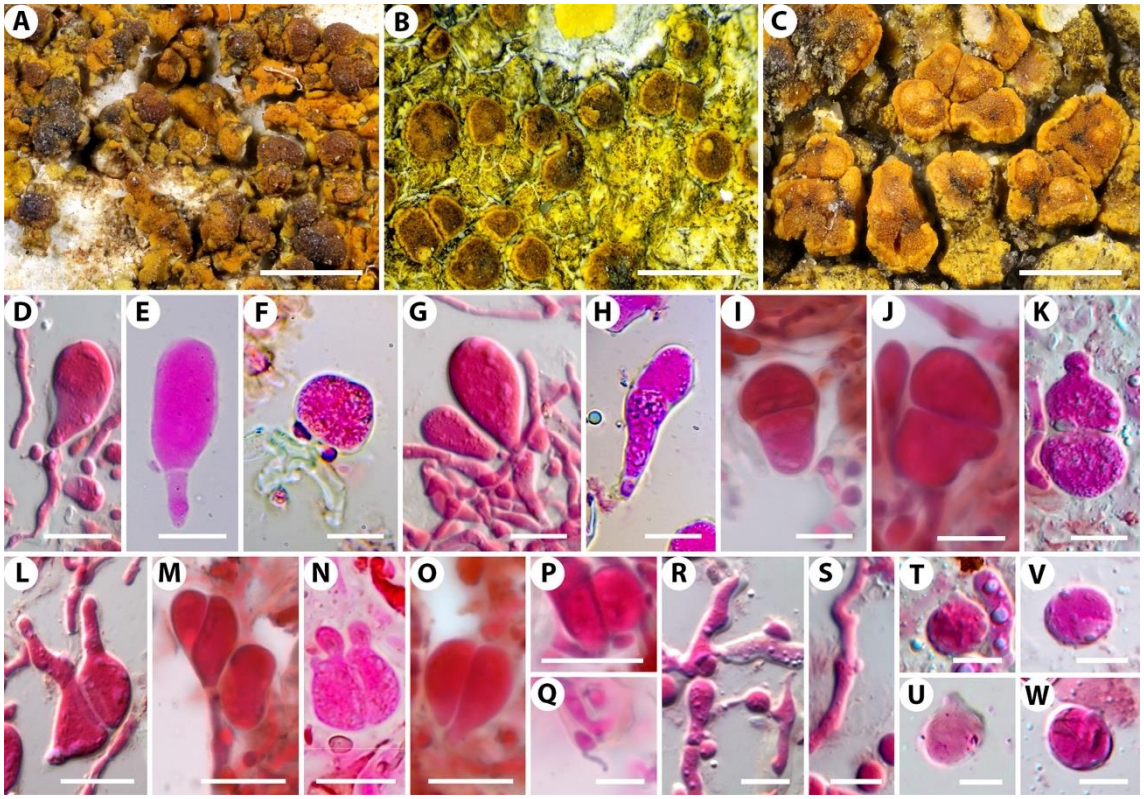

Fig. 2. *Tremella caloplacae* (A, I, J, M & O, lectotype on *Variospora aurantia*; B & E, *Diederich* 12328 on *V. flavescens*; K, T, U & W, *Freire-Rallo* S43 on *V. flavescens*; N, *Freire-Rallo* S37 on *V. flavescens*; C, D, G, L, R & S, *Sérusiaux* iv 1983 on *V. dolomiticola*; F, H, P, Q & V, *Millanes* 1371 & *Freire-Rallo* on *V. dolomiticola*). A–C, variation in gall morphology. D–G, probasidia showing basal clamp connections and haustoria. H–K, transversely septate basidia. L–M, obliquely septate basidia. N–P, longitudinally septate basidia. Q–S, haustoria and hyphal clamp connections. T–W, basidiospores. Scales: A–C = 1 mm; D–R = 10 µm; S–W = 5 µm.

Basidiomata reduced, inducing the formation of convex, sometimes hemispherical, smooth, uniform or irregular, light yellow to brown galls in the hymenium of the host, sometimes a single gall occupying the whole surface of the hymenium obscuring the thalline margin, often scattered all over the hymenium or forming conglomerates, (0.06–)0.1–0.5(–1.8) mm diam. (*n* = 404). *Hyphae* rarely with clamp connections, thin-walled, up to 3 µm diam.; haustorial branches present, tremelloid, rarely with a bifurcated filament or with two filaments, mother cell (2–)2.7–4.8(–7) µm diam. (*n* = 114), filament up to 9.5 µm in length. *Hymenium* reduced when young but developed when old, containing numerous subspherical to ellipsoid, rarely cylindrical probasidia with a basal clamp connection. *Basidia* subspherical to ellipsoid, 2-celled when mature, with one transverse, sometimes oblique, rarely longitudinal septum, when transversely septate sometimes constricted at the septum, or upper part subglobose and wider than the lower part, (11–)14.5–27.2(–46) × (5.5–)8.0–21.2(–23) µm (*n* = 508) [transversely septate: (11–)15.6–27.2(–46) × (5.5–)8.8–14.5(–21) µm (*n* = 401); obliquely septate: (12.5–)14.5–25.7(–34) × (6.5–)8.0–15.8(–22) µm (*n* = 89); longitudinally septate: (11–)15.1–22.0(–25.5) × (9–)13.3–21.2(–23) µm (*n* = 18)]; epibasidia elongate, (1.5–)2.3–5.8(–8.5) µm diam. (*n* = 77), up to at least 37 µm in length. *Basidiospores* subspherical to ellipsoid, apiculus present, (3.5–)3.8–9.6(–14) × (3–)3.8–7.9(–9.5) µm (*n* = 13).

Ecology and distribution. *Tremella caloplacae* s. str. is known to grow only in the hymenium of *Variospora aurantia*, *V. dolomiticola*, *V. flavescens* and *V. thallincola*. The species is known from France, Greece, Luxembourg, Spain, Sweden, Ukraine and the United Kingdom.

Notes. The description above refers to *Tremella caloplacae* s. str. and is based on the description by Diederich (1996), slightly modified according to the material recently studied and the exclusion of the newly described species. *Tremella caloplacae* s. str. grows exclusively on *Variospora* spp. And differs from other species in the *T. caloplacae* complex by the size of the basidiomata (often > 1 mm diam.), the absence of hyphae with clamp connections, and the subspherical to ellipsoidal probasidia. Other species growing in the hymenium of *Teloschistaceae* hosts are *T. pisutiellae* and *Tremella* sp. 11 (Diederich *et al*. 2022) but these are not known to induce galls, in contrast to the rest of intrahymenial species in the *T. caloplacae* complex. Species with a micromorphology resembling that of *T. caloplacae* s. str. (i.e. 2-celled basidia transversely, obliquely or only rarely longitudinally septate) are *T. dirinariae* Diederich *et al.*, *T. montis-wilhelmii* Diederich and *T. rinodinae* Diederich & M.S. Christ. But they differ in host selection and only *T. rinodinae* grows intrahymenially. The recent specimens of *T. caloplacae* from Crete have been collected 2.5 km NNE of the type locality and are thus likely to be genetically very similar to the type. The circumscription of *Tremella caloplacae* s. str. may be modified in the future when there is better knowledge of the species complex.

Additional specimens examined. **France:** *Pas-de-Calai*s : Audresselles, Cran-aux-Oeufs, falaise maritime, sur rochers arénacés face à la mer, on *Variospora dolomiticola*, 1983, *Sérusiaux* (BR, LG, S). *Vaucluse*: 2 km S of Gordes, col de Gordes, on *V. flavescens*, 1995, *Diederich* 12328 (BR).—**Great Britain:** *England*: **V.C. 3,** South Devon, Torbay, Berry Head, locally abundant, on *V. thallincola*, 50°25′N, 3°32′W, 1997, *Kärnefelt* 970901 (S).—**Greece:** *Crete*: S of Malia, Lasithi Plateau, 500 m NW of Tzermiado, 35°12′16″N, 25°28′58″E, 890 m, on *V. dolomiticola*, 2016, *Diederich* 18575 (BR); *ibid*., on *V. aurantia*, 2016, *Diederich* 18576 (BR).—**Luxembourg:** Luxembourg City, ancient city wall S of Rumm and W of the railway bridge, 49°36′28″N, 6°8′20″E, 245 m, on *V. flavescens*, 2016, *Diederich* 18559 (BR).—**Spain:** *Guadalajara*: Valdegrudas, Páramos de la Alcarria Occidental, cliff next to a crop area, on *V. aurantia*, 40°43′03″N, 3°00′32″W, 962 m, 2017, *Millanes* 1370 & *Freire-Rallo* (S); *ibid*., on *V. dolomiticola*, *Millanes*

1371 & *Freire-Rallo* (S).**—Sweden:** *Gotland*: Hangvar par., Irevik, along the small road towards Svarthäll on the east side of Irevik, on *V. flavescens*, 57°50′14″N, 18°36′22″E, 2014, *Westberg, Košuthová* & *Prieto* GTL24 (S). *Öland*: Jordhamn, on *V. flavescens*, 57°5′52″N, 16°53′13″E, 8 m, 2017, *Freire-Rallo* S37 (S); *ibid*., *Freire-Rallo* S41, S42, S43, S46, S50 (S).**—Ukraine:** *Khmel'nyts'ka Oblast*: Posil's'ki Tovtry National Park, , Kamyanets'-Podil's'kyi Rayon, Kitaygorod Village, 15 km SE of Kamyanets'-Podil's'kyi, canyon of Ternava River, 48°38′25″N, 26°46′59″E, 141 m, on *V. aurantia*, 2003, *Kukwa* 1851 (S).

***Tremella elegantis* Freire-Rallo, Diederich, Millanes & Wedin sp. Nov.**

MycoBank No.: MB 847663

Differs from *Tremella caloplacae* in the presence of hyphae with clamp connections, the presence of broadly fusiform probasidia, the formation of basidia with longitudinal septa in which cells elongate and grow separately, and in developing only on *Rusavskia elegans*.

Type: Norway, Finnmark, Vadsø, Store Ekkerøya, 70°04′14″N, 30°06′26″E, 50 m, on calcareous rock, on *Rusavskia elegans*, 2014, *Millanes* 1113 (S—holotype). DNA voucher: AM444; GenBank Accession nos: OQ192942 (ITS), OQ176391 (LSU).

Basidiomata reduced, inducing the formation of convex, orange or reddish orange to brown galls in the hymenium of the host, sometimes subspherical, irregular and rough, often forming gall conglomerates, sometimes a single gall occupying the whole surface of the hymenium obscuring the thalline margin, (0.1)0.13–0.68(1.15) mm diam. (n = 80). *Hyphae* sometimes with clamp connections, thin-walled, up to 3 µm diam.; haustorial branches present, tremelloid, mother cell (2)2.5–4.1(5) µm diam. (n = 34), filament up to 7 µm in length. *Hymenium* reduced when young but developed when old, containing numerous broadly fusiform to ellipsoid probasidia with a basal clamp connection. *Basidia*

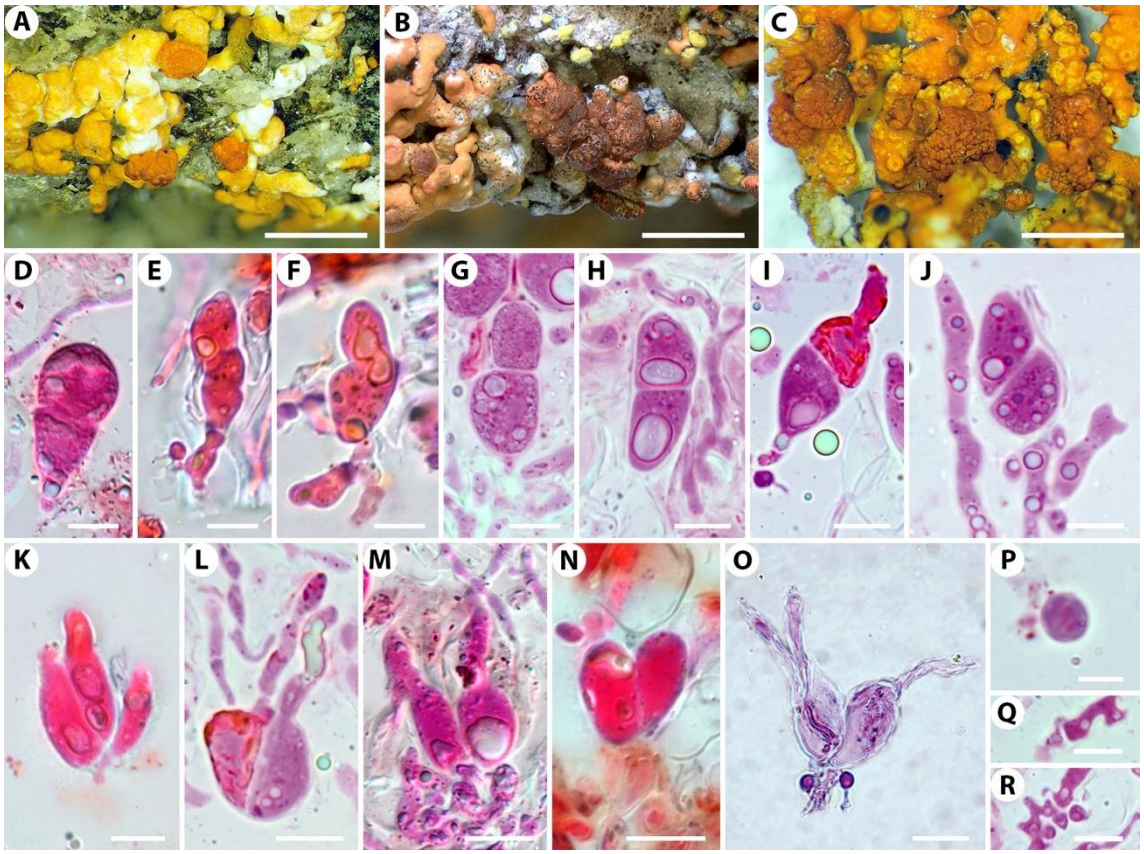

Fig. 3. *Tremella elegantis* on *Rusavskia elegans* (A, D & M, *Millanes* 808; B, E & F, *Millanes* 908; C, G, H, J, Q & P, holotype; I & L, *Millanes* 1085; K, N & R, *Millanes* 904; O, *Odelvik, Hedenäs & Rönblom* 14-453). A–C, variation in gall morphology. D–H, 2-celled transversely septate basidia with one haustorium. I–K, obliquely septate basidia with one haustorium. L–O, longitudinally septate basidia with haustoria. P, basidiospore. Q & R, hyphae with clamp connections. Scales: A–C = 1 mm; D–O = 10 µm; P–R = 5 µm.

subspherical to ellipsoid or pyriform, 2-celled when mature, with one transverse, oblique, or longitudinal septum, when transversely septate, sometimes constricted at the septa,when longitudinally septate, rarely cells elongating and growing separately, (10.5)13.6–31.8(39) × (7)8.4–15.0(23) µm (*n* = 140) [transversely septate: (13)18.0–31.8(39) × (7.5)9.7–14.2(18) µm (*n* = 38); obliquely septate: (10.5)14.8–27.4(36) × (7)8.9–15.0(21) µm (*n* = 62); longitudinally septate: (11)13.6–20.0(24.5) × (8.5)8.4–16.3(23) µm (*n* = 40)]; epibasidia elongate, (2.5)2.9–5.7(10.5) µm diam. (*n* = 68), up to at least 40 µm in length. *Basidiospores* subspherical, apiculus present, 5.5 × 6.0 µm (*n* = 1).

Etymology. From *Rusavskia elegans*, the host lichen.

Ecology and distribution. *Tremella elegantis* is known to grow only in the hymenium of *Rusavskia elegans*. The species is known from Norway and Sweden.

Notes. *Tremella elegantis* resembles *T. caloplacae* s. str. since it induces the formation of convex galls in the hymenium of its host. *Tremella elegantis*, however, grows exclusively on *Rusavskia elegans* and also differs from other species in the *T. caloplacae* complex in the size of the basidiomata (often > 1 mm diam.), the presence of hyphae with clamp connections, the ellipsoidal to broadly fusiform probasidia, and the formation of basidia with cells elongating and growing separately when they are longitudinally septate. Both *T. elegantis* and *T. sorediatae* sometimes produce basidia with longitudinal septa where the cells grow separately, but basidia are smaller in *T. elegantis*. Species also showing basidia with these characteristics are *T. christiansenii* Diederich, *T. diederichiana* Pérez-Ort. *et al.*, *T. hypocenomycis* Diederich, *T. mayrhoferi* J.C. Zamora *et al.* and *T. tuckerae* Diederich, but these differ in the larger (*T. christiansenii*, *T. diederichiana*, *T. mayrhoferi* and *T. tuckerae*) or smaller (*T. hypocenomycis*) basidia, and in that basidia can be 2–4-celled except for *T. tuckerae* (also 2-celled). The new species *T. elegantis* is phylogenetically homogeneous and is sister to *T. sorediatae*.

Additional specimens examined (all on Rusavskia elegans*)*. **Norway:** *Finnmark*: Vardø, Bukkemoltangen, Dolomites area, 70°25′33″N, 30°45′19″E, 25 m, 2014, *Millanes* 1085 (S). *Oppland*: Lom, Runningsgrende, Kleive, 61°42′57″N, 8°14′03″E, 750 m, 2013, *Millanes* 808 (S).—**Sweden:** *Ångermanland*: Grundsunda sn, Skagsudden, 2550 m S om Skags kapell, Skagsudde, 250 m WSW om fyren, 63°11′12″N, 19°01′11″E, 2 m, 2013, *Odelvik, Hedenäs* & *Rönblom* 14-453 (S). *Torne Lappmark*:

Jukkasjärvi, Mt Paddos, 68°19′8″N, 18°51′54″E, 596 m, at the base of a cliff, 2013, *Millanes* 908 (S); Jukkasjärvi, Mt Paddos, 68°19′10″N, 18°51′56″E, 625 m, 2013, *Millanes* 904 (S).

Tremella nimisiana **Freire-Rallo, Diederich, Millanes & Wedin sp. nov.**

MycoBank No.: MB 847664

Differs from *Tremella caloplacae* in the size of the basidiomata (< 1 mm diam.), the presence of hyphae with clamp connections, larger basidiospores (7.5–10.3 × 6.9–10.1 µm), and in developing only on *Xanthocarpia* spp.

Type: Spain, Guadalajara, Tendilla, Páramos de la Alcarria Occidental, 40°31′44″N, 2°58′60″W, 910 m, on calcareous rock, on *Xanthocarpia marmorata*, 2017, *Millanes* 1365 & *Freire-Rallo* (S—holotype). DNA voucher: SF291; GenBank Accession no: OQ192964 (ITS).

Basidiomata reduced, inducing the formation of convex, yellowish orange to dark brown galls in the hymenium of the host, sometimes hemispherical, irregular and rough, sometimes smooth, often forming gall conglomerates, sometimes a single gall occupying the whole surface of the hymenium, (0.08–)0.07–0.30(–0.66) mm diam. *Hyphae* sometimes with clamp connections, thin-walled, up to 3 µm diam.; haustorial branches present, tremelloid, rarely with a bifurcated filament, mother cell rarely triangular, (2.5–)2.7–5.2(–7) µm diam. (n = 74), filament up to 17 µm in length. *Hymenium* reduced when young but developed when old, containing numerous broadly fusiform to ellipsoid probasidia with a basal clamp connection. *Basidia* narrowly elongate ellipsoid to pyriform, 2-celled when mature, with one transverse, rarely oblique or longitudinal septum, when transversely septate often stalked, rarely constricted at the septa, exceptionally 3-celled, with 2 transverse septa, clavate to subcylindrical, resembling

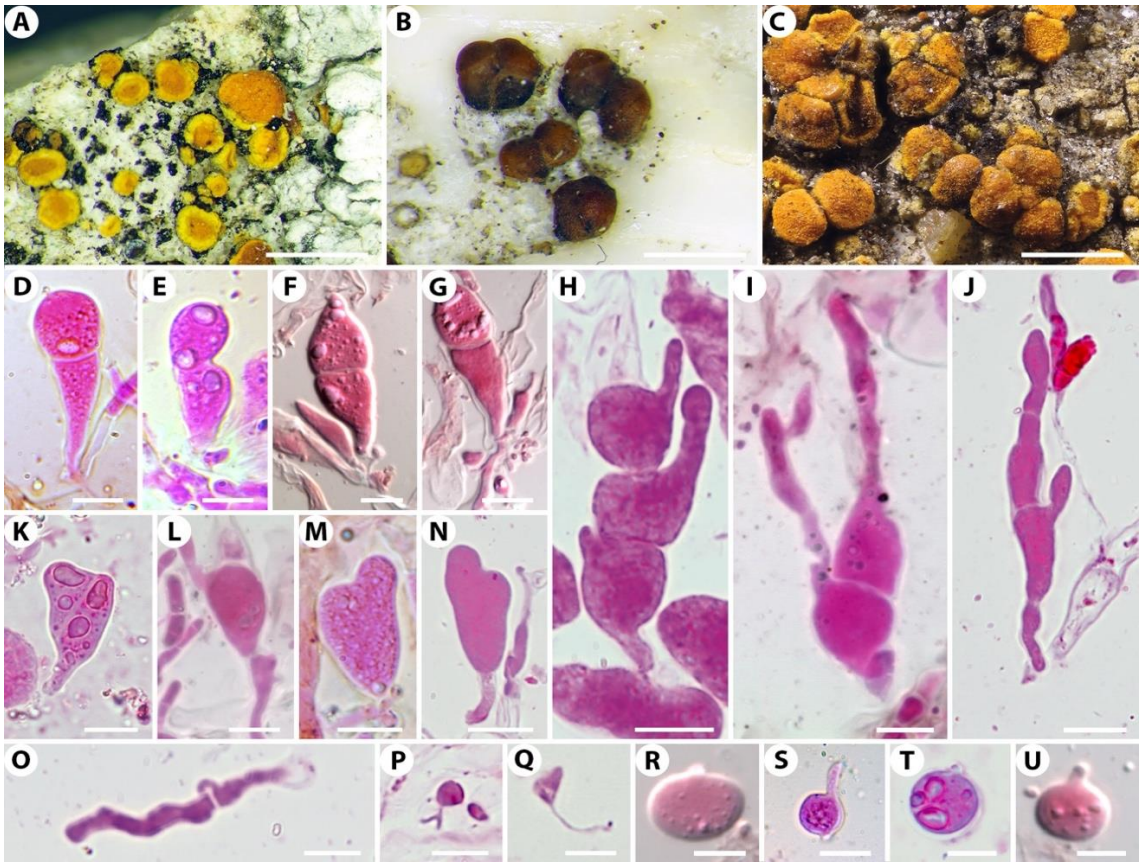

Fig. 4. *Tremella nimisiana* (A, E, M & S, holotype on *Xanthocarpia marmorata*; B, H, I, J, L, N–Q, *B. de Lesdain* 1906 on *Xanthocarpia* sp.; C, F, G, R & U, *Hafellner* 24839 on *X. lactea*; K & T, *Thor* 8202 on *X. ferrarii*). A–C, variation in gall morphology. D–G, I & J, 2-celled transversely septate basidia with haustoria and clamp connections. H, transversely septate 3-celled basidium. K & L, obliquely septate basidia. M & N, longitudinally septate basidia. O, hypha with clamp connection. P & Q, haustoria. R–U, basidiospores. Scales: A–C = 1 mm; D–N = 10 µm; O–U = 5 µm.

Biatoropsis basidia, (17–)17.1–37.9(–55.5) × (7–)9.6–13.6(–17) µm (n = 142) [transversely septate: (17–)23.8–37.9(–55.5) × (7–)9.6–13.4(–17) µm (n = 132); obliquely septate: (18–)17.1–35.2(–48) × (11–)10.6–13.6(–15) µm (n = 8); longitudinally septate: (21.5–)21.0–23.6(–23.5) × (8–)6.9–11.9(–11.5) µm (n = 2)]; epibasidia elongate, (2.5–)2.9–5.7(–11.5) µm diam. (n = 62), up to at least 40 µm in length. *Basidiospores* subspherical to ellipsoid, apiculus present, (7–)7.5–10.3(–12) × (6–)6.9–10.1(–11.5) µm (n = 38).

Etymology. Named after Pier Luigi Nimis, the Italian lichenologist, in recognition of his great work and contribution to lichenology.

Ecology and distribution. *Tremella nimisiana* is known to grow in the hymenium of *Xanthocarpia ferrarii* (Bagl.) Frödén *et al.*, *X. lactea*, *X. marmorata* (Bagl.) Frödén *et al.* and *Xanthocarpia* sp. The species is known from Austria, Estonia, France and Spain.

Notes. *Tremella nimisiana* resembles *T. caloplacae* s. str. since it induces the formation of convex galls in the hymenium of its host. *Tremella nimisiana*, however, grows exclusively on *Xanthocarpia* spp. and also differs from other species in the *T. caloplacae* complex by the size of the basidiomata (often < 1 mm diam.), the presence of hyphae with clamp connections, and the formation of 2-celled ellipsoid to pyriform basidia, or rarely 3-celled clavate to subcylindrical basidia. Other species within the *T. caloplacae* complex with occasionally 3-celled basidia are *T. parietinae*, *T. pusillae* and *Tremella* sp. 13, but these can be distinguished from *T. nimisiana* by their shorter and wider basidia. Another species commonly producing 3-celled basidia is *Tremella phaeographinae* Diederich & Aptroot, but this differs in the smaller basidia. The genetic differences among the specimens of *T. nimisiana* suggest that it is a potential species complex, which will probably be confirmed when there is a better knowledge of the species. The circumscription of *Tremella nimisiana* s. str. may therefore need to be modified in the future.

Additional specimens examined. **Austria:** *Burgenland*: Mühlgraben SW von Jennersdorf, bei den Gehöften, am Bachufer, 310 m, auf Beton einer alten Brücke, on *Xanthocarpia lactea*, 1990, *Hafellner* 24839 (GZU).—**Estonia:** *Harjumaa*: Tallinn Botanic Garden, Kloostiimetsa, 59°28′N, 24°52′E, 24 m, on *X. ferrarii* and *Xanthocarpia* sp., 1989, *Thor* 8202 (S).—**France:** *Nord*: Bray-Dunes à la frontière belge,

sur les vieilles coquilles, on *Xanthocarpia* sp., 1906, *B. de Lesdain* (ANGUC—holotype of *Caloplaca lactea* f. *ostreaeseda* (Harm.) Zahlbr.) (Navarro-Rosinés & Hladun 1996).**—Spain:** *Zaragoza*: Belchite, La Lomaza de Belchite, road to Nª Sª del Rosario, 625 m, calcareous knoll, on *X. marmorata*, 2003, *Etayo* 20387 (hb. Etayo).

Tremella parietinae **Freire-Rallo, Diederich, Millanes & Wedin sp. nov.**

MycoBank No.: MB 847665

Differs from *Tremella caloplacae* in the presence of hyphae with clamp connections, the broadly fusiform probasidia, the rarely 3-celled basidia, bigger basidiospores (6.5–11.9 × 5.7–11.9 µm), and in developing only on *Xanthoria parietina*.

Type: Spain, Madrid, Villaviciosa de Odón, Área Recreativa El Sotillo, 40°22′03″N, 3°56′44″W, 580 m, on the bark of *Fraxinus* sp., on *Xanthoria parietina*, 2017, *Millanes* 1328 (S—holotype). DNA voucher: SF390; GenBank Accession no: OQ418450 (ITS).

Basidiomata reduced, inducing the formation of convex, yellow to orange galls in the hymenium of the host, often hemispherical, smooth, uniform and scattered all over the hymenium, sometimes irregular and forming conglomerates, (0.05–)0.10–0.42(–1.18) mm diam. (*n* = 330). *Hyphae* sometimes with clamp connections, thin-walled, up to 3 µm diam.; haustorial branches present, tremelloid, rarely with a bifurcated filament, mother cell (2–)3.0–5.3(–6.5) µm diam. (*n* = 116), filament up to 10 µm in length. *Hymenium* reduced when young but developed when old, containing numerous broadly fusiform to ellipsoidal probasidia with a basal clamp connection. *Basidia* subspherical, pyriform or ellipsoid, 2-celled when mature, with one transverse, sometimes oblique, rarely longitudinal septum, when transversely septate sometimes constricted at the septum, or upper part subglobose and wider than the lower part, rarely basidia 3-celled

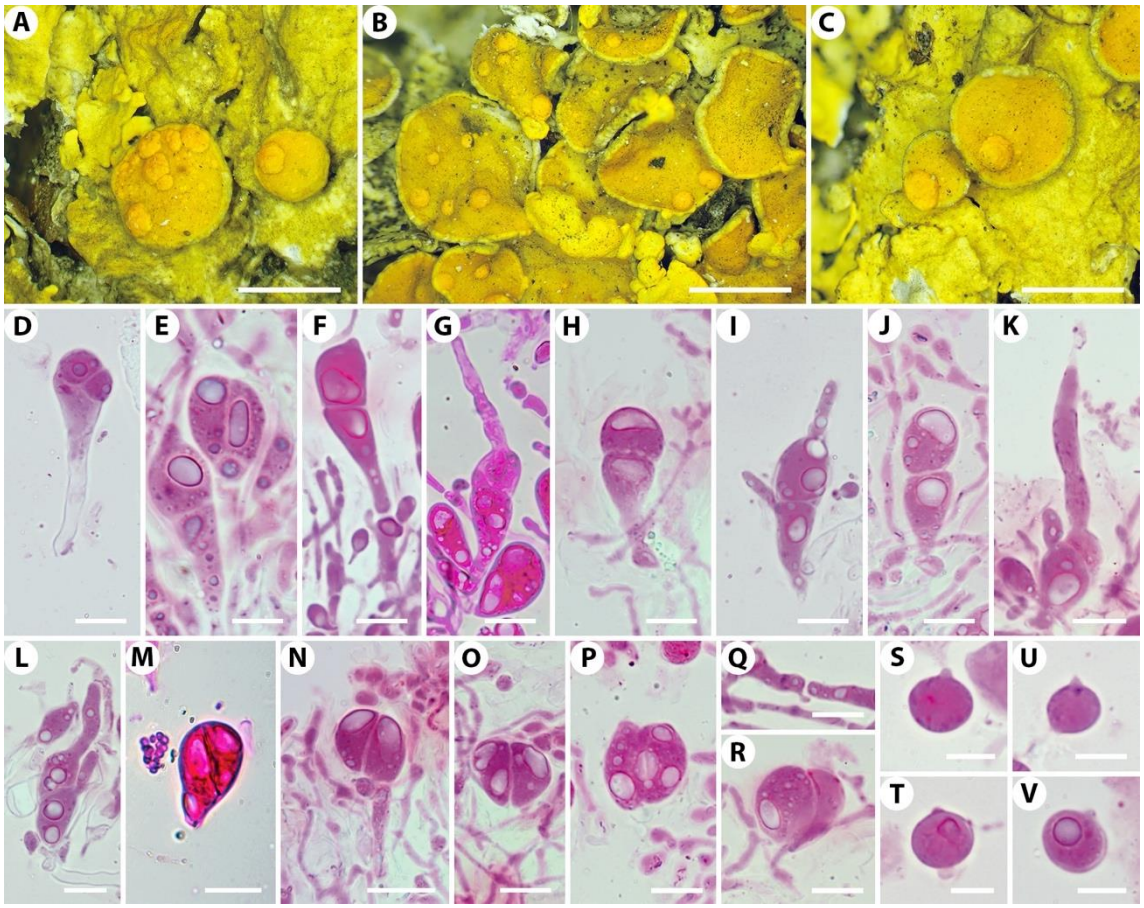

Fig. 5. *Tremella parietinae* on *Xanthoria parietina* (A, D, E, K, T & U, *Diederich* 17385; B, F, J, L, P & S, holotype; C, H, I, N, O & R, *Diederich* 17740; G, M, Q & V, *Obermayer* 12148a). A–C, variation in gall morphology. D & E, 3-celled basidia and transversely septate 2-celled basidium. F–J, transversely septate basidia with haustoria and clamp connections. K–N, obliquely septate basidia with haustoria and clamp connections. O, P & R, longitudinally septate basidia. Q, hypha with clamp connection. S–V, basidiospores. Scales: A–C = 1 mm; D–R = 10 µm; S–V = 5 µm.

with two oblique septa or one transverse and another oblique septum, rarely with a distinct stalk, (11–)14.0–34.3(–47.5) × (5–)9.7–21.5(–22) µm (*n* = 408) [transversely septate: (12–)20.7–34.3(–47.5) × (5–)10.1–14.3(–18.5) µm (*n* = 315); obliquely septate: (15–)19.6–30.3(–40) × (7.5–)9.7–14.6(–20.5) µm (*n* = 84); longitudinally septate: (11–)14.0–21.2(–23) × (14–)16.7–21.5(–22) µm (*n* = 9)]; epibasidia elongate or cylindrical, (2–)2.9–5.1(–7.5) µm diam. (*n* = 136), up to at least 43.5 µm in length. *Basidiospores* subspherical, apiculus present, (4–)6.5–11.9(–14.5) × (3–)5.7–11.9(–14.5) µm (*n* = 46).

Etymology. From *Xanthoria parietina*, the host lichen.

Ecology and distribution. *Tremella parietinae* is known to grow only in the hymenium of *Xanthoria parietina*. The species is known from Austria, Luxembourg, Portugal, Slovenia, Spain and Sweden but probably has a broader distribution as it seems not to be rare where *Xanthoria parietina* is present.

Notes. *Tremella parietinae* resembles *T. caloplacae* s. str. since it induces the formation of convex galls in the hymenium of its host, but it grows exclusively on *Xanthoria parietina* and also differs from other species in the *T. caloplacae* complex by the size of the basidiomata (often > 1 mm diam.), the presence of hyphae with clamp connections, the ellipsoidal to broadly fusiform probasidia, and the formation of 2-celled or rarely 3-celled basidia. *Tremella occultixanthoriae* is another species growing on *X. parietina*, but it grows on the lower surface of the thallus and produces 4-celled basidia with longitudinal septa devoid of epibasidia. Differences among *T. nimisiana*, *T. parietinae*, *T. pusillae* and *Tremella* sp. 13 are discussed in the notes of *T. nimisiana*. The new species *T. parietinae* is phylogenetically homogeneous.

Additional specimens examined (all on Xanthoria parietina*)*. **Austria:** *Kärnten*: Zentralalpen, 46°50′05″N, 14°47′10″E, 550 m, 2010, *Hafellner* 77065 (GZU). *Steiermark*: Nordalpen, Dachstein-Gruppe, Ramsau, 47°25′16″N, 13°38′10″E, 1170 m, 2011, *Obermayer* 12148a (GZU); *ibid*., 2012, *Obermayer* 12446 (GZU); *ibid*., 47°25′35″N, 13°39′10″E, 1180 m, 2011, *Obermayer* 12147 (GZU); Oststeirisches Hügelland, Graz, Andritz, Pfanghofweg 40a, 47°06′57″N, 15°26′18″E, 410 m, 2011, *Pinter* 11010 (GZU); Oststeirisches Hügelland, Graz, Ragnitztal, 47°4′35″N, 15°28′50″E, 380 m, 2010, *Hafellner* 77075 (GZU).—**Luxembourg:** Pétange, Fuussbësch, 2012, *Diederich* 17385 (BR); Belvaux, Metzerbierg, 2012, *Diederich* 17455 (BR); Belvaux, Kiemreech, 2012, *Diederich* 17473 (BR); Strassen, Tossebierg, 2014, *Diederich* 17740 (BR).—**Portugal:** *Lisboa*: Monsanto Natural Park, 38°43′44″N, 9°10′54″W, 180 m,

2019, *Etayo* 31826 (hb. Etayo).—**Slovenia:** Southern Alps, Julian Alps, Cezsoča S of Bovec SE above the village, 46°19′10″N, 13°33′20″E, 380 m, 2003, *Hafellner* 77507 (GZU).—**Spain:** *Castilla y León*: Burgos, Santo Domingo de Silos, 41°57′30″N, 3°24′00″W, 1130 m, 2019, *Etayo* 31851 (hb. Etayo); Burgos, between Santo Domingo de Silos and Espinosa de Cervera, 41°54′16″N, 3°28′04″W, 1145 m, 2019, *Etayo* 31889 (hb. Etayo); Burgos, road from Lerma to Santo Domingo de Silos, 2019, *Etayo* 31970 (hb. Etayo); Segovia, San Ildefonso, 40°52′22″N, 4°01′07″W, 1220 m, 2010, *Zamora*, *Zamora* & *Señoret* 2010 (G). *Extremadura*: Cáceres, Monfragüe National Park, Villareal de San Carlos, 39°50.91′N, 6°02.48′W, 290 m, 2014, *Millanes* 1192 (S); Cáceres, Monfragüe National Park, from Salto del Gitano to Fuente del Francés, 39°49′42″N, 6°03′02″W, 320 m, 2014, *Millanes* 1197 (S). *Madrid*: El Escorial, 2011, *Vivas, Zamora* & *Zamora* (G); Pinilla, 40°55′42″N, 3°48′57″W, 1100 m, 2014, *Millanes* 1190 (S); same locality as the type, 2017, *Millanes* 1297, 1304, 1364 (S); 2019, *Freire-Rallo* S128 & *Millanes* (S); *ibid*., 2011, *Zamora* (BR).—**Sweden:** *Östergötland*: Nässja Parish, 58°27′53″N, 14°48′47″E, 109 m, 2013, *Millanes* 833, 849 (S).

Tremella pusillae **Freire-Rallo, Diederich, Millanes & Wedin sp. nov.**

MycoBank No.: MB 847666

Differs from *Tremella caloplacae* in the presence of ellipsoid probasidia, the rare presence of 3-celled basidia, the size of the basidiospores (7.6–9.2 × 6.7–10.2 µm), and in developing only on *Calogaya pusilla*.

Type: Sweden, Öland, Jordhamn, 57°05′53″N, 16°53′13″E, 6 m, on calcareous rock, on *Calogaya pusilla*, 2017, *Freire-Rallo* S33 (S—holotype). DNA voucher: SF234; GenBank Accession nos: OQ192934 (ITS), OQ176384 (LSU).

Basidiomata reduced, inducing the formation of convex, light orange to brown galls in the hymenium of the host, hemispherical, irregular and rough, rarely smooth and uniform, often forming gall conglomerates in the hymenium of the host, sometimes the thalline margin of the host is not visible, (0.13–)0.14–0.55(–1.5) mm diam. (*n* = 84). *Hyphae* thin-

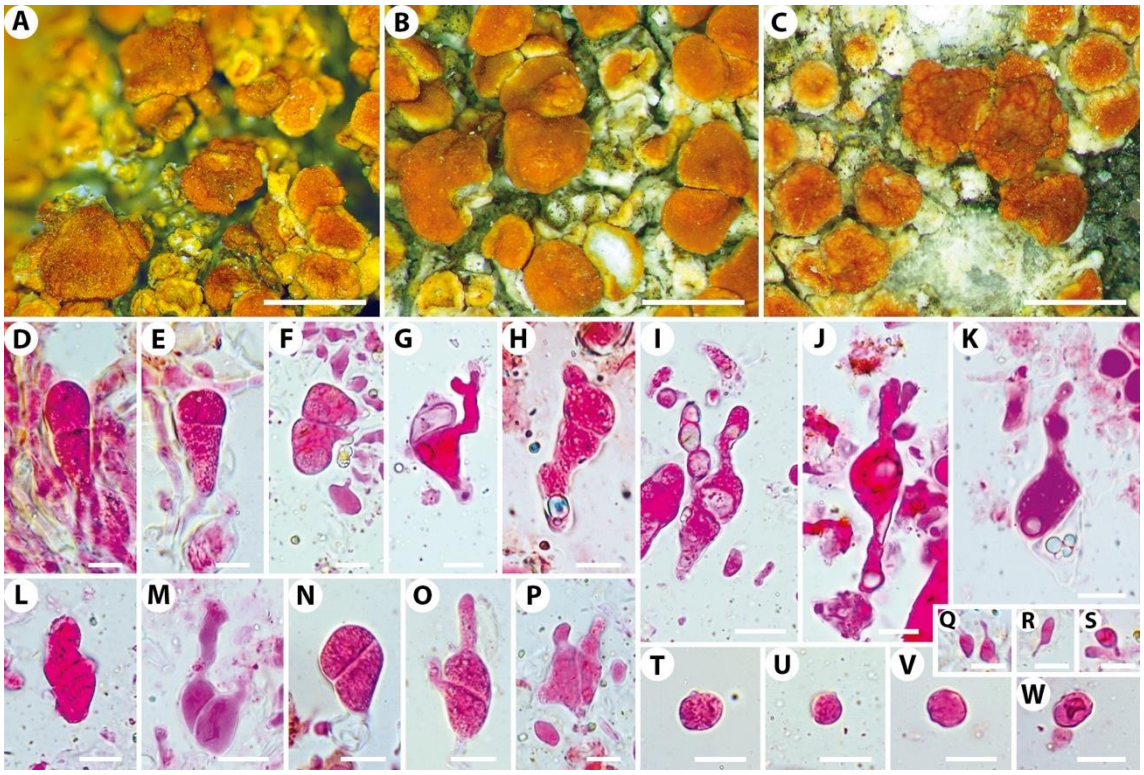

Fig. 6. *Tremella pusillae* on *Calogaya pusilla* (A, F, G, I, J, L, P–S, holotype; B, D, E, N, O, T, U & W, *Freire-Rallo* S37; C, K & M, *Freire-Rallo* S44; H & V, *Freire-Rallo* S45). A–C, variation in gall morphology. D–J, 2-celled transversely septate basidia. K–O, obliquely septate basidia. P, longitudinally septate basidium. Q–S, haustoria. F, T–W, basidiospores. Scales: A–C = 1 mm; D–P, T–W = 10 µm; Q–S = 5 µm.

walled, up to 3 µm diam., clamp connections not observed; haustoria rarely observed, haustorial branches tremelloid, mother cell (2.5–)2.3–4.8(–5) µm diam. (n = 3), filament up to 4 µm in length. *Hymenium* reduced when young but developed when old, containing numerous ellipsoid probasidia. *Basidia* ellipsoid or pyriform, 2-celled when mature, rarely 3-celled, often with one transverse or oblique, rarely longitudinal septum, when transversely septate, sometimes constricted at the septum, (13–)15.6–26.7(–30) × (6.5–)9.5–19.3(–18.5) µm (n = 58) [transversely septate: (13–)18.6–26.7(–30) × (6.5–)9.5–13.0(–15.5) µm (n = 38); obliquely septate: (13.5–)19.2–26.2(–28) × (9–)9.6–14.4(–18.5) µm (n = 18); longitudinally septate: (17–)15.6–23.3(–22.5) × (15–)14.2–19.3(–18.5) µm (n = 2)]; epibasidia cylindrical to elongate, (2.5–)2.9–5.2(–6) µm diam.

(*n* = 16), up to at least 34 µm in length. *Basidiospores* subspherical, apiculus present, (7–)7.6–9.2(–9.5) × (7–)6.7–10.2(–11) µm (*n* = 10).

Etymology. From *Calogaya pusilla*, the host lichen.

Ecology and distribution. *Tremella pusillae* induces galls in the hymenium of *Calogaya pusilla*. It is known only from Sweden.

Notes. *Tremella pusillae* resembles *T. caloplacae* s. str. since it induces the formation of convex galls in the hymenium of its host. *Tremella pusillae*, however, grows exclusively on *Calogaya pusilla* and also differs from other species in the *T. caloplacae* complex by the size of the basidiomata (often > 1 mm diam.), the absence of hyphae with clamp connections, the subspherical to ellipsoidal probasidia, and the formation of 2-celled or rarely 3-celled basidia. Differences among *T. nimisiana*, *T. parietinae*, *T. pusillae* and *Tremella* sp. 13 are discussed in the notes of *T. nimisiana*. The new species *T. pusillae* is phylogenetically homogeneous and is closely related to *Tremella* sp. 13.

Additional specimens examined (all in Calogaya pusilla*)*. **Sweden:** same locality as the type, 2017, *Freire-Rallo* S34, S44, S45 (S). *Östergötland*: Nässja parish, 58°27′53″N, 14°48′47″E, 112 m, on calcareous rocks on the church wall, 2013, *Millanes* 835 (S).

***Tremella sorediatae* Freire-Rallo, Diederich, Millanes & Wedin sp. nov.**

MycoBank No.: MB 847667

Differs from *Tremella caloplacae* and from other species in the *T. caloplacae* complex in the development of basidiomatal galls on the host thallus instead of the hymenium, and

in the presence of basidia with longitudinal septa with cells elongating and growing separately.

Type: Greenland, Qagssiarsuk, open area, 61°10′N, 45°35′W, on rock, on *Rusavskia sorediata*, 2005, *Kukwa* 4385a (UGDA—holotype; BR, S—isotypes). DNA voucher: AM32; GenBank Accession nos: OQ192945 (ITS), OQ176393 (LSU).

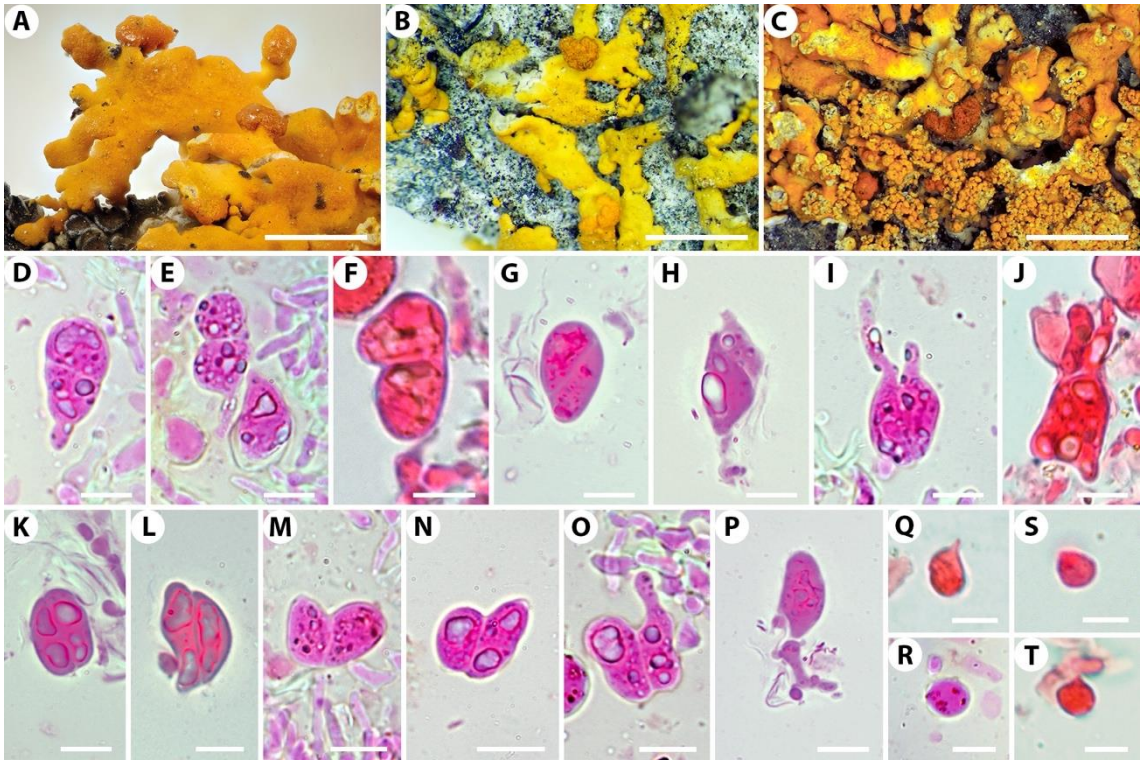

Fig. 7. *Tremella sorediatae* on *Rusavskia sorediata* (A, C, F, G, J, K, L, P, Q, S & T, holotype; B, D, E, H, I, M, N, O & R, *Goward* 01-608). A–C, morphological variation in gall morphology. D–F, 2-celled transversely septate basidia. G–J, obliquely septate basidia. K–O, longitudinally septate basidia. P, probasidium with haustorium and clamp connection. Q–T, basidiospores. Scales: A–C = 1 mm; D–P = 10 µm; Q–T = 5 µm.

Basidiomata growing on the host thallus, inducing the formation of convex, orange to brown galls, more or less subspherical, irregular and rough, often forming gall conglomerates, (0.19–)0.15–0.66(–1.34) mm diam. (n = 29). *Hyphae* thin-walled, up to 3 µm diam., clamp connections not observed; haustorial branches present, tremelloid,

mother cell (2–)2.2–3.9(–4.5) µm diam. (*n* = 21), filament up to 7 µm in length. *Hymenium* reduced when young but developed when old, containing numerous ellipsoid probasidia with a basal clamp connection. *Basidia* ellipsoid, pyriform, 2-celled when mature, often with one transverse or oblique, rarely longitudinal septum, when transversely septate, sometimes constricted at the septum, when longitudinally septate, sometimes cells elongating and growing separately, (12–)15.0–22.6(–24.5) × (7.5–)8.7–16.2(–16) µm (*n* = 42) [transversely septate: (12–)16.5–22.6(–24.5) × (7.5–)8.7–12.7(–14.5) µm (*n* = 18); obliquely septate: (12.5–)15.0–21.3(–24.5) × (8–)8.8–13.0(–15) µm (*n* = 20); longitudinally septate: (16–)16.7–19.3(–19) × (13–)13.2–16.2(–16) µm (*n* = 4)]; epibasidia elongate, (2–)2.1–4.1(–4) µm diam. (*n* = 20), up to at least 25 µm in length. Sterigma not observed. *Basidiospores* subspherical to ellipsoid, apiculus present, (4–)4.1–5.7(–6) × (3–)3.0–4.5(–5) µm (*n* = 8).

Etymology. From *Rusavskia sorediata*, the host lichen.

Ecology and distribution. *Tremella sorediatae* induces galls on the thallus of *Rusavskia sorediata*. The species is known from Greenland and Canada.

Notes. *Tremella sorediatae* grows exclusively on *Rusavskia sorediata* and differs from other species in the *T. caloplacae* complex by being the only species that induces galls on the thallus of its host. It is similar to *T. xanthomendozae*, a species apparently confined to *Xanthomendoza weberi*, that induces the formation of galls on the thallus of its host, but differs in the smaller size of its basidia and basidiospores. Differences with *T. elegantis* are discussed in the notes of this species. We have studied only two specimens of *T. sorediatae* and they form a supported monophyletic lineage. However, the genetic

differences between the two samples suggest that further material and studies are needed to clarify whether this is a single species or a species complex.

Additional specimen examined. **Canada:** *British Columbia*: Crown Lake, Marble Canyon Provincial Park, 25 km NE of Lillooet, 600 m, on *Rusavskia sorediata*, 2001, *Goward* 01-168 (BR, UBC).

***Tremella* sp. 13** (on *Calogaya biatorina*)

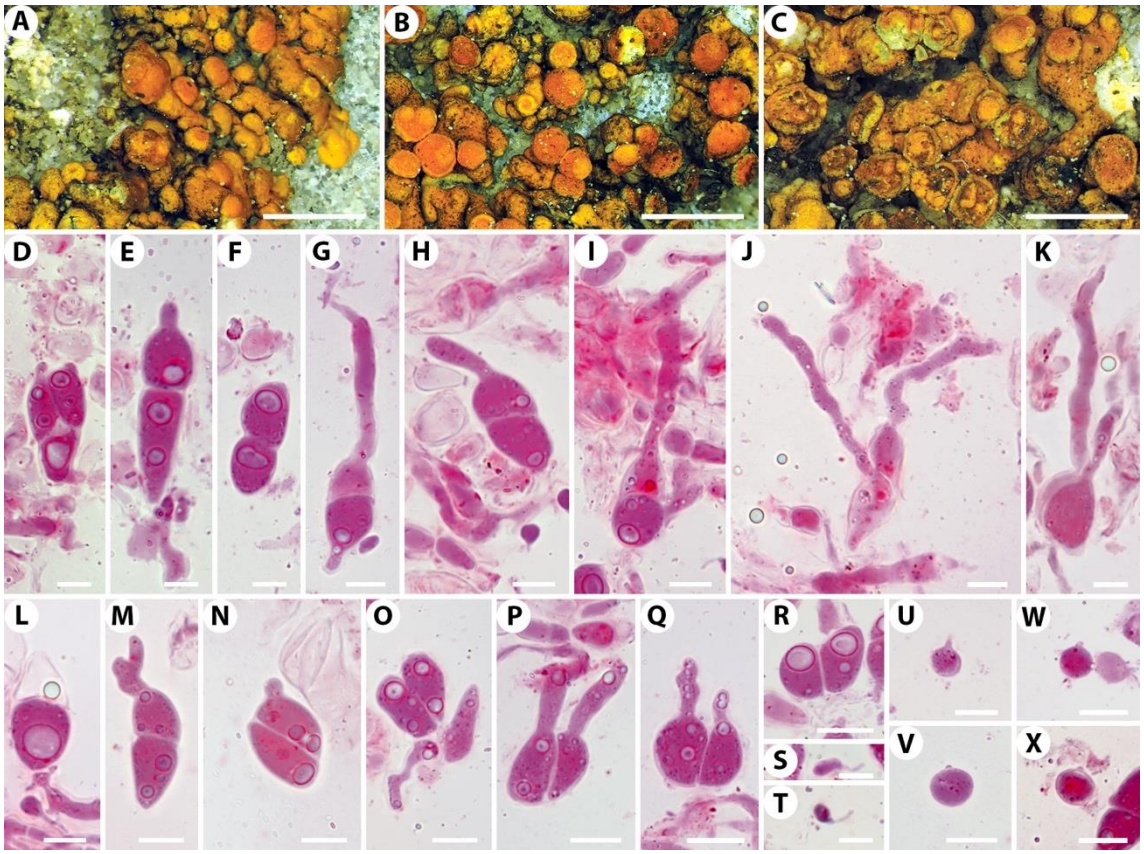

Fig. 8. *Tremella* sp. 13 on *Calogaya biatorina* (*Etayo* 20762). A–C, variation in gall morphology. D, 3-celled basidium. E–J, L & M, 2-celled transversely septate basidia with haustoria and clamp connections. N & O, obliquely septate basidia. K, P–R, longitudinally septate basidia. S & T, haustoria. U–X, basidiospores. Scales: A–C = 1 mm; D–R = 10 µm; S–X = 5 µm.

Basidiomata reduced, inducing the formation of convex, light to dark orange galls in the hymenium of the host, hemispherical, smooth and uniform, often several galls on the same apothecium but not forming aggregates, (0.12–)0.17–0.35(–0.48) mm diam. (n = 35). *Hyphae* thin-walled, up to 3 µm diam., clamp connections not observed;

haustoria rarely observed, haustorial branches tremelloid, mother cell (3.5–)3.6–5.5(–6) µm diam. (*n* = 11), filament up to 5 µm in length. *Hymenium* reduced when young but developed when old, containing numerous broadly fusiform to ellipsoid probasidia. *Basidia* narrowly elongate ellipsoid to subspherical, pyriform, 2-celled when mature, rarely 3-celled, often with one transverse septum, rarely with oblique or longitudinal septum, when transversely septate, sometimes constricted at the septum, (19–)20.0–33.3(–41.5) × (10.5–)11.4–16.8(–17) µm (*n* = 47) [transversely septate: (19–)24.2–33.3(–41.5) × (10.5–)11.4–14.5(–17.5) µm (*n* = 38); obliquely septate: (23.5–)24.0–31.2(–33.5) × (12.5–)12.6–16.2(–17) µm (*n* = 6); longitudinally septate: (20–)20.0–21.4(–21.5) × (14.5–)14.6–16.8(–17) µm (*n* = 3)]; epibasidia elongate, (3.5–)4.1–5.8(–7) µm diam. (*n* = 29), up to at least 61 µm in length. *Basidiospores* subspherical, apiculus present, (8.5–)8.7–10.2(–10.5) × (8–)8.2–10.3(–10.5) µm (*n* = 5).

Ecology and distribution. *Tremella* sp. 13 induces galls in the hymenium of *Calogaya biatorina*. It is known only from Spain.

Specimen examined. **Spain:** *Huesca*: ascent slope to Góriz, 1990 m, on *Calogaya biatorina*, 2003, *Etayo* 20762 (hb. Etayo).

***Tremella* sp. 14** (on *Calogaya decipiens*)

Basidiomata reduced, inducing the formation of convex, dark yellow to light brown galls in the hymenium of the host, subspherical, irregular, (0.09–)0.08–0.39(–0.81) mm diam. (*n* = 23). *Hyphae* up to 3 µm diam., clamp connections not observed; haustoria rarely observed, haustorial branches tremelloid, mother cell (2–)2.0–4.1(–4) µm diam. (*n* = 2), filament up to 3 µm in length. *Hymenium* reduced when young but developed when old,

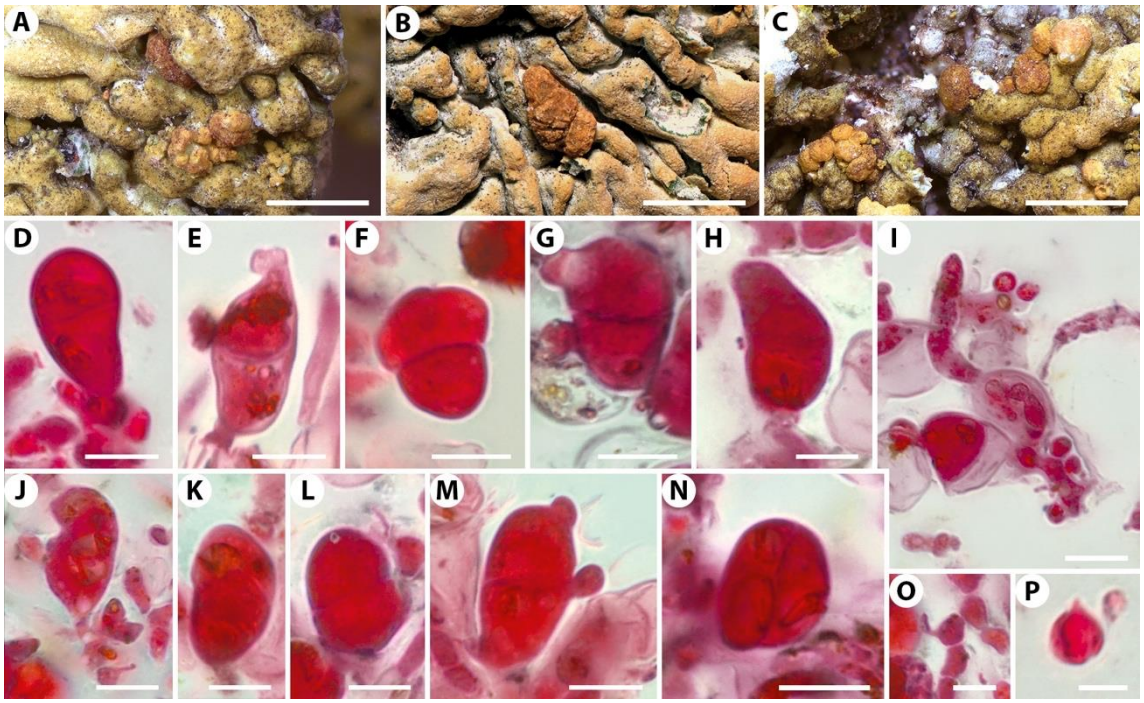

Fig. 9. *Tremella* sp. 14 on *Calogaya decipiens* (*Millanes* 850). A–C, variation in gall morphology. D–K, 2-celled transversely septate basidia with haustoria and clamp connections. L & M, obliquely septate basidia. N, longitudinally septate basidium. O, haustorium. P, basidiospore. Scales: A–C = 1 mm; D–N = 10 µm; O & P = 5 µm.

containing numerous ellipsoid probasidia. *Basidia* ellipsoid to subspherical, 2-celled when mature, with one transverse septum, rarely obliquely or longitudinally septate, when transversely septate, sometimes upper part subglobose and wider than the lower part, (11–)13.2–19.4(–23) × (8–)8.3–11.4(–14.5) µm (*n* = 39) [transversely septate: (11–)13.2–18.9(–23) × (8–)8.4–11.1(–14.5) µm (*n* = 33); obliquely septate: (16.5–)16.0–19.4(–20) × (8.5–)8.3–10.4(–10.5) µm (*n* = 4); longitudinally septate: (14–)13.7–16.9(–16.5) × (9–)8.8–11.4(–11) µm (*n* = 2)]; epibasidia elongate, (2–)2.4–3.2(–3.5) µm diam. (*n* = 16), up to at least 24 µm in length. *Basidiospores* subspherical, apiculus present, 5.5 × 4.5 µm (*n* = 1).

Ecology and distribution. *Tremella* sp. 14 induces galls in the hymenium of *Calogaya decipiens*. It is known only from Sweden.

Specimen examined. **Sweden:** *Östergötland*: Nässja parish, historically interesting area with an ancient stone circle, a churchyard, and a church, 58°27′53″N, 14°48′47″E, 109 m, on *Calogaya decipiens*, 2013, *Millanes* 850 (S).

***Tremella* sp. 15** (on *Polycauliona*)

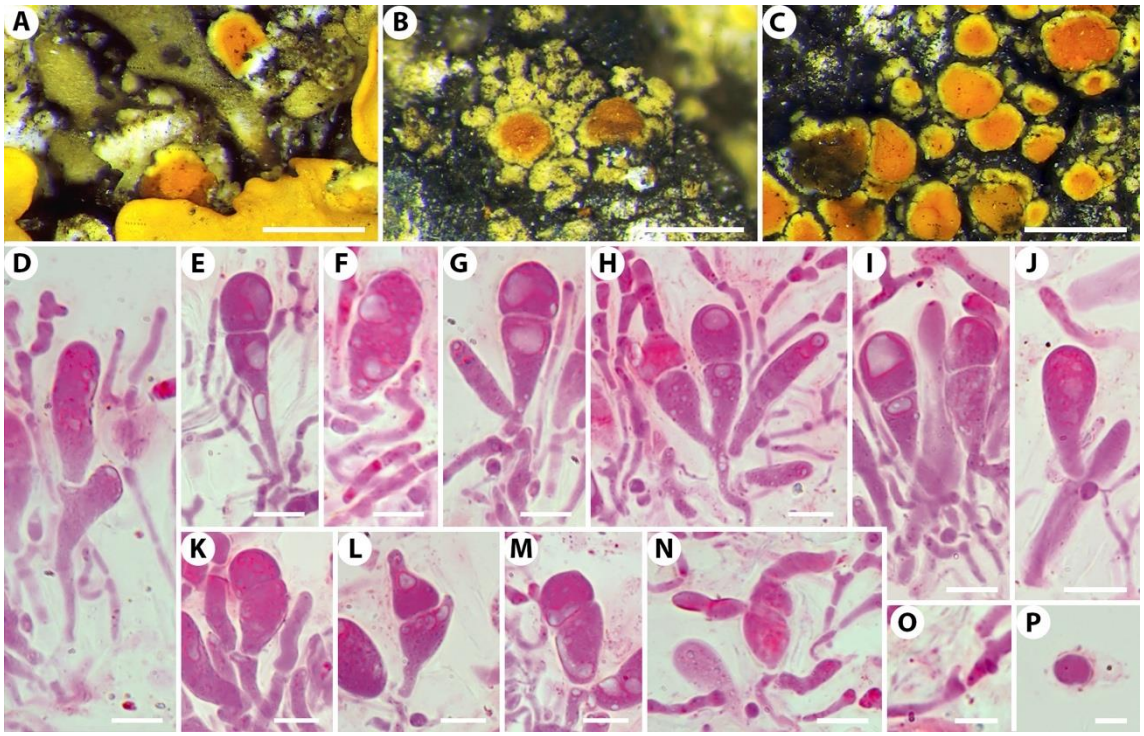

Fig. 10. *Tremella* sp. 15 on *Polycauliona* sp. (*Etayo* 19125). A–C, variation in gall morphology. D–I, 2-celled transversely septate basidia with haustoria and clamp connections. K–N, obliquely septate basidia with haustoria and clamp connections. J, probasidia with haustorium. O, hypha with clamp connection. P, basidiospore. Scales: A–C = 1 mm; D–N = 10 µm; O & P = 5 µm.

Basidiomata reduced, inducing the formation of convex, dark orange galls in the hymenium of the host, hemispherical, smooth and uniform, sometimes irregular, scattered all over the hymenium but sometimes forming gall conglomerates, (0.07–)0.08–0.25(–0.82) mm diam. (*n* = 18). *Hyphae* sometimes with clamp connections, up to 3 µm diam.; haustoria rarely observed, haustorial branches tremelloid, rarely with two filaments or one bifurcated filament, mother cell (2–)2.6–4.7(–5.5) µm diam. (*n* = 13), filament up to 7.0 µm in length. *Hymenium* reduced when young but developed when old, containing

numerous broadly fusiform to ellipsoid, pyriform probasidia. *Basidia* narrowly elongate ellipsoid to ellipsoid, 2-celled when mature, with one transverse septum, rarely obliquely septate, when transversely septate, sometimes stalked, sometimes laterally elongated cells resembling *Biatoropsis* basidia, (20.5–)26.5–40.5(–52) × (8–)10.5–13.6(–15) µm (*n* = 54) [transversely septate: (20.5–)26.5–40.5(52) × (8–)10.5–13.6(–15) µm (*n* = 53); obliquely septate: 25.5 × 13.5 µm (*n* = 1)]; epibasidia elongate, (2.5–)3.3–4.9(–5) µm diam. (*n* = 19), up to at least 37 µm in length. *Basidiospores* subspherical, apiculus present, 6.5 × 7.0 µm (*n* = 1).

Ecology and distribution. *Tremella* sp. 15 induces galls in the hymenium of *Polycauliona* sp. It is known only from Spain.

Specimen examined. **Spain:** *Teruel*: between Rubielos and Mora, Fuentes del Ocino, 1100 m, on *Polycauliona* sp., 2002, *Etayo* 19125 (hb. Etayo).

Chapter 3

Insights into the life cycle of *Tremella caloplacae* s. l.

Sandra Freire-Rallo, Veera Tuovinen Nogerius, Mats Wedin and Ana M. Millanes

Abstract

Complex life cycles are common among fungi. Life-cycle dimorphism –which involves the alternance of a unicellular yeast phase and a filamentous dykariotic phase – is particularly frequent in Basidiomycota and is often a characteristic of parasite species of plants, animals, and other fungi (including lichens). Lichen-inhabiting (lichenicolous) fungal species are represented both in Ascomycota and Basidiomycota, but lichen-inhabiting dimorphic basidiomycetes have recently attracted much interest as more is discovered about their diversity and biology. Among the lichenicolous dimorphic basidiomycetes, the Tremellomycetes are the most diverse group and include species that are very specific towards the lichens they live on, on which they often induce the formation of galls or deformations. The filamentous stage of these fungi has been traditionally more studied than the yeast stage, and species are usually described based mainly on this filamentous stage. *Tremella parietinae* (Tremellomycetes, Basidiomycota) is a lichen-inhabiting species that belongs to the *Tremella caloplace* complex, and which filamentous phase is known to develop exclusively in the hymenium of its hosts, the widespread lichen *Xanthoria parietina* (Teloschistaceae, Lecanoromycetes, Ascomycota). It is unknown, however, if the yeast phase of this species is also restricted to the same location within the lichen. Using molecular methods (Polymerase chain reaction (PCR) and Sanger sequencing) and fluorescent *in situ* hybridization coupled to confocal laser scanning microscopy (FISH-CLSM), we investigate the life cycle of *T. parietinae* and the distribution of the yeast and filamentous phases of this species, within the host lichen. Contrarily to the filamentous phase, the yeast phase is not restricted to the hymenium but grows also in the thallus of the lichen. Using molecular methods, we also achieve a pilot investigation of the presence of asymptomatic *Tremella* yeasts in other lichen species of the Teloschistaceae. Our results indicate that at least the yeast phase of *Tremella caloplacae* s. str. is less specific towards the lichen than its filamentous phase.

Keywords: basidiomycete, confocal laser scanning microscopy, fluorescent *in situ* hybridization, life cycle, yeast

Introduction

Fungal diversity is represented by a wide variety of morphologies, lifestyles and nutritional habits (Hawksworth & Lücking, 2017). Concerning nutritional habits, two big groups could be considered: free living species and others that need to live in association with other organisms. Among the last, the lichenized fungi live in symbiosis with algae or cyanobacteria, forming lichens (Grube & Wedin, 2016). However, a great variety of other fungi living in association with lichens are also known, the so-called mycobiome (Grimm et al. 2021). Lichenicolous fungi form part of this mycobiome, developing their life cycle in or on lichens in an obligate manner (Grube & Wedin, 2016). They show from symbiotic to parasitic behavior towards the lichen (Diederich et al. 2022) and are either Ascomycetes (96%) or Basidiomycetes (4%) (Diederich et al. 2018). Within the comparingly small amount of existing lichenicolous Basidiomycetes, one of the most diverse groups is the Tremellomycetes. These are generally dimorphic which means that they can alternate a monokaryotic unicellular yeast and a dikaryotic filamentous phase, presumably depending on the physical or chemical conditions of their environment (Bahn et al. 2005, Sia et al. 2000, Lin 2009, Hou et al. 2011). The filamentous stage has been traditionally more studied than the yeast stage in jelly fungi and in lichenicolous species. In particular, numerous Tremellomycete lichenicolous species have been described in the last decades by studying the basidiomata (sexual fruiting bodies) that they form in their lichen host (Diederich et al. 2018, 2022).

The presence of a yeast stage of Basidiomycete fungi in Ascomycete lichens has been largely known (add references) but also overlooked and seldom isolated (Girlanda et al. 1997; Prillinger et al. 1997; U'Ren et al. 2012; Muggia et al. 2016; Cometto et al. 2022). However, new studies on Basidiomycetes yeasts in lichens have emerged in recent years (Fernandez-Mendoza et al. 2017; Lendemer et al. 2019; Mark et al. 2020; Smith et al.

2020; Tagirzhanova et al. 2021; He et al. 2022) including studies using Fluorescent *in situ* hybridization (FISH) to specifically bind target species (Spribille et al. 2016; Tuovinen et al. 2019, 2021). Spribille et al. (2016) found *Cyphobasidium* (Cystobasidiomycetes, Pucciniomycotina) yeasts in a great variety of macrolichen taxonomical groups, specially in the family Parmeliaceae, and this led the authors to question the role of these Basidiomycete yeasts in the lichen symbiosis. They also hypothesized that the presence of *Cyphobasidium* yeasts correlates with phenotypic variations in *Bryoria* species. In *Bryoria* and *Letharia* species, the *Cyphobasidium* yeasts were visualized in the cortex of specimens without presence of galls (deformations induced in the lichen host by the Tremellomycete basidiomata or sexual fruiting bodies). *Tremella lethariae* induces galls in *Letharia* lichens, but its yeast stage has also been detected in the cortex of several species of *Letharia* specimens without galls (Tuovinen et al. 2019). Something similar occurs with *T. macrobasidiata* and *T. variae,* described inducing galls in *Lecanora chlarotera* and *L. varia*, respectively (Zamora & Perez-Ortega, 2011; Zamora et al. 2016), but which yeast stage has been visualized also in the cortex or the hymenia of specimens without galls (Tuovinen et al. 2021). These studies on *Tremella* yeasts also suggested the yeast phase to be less host specific that the filamentous phase and reported sequences from different *Tremella* species in the same lichen specimen. Sequences of *T. lethariae* and of a different lineage of *Tremella* denominated *Tremella* sp. B (Lindgren et al. 2015) were obtained from the same *Letharia* specimens (Tuovinen et al. 2019). Likewise, *T. macrobasidiata* and *T. variae* yeasts were detected by PCR and FISH-CLSM analysis indistinctly in *L. chlarotera* or *L. varia* lichens with or without galls, but its filamentous phases and sexual structures were restricted to their corresponding lichen hosts (Tuovinen et al. 2021). This same pattern occurs in the case of *Cyphobasidium hypogymniicola*, whose yeast phase has been reported in several species of macrolichens (Mark et al.

2020), but so far, its filamentous phase is only known from *Hypogymnia physodes* (Millanes et al. 2016; Diederich et al. 2022).

Tremella comprises a great number of lichenicolous species from which there are still knowledge-gaps about their life cycles, reproductive strategies, or species boundaries (Diederich 1996; Zamora et al. 2016; Tuovinen et al. 2019, 2021; Freire-Rallo et al. 2023a). *Tremella caloplacae* s. l. is one of these species for which there is only known its filamentous stage (Zahlbruckner 1906; Diederich 1996, 2007; Sérusiaux et al. 2003). This species was erroneously described as a Hyphomycete in 1906 (Zahlbruckner 1906), almost a century later it was formally described as a lichenicolous Tremellomycete (Sérusiaux et al. 2003) and took two more decades to verify that it was a species complex (Freire-Rallo et al. 2023a,b) that will continue to expand with all probability. Five new species were formally described based on basidiomata morphology within the *T. caloplacae* species complex and *T. caloplacae* s. str. was circumscribed to specimens growing on *Variospora* spp (Freire-Rallo et al. 2023b). *Tremella elegantis* induce the formation of galls in the hymenium of *Rusavskia elegans*, *T. nimisiana* in the hyenium of *Xanthocarpia* spp., *T. parietinae* in the hymenium of *X. parietina*, *T. pusillae* in the hymenium of *Calogaya pusilla* and *T. sorediatae* induce galls on the thallus of *R. sorediata* (Freire-Rallo et al. 2023b). There is still a poor knowledge of these species, and it has been assumed that, like other Tremellomycetes and lichenicolous *Tremella* species they have a dimorphic life cycle (Diederich 1996; Chen 1998; Tuovinen et al. 2019, 2021), but it is interesting to investigate their life cycle further. Among the species in the complex, *Tremella parietinae* is particularly suited for such studies, since galls are only induced in the hymenium of *Xanthoria parietina*, which is a common and widespread lichen species.

In this work we wanted to study the different phases of the life cycle of *T. parietinae* and its location within *X. parietina*. The yeast stage of *T. parietinae* has never been detected by optical microscopy, so we also aimed to detect the presence of *T. parietinae* yeasts in *X. parietina* specimens with and without galls. We performed FISH-CLSM and PCR analysis and Sanger sequencing in samples from different areas of the lichen host, in specimens with and without galls collected in Spain (population A) (Table S1). More specifically, we aimed to 1) study if the filamentous phase of *T. parietinae* was restricted to the hymenium of *X. parietina*, 2) confirm that there is a yeast stage in the life cycle of *T. parietinae*, 3) investigate if *T. parietinae* completes its life cycle in the lichen host and, if so, 4) identify where the different phases are located within *X. parietina*. We also wanted to preliminary study the presence of a yeast stage in different localities, in different species of *T. caloplacae* s. l. and in different potential host lichens of the Teloschistaceae. For this we performed a pilot study and collected samples in Sweden (Stockholm, population B; several localities in Öland) (Table S1) and analyzed the samples by PCR and Sanger sequencing.

Materials and methods

Sampling and DNA extraction

To study the location of the different phases of the life cycle of *Tremella parietinae* within *Xanthoria parietina*, and to investigate the presence of *T. parietinae* in specimens without galls, we selected a population of *X. parietina* (population A, Spain, Table S1) with a high incidence of specimens showing *Tremella* galls. This population is situated in "El Sotillo" Recreational Area, within the Regional Park of the Middle Course of the Guadarrama River (www.comunidad.madrid.es). It is an ecological corridor that forms a gradient of riparian vegetation from the river and its alluvial deposits with areas of *Arundo donax*, *Phragmites* spp., *Typha* spp., *Scirpus* spp., *Populus nigra*, *P. alba*, *Salix* spp., *Ulmus*

minor, and *Fraxinus angustifolia* to forests of *Pinus pinea*, *P. pinaster* or *Quercus faginea*, and *Q. rotundifolia* typical of Mediterranean forests that can be found further away from the Guadarrama River. Samples were collected in an easily accessible area (40°21'59.4"N 3°56'43.1"W), dominated by *Fraxinus*, *Populus* and *Ulmus* along the Guadarrama river, located in the vicinity of the M-501 motorway and on the outskirts of Villaviciosa de Odón (Madrid, Spain). The recreational area is a busy area, crossed by several trekking paths, with picnic and children's areas.

In this population we collected 22 fresh specimens of *X. parietina*, 6 with and 16 without galls induced by *T. parietinae*. (Table S1). In addition, to achieve a pilot screening both in alternative localities of *X. paritietina* and in other lichen species in the *T. caloplacae* complex we collected 8 samples in a second population of *X. parietina* (population B, Sweden, Table S1) in which *Tremella* galls were not observed and we also collected 15 specimens of other lichen species in the Teloschistaceae (Öland, Sweden, Table S1) that were thoroughly examined for the presence of *T. caloplacae* s. l. Of the total number of these lichens, 4 had galls of *T. caloplacae* s. l. Population B is in Stockholm (Sweden), in an urban area in the vicinity of the National Museum of Natural History, the University of Stockholm and the metro station Universitetet. The vegetation is typical of urban areas, made up of *Acer platanoides*, *Betula pendula*, *Pinus sylvestris*, *Populus tremula*, *Sorbus* spp. and *Tilia* spp. This area is heavily used by pedestrians and road traffic, the latter mainly coming from the Roslagsvägen highway. Other Teloschistaceae samples were collected in different localities of Öland (Sweden). They come from calcareous cliff rocks in coastal environments and from the designated World Heritage Site by UNESCO, Stora Alvaret (the Great Alvar). This is an area formed by a bedrock of sedimentary calcareous limestones where higher plants are scarce and the soil-crust is dominated by *Cladonia*

spp., *Thamnolia vermicularis*, *Squamarina cartilagínea*, *Fulgensia* spp. and *Psora decipiens* lichens (Albertson 1950; Fröberg 1999; Büdel et al. 2014).

In all the localities where samples were collected, there were specimens with and without galls except for the locality with *Xanthoria parietina* in Sweden (Locality B) where no galls of *T. parietinae* were found.

Material fixation for FISH

Of the *X. parietina* lichens collected in Spain, two specimens with galls of *T. parietinae* and three samples without galls were selected for FISH analysis. Samples were collected after a period of rain and subsequently treated following the instructions in Tuovinen et al. (2021) to ensure the lichens were metabolically active. Petri dishes were used to place the specimens with moisturized filter paper, sprayed with $_{dd}H_2O$ and loosely covered with a lid. Once they get dried samples were moisturized again, replicating the process for seven days. Afterwards, samples were moisturized one final time and after that we cut by hand pieces of thalli, apothecia without galls and apothecia with galls of *T. parietinae* from *X. parietina* specimens with galls. From *X. parietina* specimens without galls, we cut pieces of thalli and apothecia. Lichen pieces were placed in Eppendorf tubes with 1 ml of 1 × PBS (pH 7.4) in a vacuum chamber. Vacuum was applied to the tubes with open lids for c.a. 10 s, then the tubes were closed and carefully tapped to help to remove the air from the samples. Cycles of vacuum were applied until the lichen pieces sunk to the bottom of the tubes. PBS were replaced by 4% formaldehyde (methanol free) for fixation and incubated for 2 h at 4 °C. Samples were then washed three times in 1 × PBS for 10 min each, to remove traces of formaldehyde. Afterwards, samples were incubated twice in acetone for 8 h to dissolve strongly autofluorescent secondary metabolites. The

acetone was washed with 1 × PBS as previously done, the PBS was removed and the samples were stored at −20 °C until FISH.

Design of FISH probes

Specific probes for Tremellales (this study) and Lecanoromycete (Spribille et al. 2016; Tuovinen et al. 2019) species based on nuLSU sequences from *T. parietinae* and *X. parietina*, were designed and used following Tuovinen et al. (2021) (Table 1). Probes for Tremellales match multiple *Tremella* species from the *T. caloplacae* complex but not *X. parietina*; likewise, probes for the Lecanoromycete match *X. parietina* while not *Tremella* species. Since the probes could bond to any *T. caloplacae* s. l. species, PCR and sequencing were used to investigate the presence of the target species *T. parietinae*. *Tremella* species has strong autofluorescence in green wavelengths, whilst *X. parietina* and its photobiont are autofluorescent in red (hyphae and algae) and green (paraphyses, ascospores and algae) wavelenghts. To enhance the fluorescent signal of the Tremellales probes a double labelling with 6-FAM at 5' end and FITC at 3' end, with signal in green wavelengths, was performed. Each of the probes for the Lecanoromycete were mono labelled with Cy5 at 5' end, with signal in red wavelengths.

Fluorescent* in situ *hybridization

Fixed material of *X. parietina* was treated for permeabilization and hybridization as described by Tuovinen et al. (2019). To ensure that the probes entered the cells, the material was permeabilized for 1 h in 1 × PBS at 36 °C with 0.1 mg/ml (0.04 U) chitinase and 1% SDS. Samples were then washed thrice in 1 × PBS for 10 min, subsequently dehydrated in 50%, 80% and 99.7% ethanol and let air-dry. For hybridization, the fragments were incubated for 3 h at 46 °C in a solution of 1 µM of each probe and helper probe, 0.5 µM Hoechst 333442 for staining nuclei, and hybridization buffer containing

Table 1. Probes, helper probes and laser lines used for fluorescent *in situ* hybridization and confocal laser scan microscopy.

Xanthoria parietina	Probe sequence 5'–3'	Laser line	Target/comment	Source
Tm_28SCalo	6-FAM-TATCACAGAGCGCCGGTC-FITC	488	Tremellomycete	This study
Tm28SCalo_hlprA	GTCGTGTCAAGTACGGGA		Used with Tm_28SCalo	This study
Tm28SCalo_hlprB	CTCGACTCGTAGAAGA		Used with Tm_28SCalo	This study
Lec28S_1_Xan	Cy5-TCTGGTTGCAAGCGCTTC	633	Lecanoromycete	Tuovinen et al. 2019
Lec28S_1_Xan_hlprB	ACGGCTGATCACCCGCGG		Used with Lec28S_1_Xan	Tuovinen et al. 2019
Lec28S_170_Xan	Cy5-CGAAGGGGTTTCACAC	633	Lecanoromycete	Spribille et al. 2016
Lec28SXan_170_hlprB	TGAGCTGCATTCCCAAAC		Used with Lec28S_170_Xan	Spribille et al. 2016
Hoechst 333442		405	Nuclei	Thermo Fisher Cat#62249

10% formamide, 0.9 M NaCl, 0.01% SDS and 20 mM Tris-HCl (1 M pH 7.2). For each hybridization reaction, a negative control without probes was included. Hybridization solution was removed and the samples were incubated for 20 min at 48 °C in 0.45 M NaCl, 0.02 M Tris-HCl, 0.01% SDS washing buffer. Then, the fragments were washed in 1 × PBS for 10 min and let air-dry. Mounting of the hybridized samples was carried out with ProLong Glass Antifade (ThermoFisher) on microscopy slides.

Confocal laser scanning microscopy and image processing

The CLSM scanning of the hybridized slides were carried out by using an inverted IX83 Olympus confocal microscope with Olympus X-Line 60× oil immersion objective. Excitation of Hoechst, 6-FAM/FITC and Cy5 fluorophores was made with 405, 488 and 633 laser lines, respectively, with detection wavelengths of 426–476 nm for Hoechst, 493–598 nm for green and 638–797 nm for red. As both *T. parietinae* and *X. parietina* have strong autofluorescence in green and red, negative controls were used to set laser intensities and gain values to avoid interpretation of autofluorescence of the sample as hybridization signal. Simultaneous scanning was used for Hoechst and Cy5 and sequential scanning for 6-FAM. Z stacks of the samples were acquired with the microscope CellSens Dimensions software (Olympus).

Images were processed with Fiji (Schindelin et al. 2012) and ImageJ (Schneider et al. 2012). Sum slices projections and 3D projects were made, and color balance was adjusted for the clarity of presentation.

DNA extraction and amplification

From *X. parietina* specimens with galls, we performed DNA extractions of galls, thalli, hymenia and complete apothecia without galls; from *X. parietina* specimens without galls the DNA extractions were carried out from the thalli, hymenia and complete apothecia (Table S1). We followed the same criteria for DNA extractions of other lichens in the Tesloschistaceae, but no samples were selected from galls in those specimens with galls. Material was DNA extracted by using the Qiagen DNeasy Plant MiniKit, following manufacturer's instructions but eluting twice with 50 µl of ultrapure water as final step.

PCR amplification of all DNA extractions was performed using specific primers for *Tremella,* targeting the internal transcribed spacer I (ITS1), the 5.8S rDNA gene, the internal transcribed spacer II (ITS2) and a portion of ca. 900 nucleotides of the nuLSU rDNA gene. PCRs were made by combining the specific primers BasidLSU3-3, BasidLSU1-5 (Millanes et al., 2011), TRMcal_R2 and TRMLSU_1F (Freire-Rallo et al. 2023a) with general fungal primers ITS1F (Gardes and Bruns, 1993) and LR5 (Vilgalys and Hester, 1990).

PCRs were performed using Illustra^{TM} Hot Star PCR beads, according to the manufacturer's instructions, with the following settings for each primer combination: 1) for ITS1F/BasidLSU3-3, ITS1F/TRMcal_R2, TRMLSU_1F/LR5 we run an initial denaturing at 95 °C for 5 min; 4 cycles of 95 °C for 40 s, 53 °C for 40 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; 32 cycles of 95 °C for

30 s, 47 °C for 30 s and 72 °C for 90 s; final extension step of 72 °C for 10 min, and 2) for BasidLSU1-5/LR5 and BasidLSU3-5/LR5 we used an initial denaturing at 95 °C for 5 min; 4 cycles of 95 °C for 40 s, 56 °C for 40 s and 72 °C for 90 s; 4 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s; 32 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; final extension step of 72 °C for 10 min. DNA amplification was performed by adding 5 µl of DNA extraction and 0.5 µl of each primer of a primer concentration of 10 µM, except for the gall samples were we added 2 µl of DNA extraction. PCR amplification products were purified prior to sequencing with Exo-sap-ITTM (USB Corporation, Cleveland, Ohio, USA). Samples were sent for Sanger sequencing to Macrogen Spain (Barajas, Spain). These sequences, together with those from Freire-Rallo et al. (2023b) (Table 1), were used for the phylogenetic analysis.

Sequences alignment and phylogenetic analysis

Assembling and editing of newly produced sequences was performed with Geneious Prime® 2021.0.3. (www.geneious.com). We created a data matrix for the ITS1, 5.8S, ITS2 and a portion of the nuLSU regions, combining the newly produced sequences with the sequences of *T. caloplacae* s. l. from Freire-Rallo et al. (2023b). For the phylogenetic analysis *T. candelariellae* was used as outgroup based on previous literature (Millanes et al. 2011; Liu et al. 2015; Freire-Rallo et al. 2023a). Sequences were aligned using the L-INS-I algorithm implemented in MAFFT (Katoh et al., 2002, 2005; Katoh and Standley, 2013). We applied GBlocks v.0.91b (Castresana, 2000) to identify and exclude misaligned positions, major insertions and ambiguous and/or divergent regions, with a relaxed selection of blocks as suggested by Talavera and Castresana (2007). We used Mesquite v.3.6 (Maddison and Maddison, 2018) to check the alignments and convert terminal gaps to missing data. Maximum likelihood (ML) analysis were performed with IQTree (Nguyen et al. 2015) considering four independent partitions, ITS1, 5.8S, ITS2 and

nuLSU. Conflicts among each individual partition were assessed with maximum likelihood ultrafast bootstrap in IQTree, considering highly supported clades (IQTree UF-BS > 95%) in disagreement an indication of conflict (Mason-Gamer & Kellogg, 1996; Hoang et al. 2018). No conflict was detected in our data matrices; thus, we combined our four partitions in a single data set for subsequent analysis. ModelFinder in IQTree (Kalyaanamoorthy et al. 2017) was used to select the model for each partition, with the corrected Akaike information criterion (AICc): the TIMe + I model was selected for the ITS1, the K2P model for the 5.8S, the TIM2e + Γ4 for the ITS2, and the TIM3 + F + Γ4 for the nuclear LSU. Finally, a maximum likelihood search was performed and support achieved by standard bootstrap using 1000 bootstrap pseudorreplicates.

Results

DNA amplification and phylogenetic analysis

We performed a total of 92 PCR reactions on 22 specimens of *Xanthoria parietina* from Population A (6 with galls and 16 without galls), using *Tremella* specific primers for the ITS1, 5.8S, ITS2 regions. We detected *Tremella parietinae* in 5 (ca. 31%) specimens of *X. parietina* without galls and in 5 (ca. 83%) specimens with galls (Table 2). Using 8 specimens of *X. parietina* without galls collected in Sweden (Population B, Table S1), we carried out 36 PCR reactions also with specific primers of *Tremella* but for the ITS1, 5.8S, ITS2 and nuLSU regions. No *T. parietinae* was detected in any of these samples. We performed 49 PCR reactions for the 15 specimens of lichens of Teloschistaceae (11 without galls and 4 with galls of *T. caloplacae* s. l.) collected in Öland (Table S1), for which we detected *T. caloplacae* s. l. in 12 (80%) specimens (10 (91%) without galls and 2 (50%) with galls).

Table 2. PCR detection of *Tremella parietinae* on *Xanthoria parietina* specimens collected in El Sotillo locality (Madrid, Spain) (population A). The term "sample" refers to each of the fragments that were selected for the PCR analyses; the term "specimen" refers to the entire lichen.

Location in the lichen	**Percentage (%) of samples with *Tremella parietinae* detected by PCR**	**Number of specimens checked**
Sample without galls (thallus)	19	16
Sample without galls (whole apothecium)	19	16
Sample without galls (hymenium)	0	3
Sample with galls (thallus)	50	6
Sample with galls (whole apothecium without galls)	50	6
Sample with galls (hymenium without galls)	100	2
Galls	50	2
PCR detection		
TOTAL without galls	31	16
TOTAL with galls	83	6

We generated 39 sequences corresponding to *T. caloplacae* s. str., *T. candelariellae*, *T. dendrographae*, *T. parietinae* and *T. pusillae*. Allthough these sequences obtained had quality enough for Blast comparison, only 32 sequences of *T. caloplacae*, *T. parietinae* and *T. pusillae* (29 of the ITS and 3 of the nuLSU regions) had quality enough for the phylogenetic analysis, 10 sequences from specimens with galls and 22 sequences from specimens without galls. Four of the 32 *T. parietinae* sequences obtained were from samples also studied by FISH-CLSM (Table S1): one sequence from apothecia of *X. parietina* without galls, one sequence from apothecia without galls of one specimen of *X. parietina* with *T. parietinae* galls, one sequence from *T. parietinae* galls, and one sequence from *X. parietina* thallus. All new generated sequences were deposited in GenBank (Table S1).

No incongruence was found among DNA regions; thus, sequences were concatenated into a dataset for the ITS1, 5.8S, ITS2 and nuLSU regions including the newly produced sequences (Table S1) in combination with ITS and nuLSU sequences from Freire-Rallo et al. (2023b). A data matrix was generated with a total of 1190 characters (ITS1: 1–119;

5.8S: 120–277; ITS2: 278–422; nuLSU: 423–1190). The best ML tree (Fig. 1) obtained had a ln-likelihood value of -2694.561. Sequences of *Tremella* coming from specimens of *X. parietina* with or without galls are homogeneously distributed within the *T. parietinae* clade. The two sequences of *Tremella* from *Calogaya pusilla* with galls are placed within the *T. pusillae* clade, whilst the *Tremella* samples coming from *Cg. pusilla* without galls are placed within the *T. caloplacae* s. str. clade. The rest of the *Tremella* sequences extracted from different Teloschistaceae lichens without galls are all placed within the *T. caloplacae* s. str. clade regardless of whether they come from thalli or apothecia fragments (Fig. 1).

***FISH-CLSM studies in* Tremella parietinae**

Nuclei and cells of *X. parietina* and *T. caloplacae* s. l. were specifically stained by fluorescent *in situ* hybridization in galls, apothecia without galls, and thalli from *X. parietina* specimens with and without galls. Hybridized slides and negative controls were visualized with confocal laser scanning microscope. The presence of *Tremella* was verified in the 12 studied samples corresponding to thalli and apothecia from specimens of *Xanthoria parietina* with or without galls (Figs. 2, S1, S2 and S3). Yeasts of *Tremella parietinae* were intermixed with hyphae of *X. parietina*, usually in superficial areas of both the upper and lower cortices of the thalli, the thalline margins of the apothecia, or the hymenium (Figs. 2, S1, S2 and S3). The yeasts were distributed either grouped in small patches (Figs. 2A; S1A-C,H,I,L; S3F) or in isolation (Figs. 2B,C,F; S1D-G,J,K; S2; S3C-E). The filamentous stage of *Tremella* was observed only in specimens with galls: in galls and in an apothecium without galls from one specimen of *X. parietina* with galls (Figs. 2D,E; S3A,B). In these samples we could observe hyphae, basidia, haustoria and clamp connections of *Tremella* (Fig. 2D,E), while basidiospores were only observed from

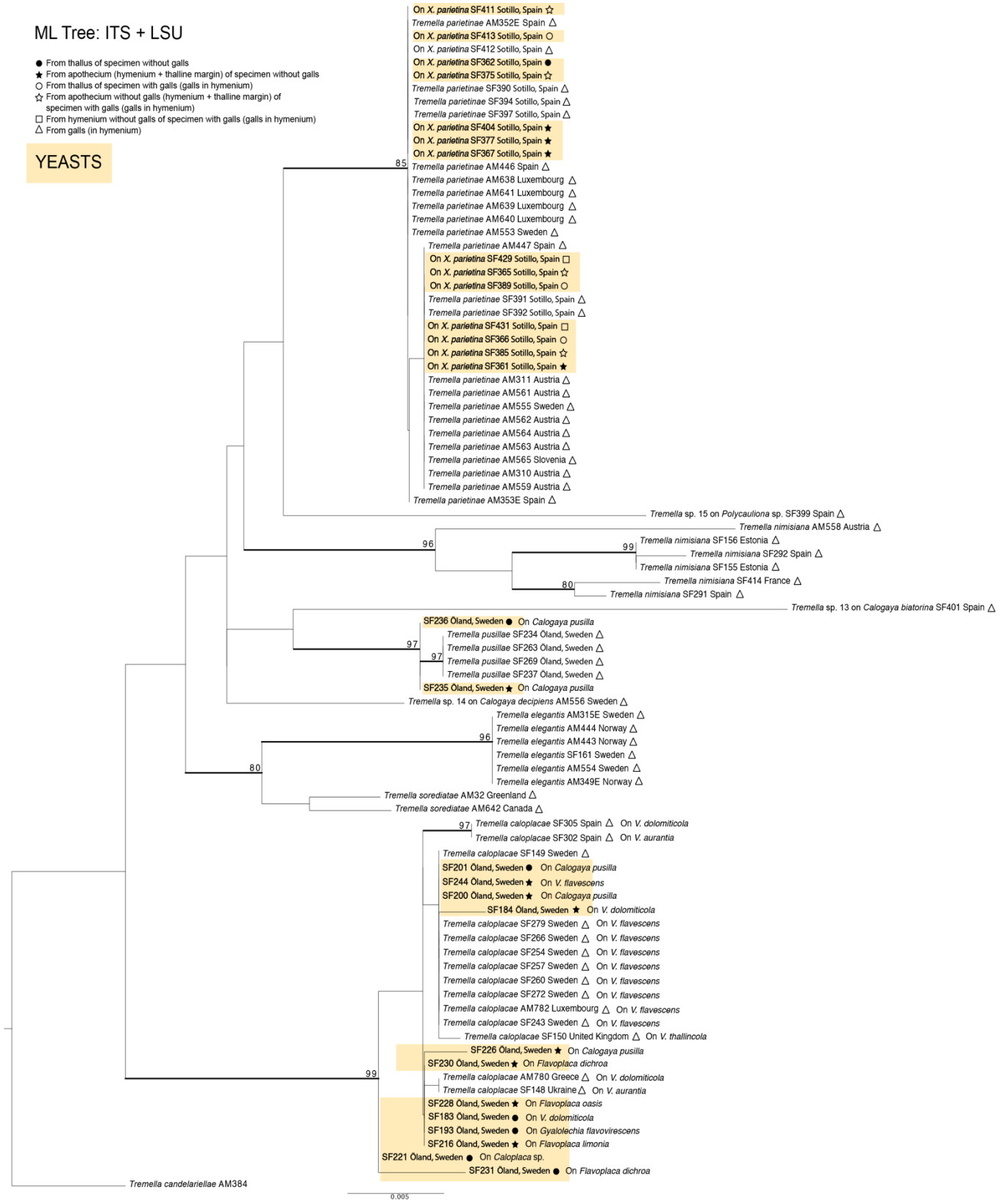

Figure 1. Phylogram based on ITS and nuLSU sequences, corresponding to the best tree recovered in the maximum likelihood analysis. A legend with the symbols used in the terminals of the tree is included in the left upper corner of the figure. Based on our FISH-CLSM results, we assume that sequences obtained from galls correspond to the filamentous phase, whereas sequences obtained from specimens or areas without galls, correspond to the yeast phase. ML-BS values ≥ 70 are indicated above boldface branches. Branch lengths are scaled to the expected number of substitutions per site.

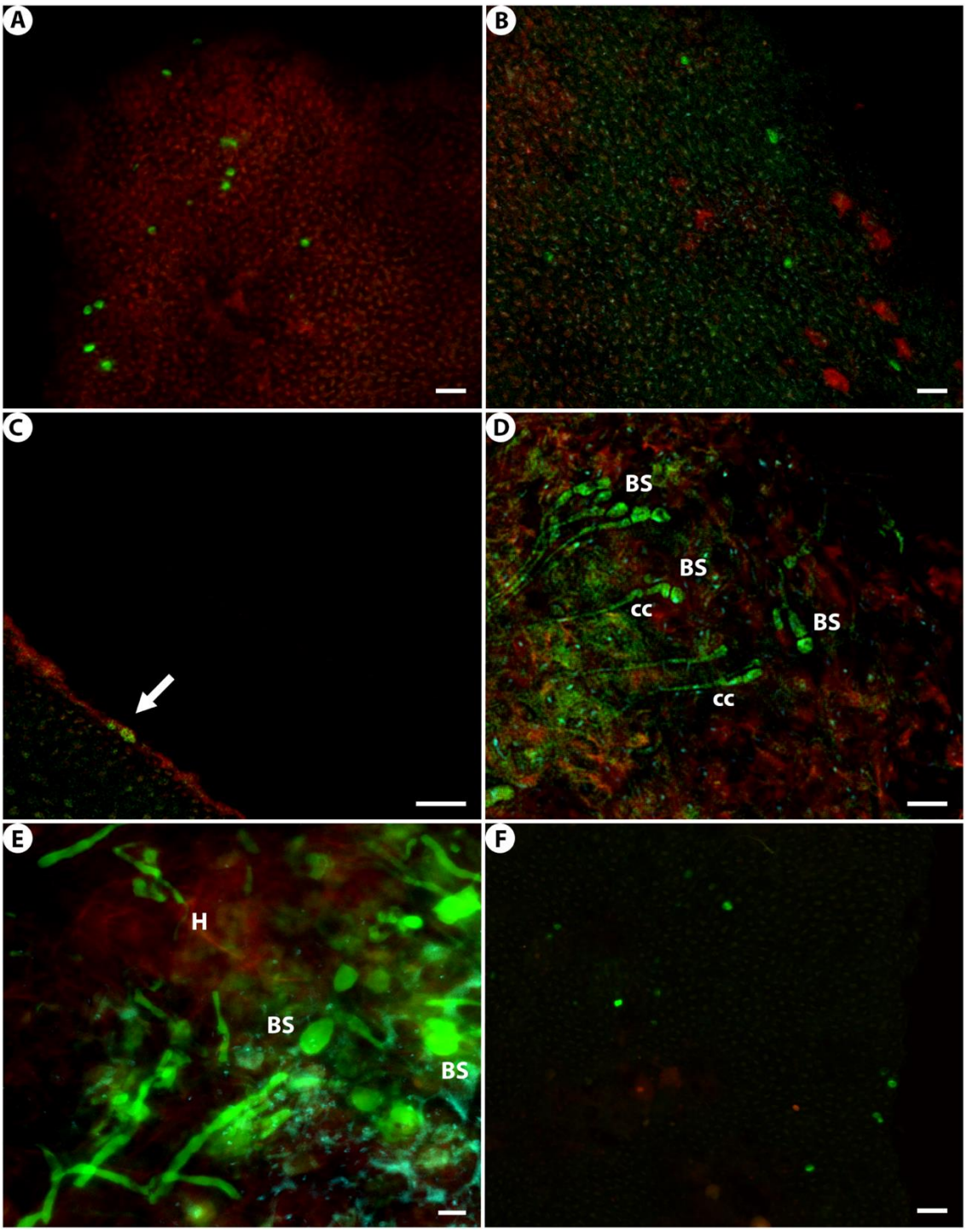

Figure 2. *Tremella parietinae* on *Xanthoria parietina* specimens, sum slides projection. Green: *T. parietinae*, red: algal autofluorescence and *X. parietina*. A) *T. parietinae* yeasts in the lower cortex (thallus) of *X. parietina* (specimen without galls). Scale bar: 5 µm. B) *T. parietinae* yeasts in the upper cortex (thallus) of *X. parietina* (specimen without galls). Scale bar: 5 µm. C) *T. parietinae* yeasts in the thalline margin of an apothecium without galls from a specimen of *X. parietina* in which other apothecia had galls. Arrow indicates the position of yeasts. Scale bar: 5 µm. D) *T. parietinae* filamentous stage in the hymenium of an apothecium without galls from a specimen of *X. parietina* in which other apothecia had galls. Scale bar: 10 µm. E) *T. parietinae* filamentous stage in the hymenium of a *X. parietina* apothecium with galls. Scale bar: 5 µm. F) *T. parietinae* yeasts in the upper cortex (thallus) of *X. parietina* (specimen with galls). Scale bar: 5 µm. BS: basidium. cc: clamp connection. H: Haustorium.

Tremella gall samples (Fig. S3A,B). Differences in fluorescent signal among different areas of both the host lichen and the *Tremella* within the same sample were observed during CLSM.

Discussion

The yeast phase of *Tremella parietinae* is present in the thalli and the apothecia of specimens of *Xanthoria parietina* with and without galls (Figs. 2, S1, S2 and S3), while the filamentous phase has been found exclusively inside the hymenia, with and without galls, of *X. parietina* specimens with galls (Figs. 2 and S3). That *Tremella parietinae* completes its life cycle within its host lichen was expected and is in line with the results obtained by Tuovinen et al. (2019, 2021) on other lichenicolous *Tremella*.

Dimorphic species are abundant and widespread among fungi, including the Tremellomycetes, as known from mycoparasitic species (Bandoni 1963, 1995; Chen 1998). Budding germination of basidiospores in lichenicolous species have been documented in observations through optical microscopy (Diederich 1996; Zamora et al. 2016) and the frequency and abundance of the yeast phase of lichenicolous basidiomycete species, within the lichen thalli, was made dramatically evident in the studies by Spribille et al. (2016) and Tuovinen et al. (2019, 2021). The yeast stage of *T. parietinae* in specimens of *X. parietina*, has been confirmed by PCR with specific primers and by FISH-CLSM, independently of the presence or absence of galls (Figs. 2, S1, S2 and S3; Tables 2 and S1). Nevertheless, PCR detection of *T. parietinae* in *X. parietina* samples without galls has been low. Negative results in PCR analysis could be attributed to multiple factors related to the type of samples and their preparation: presence of polysaccharides, polyphenols, terpenoid compounds, dead biomass, presence of salts, low target DNA concentration or human error during samples preparation (Ijzerman et al.

1997; Ekman 1999; Burkardt 2000; Wei et al. 2008; Schrader et al. 2012). Despite having negative PCR results, the target species may be present in the samples and undetectable false negatives may be obtained (Burkardt 2000; Bacich et al. 2011). Detection of *T. parietinae* in specimens of *X. parietina* with galls from population A (El Sotillo, Spain) is dramatically higher than detection in specimens from population B (Stockholm, Sweden). In population A there is a high abundance of *X. parietina* and these have an unusual high presence of *Tremella* galls. The causes behind this high incidence of galls remain unknown. To our knowledge, there are no studies on factors influencing the induction of galls by *Tremella*. The high incidence of *T. parietinae* yeasts detected in population A combined with a hypothetical optimal combination of environmental factors could favor the high incidence of induced galls. Potential yeast phase has also been detected in thalli and apothecia samples without galls from Teloschistaceae lichen specimens with and without *Tremella* galls, analyzed with PCR in the pilot study (Table S1).

Tremella parietinae can develop basidiomata without inducing gall formation in its host lichen. Using FISH-CLSM, hyphae and a basidioma of *T. parietinae* have been observed in a hymenium without galls from a specimen of *X. parietina* with galls (Fig. 2D). Other lichenicolous species of *Tremella* can develop basidiomata without inducing the formation of galls. This is known in other lichenicolous *Tremella* species, such as *T. abrothalli*, *T. graphidastrae*, *T. pisutiellae*, *T. protoparmeliae*, *Tremella* sp. 2 on *Lecanora rimicola* or *Tremella* sp. 11 on *Pyrenodesmia chalybaea* (Diederich et al. 2022). Similarly to *Tremella parietinae*, other species as *T. candelariealle*, *T. endosporogena* or *T. rinodinae* develope intrahymenial basidiomata that will induce galls when mature (Diederich et al. 2022). In *Tremella parietinae*, the observed basidiomata have two-celled mature basidia that match the morphology of the basidiomata observed in galls (Freire-

Rallo et al. 2023b), but from which no epibasidia have yet developed. Haustoria were not observed in this specimen, but clamp connections were present at the base of the mature basidia (Fig. 2D), which indicates the probable presence of haustoria (Oberwinkler et al. 1984; Bauer & Oberwinkler 1990; Zugmaier & Oberwincker, 1995). Haustoria and basidiospores were only observed in basidiomata from galls (Fig. 2E). The different structures seen in basidiomata from hymenium without galls and from gall samples could be attributed to the less development of the first, that will probably end up inducing the formation of a gall when mature. Also, differences in cell fluorescent signal detected during CLSM observations, sometimes within the same sample, need to be considered. Low levels of cell metabolic activity or errors in the fixation, permeabilization, or hybridization process of the samples could be related with heterogeneous fluorescent signal of the samples (Amann et al. 1995; Yilmaz & Noguera, 2004), making difficult to visualize less fluorescent structures.

The yeast stage of *Tremella* can be present in thalli (including thaline margin) and hymenia of samples without galls, both in *X. parietina* and in other Teloschistaceae. This has been verified with FISH for five *X. parietina* specimens from population A and with PCR for all samples studied. It is widely known that the yeast stage of the Basidiomycetes forms part of the lichen mycobiome (Prillinger et al. 1997; Ekamn 1999; Schneider et al. 2011; Spribille et al. 2016; Fernandez-Mendoza et al. 2017; Banchi et al. 2018; Tuovinen et al. 2019, 2021; Tagirdzhanova et al. 2021; Cometto et al. 2022). The role they play in the lichen is something that remains to be clarified. Spribille et al. (2016) suggested that the Basidiomycete yeast, particularly *Cyphobasidium* yeasts, could be part of the lichen symbiosis or to have a relevant role in lichens. A debate on this is open since other studies offer contrast to the first (Oberwinkler 2017; Lendermer et al 2019, Mark et al. 2020), opening a debate that has not yet been resolved. Dimorphic fungi switching between yeast

and filamentous stages is a strategy widely used to occupy a wide range of niches and is probably a response to stress dependent on the environmental conditions (Lin et al. 2014; Brown et al. 2017). *Cyphobasidium hypogymniicola*, *Tremella macrobasidiata* and *T. variae* are dimorphic lichenicolous fungi (Spribille, et al. 2016; Tuovinen et al. 2019, 2021), whose yeast phase has been found in other lichen species than the one in which they develop their filamentous phase (Diederich 1996; Zamora & Perez-Ortega, 2011; Millanes et al. 2016; Zamora et al. 2016). The mechanisms behind the formation of the basidioma in these species remain unknown, as so the optimal conditions for the formation of sexual reproductive structures.

In the pilot study carried out on Teloschistaceae lichens, the presence of *T. caloplacae* s. l. was detected by PCR in samples of thalli and apothecia without galls from specimens with and without galls. Moreover, *Tremella* sequences obtained from specimens of *Calogaya*, *Flavoplaca* and *Gyalolechia* are placed within *Tremella caloplacae* s. str., which filamentous phase is restricted to grow on species of *Variospora*. Dimorphic Basidiomycete yeasts are considered to be widespread and less specific than their filamentous phase (Fernandez-Mendoza et al. 2017; Lendemer et al. 2019; Tuovinen et al. 2019, 2021; Mark et al. 2020). Our results are in line with this, as phylogenetic analyses show that *Tremella* species detected in samples without galls and from specimens without galls are less specific than the same coming from samples with galls. *Tremella pusillae* and *T. caloplacae* s. str. were detected in specimens of *Calogaya pusilla* and *Variospora* spp. with galls, respectively, from fragments of thalli and apothecia without galls. However, in specimens of *Cg. pusilla*, *Variospora* spp. and *X. parietina* without galls, the presence of other lichenicolous *Tremella* species such as *T. dendrographae*, *T. candelariellae* or *T. caloplacae* s. str. has been detected (Table S1). Within the same lichen specimen, more than a single lichenicolous Tremellomycete

species could be present (Tuovinen et al. 2019, 2021). The detection of more than one of these species is limited when performing PCR analyses and the presence of several species is misinterpreted as contaminations (Ekman 1999; Cernajova & Skaloud, 2019). DNA proportion of a *Tremella* that is developing basidiomata within a lichen specimen could mask the possible presence of other lichenicolous *Tremella* species, and thus resulting in a lower detection rate (Tuovinen et al. 2021).

Conclusions

Tremella parietinae is a dimorphic species, developing its filamentous phase exclusively in the hymenium of *Xanthoria parietina* and its yeast phase in both the hymenium and the thallus. Specific probes designed to detect *T. parietinae* hybridize with the target species making possible to discriminate between the fluorescent signal of the probes and the autofluorescence of *T. parietinae* itself. PCR analyses with specific primers for lichenicolous *Tremella* species are useful to detect the presence of *Tremella parietinae* in areas of the lichen – or in specimens – devoid of galls. The yeast phase of *T. caloplacae* s. l. is less host specific than its filamentous phase.

Supplementary material – Freire-Rallo et al. – Chapter 3

Insights into the life cycle of *Tremella caloplacae* s. l.

Sandra Freire-Rallo, Veera Tuovinen Nogerius, Mats Wedin and Ana M. Millanes

Table S1. Lichen specimens and Tremella data with GenBank accession numbers for newly produced sequences.

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF163	Apothecium	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: NRM gardens (first tree), on the left of the main door. 59°22'8.90"N 18° 3'8.91"E. 15-03-2017. Sandra Freire Rallo (S1).
SF164	Thallus		None		-	-		
SF165	Apothecium	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: Frescativägen, between NRM and metro station. 59°22'0.63"N 18° 3'12.75"E. 15-03-2017. Sandra Freire Rallo (S2).
SF166	Thallus		None		-	-		
SF167	Apothecium	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: Frescativägen, between NRM and metro station. 59°21'59.39"N 18° 3'13.87"E. 15-03-2017. Sandra Freire Rallo (S3).
SF169	Thallus		None		-	-		
SF170	Thallus	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: outside the Universitetet metro station. 59°21'57.70"N 18° 3'15.79"E. 15-03-2017. Sandra Freire Rallo (S4).
SF171	Apothecium	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: University campus. 59°21'55.19"N 18° 3'28.50"E. 15-03-2017. Sandra Freire Rallo (S5).
SF172	Thallus		None		-	-		
SF174	Thallus	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: Path between Svante Arrhenius väg and Bergiusvägen (behind the NRM area). 59°22'0.59"N 18° 3'30.37"E. 15-03-2017. Sandra Freire Rallo (S6).
SF176	Thallus	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: Path between Svante Arrhenius väg and Bergiusvägen (behind the NRM area). 59°22'5.39"N 18° 3'33.60"E. 15-03-2017. Sandra Freire Rallo (S7).
SF177	Apothecium	*X. parietina*	None	None	-	-		SWEDEN, Stockholm: Backyards of th NRM. Behind palaeobiology building. 59°22'7.29"N 18° 3'23.59"E. 15-03-2017. Sandra Freire Rallo (S8).

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF182	Apothecium	*A. holocarpa*	None	None	-	*T. caloplacae*		SWEDEN, Öland, West Gösslunda. Alt. 25 m, 56°29.609' N 016°30.680' E. Stora Alvaret, on limestone (calcareous rock). 13-06-2017. Sandra Freire Rallo (S11).
SF183	Thallus	*V. dolomiticola*	None	None	-	*T. caloplacae*		SWEDEN, Öland, West Gösslunda. Alt. 26 m, 56°29.603' N 016°30.683' E. Stora Alvaret, on limestone (calcareous rock). 13-06-2017. Sandra Freire Rallo (S10).
SF184	Apothecium		None		-	*T. caloplacae*		
SF192	Apothecium	*G. flavovirescens*	None	None	-	-		SWEDEN, Öland, West Gösslunda. Alt. 27 m, 56°29.740' N 016°30.644' E. Stora Alvaret, on limestone (calcareous rock). 13-06-2017. Sandra Freire Rallo (S14).
SF193	Thallus		None		-	*T. caloplacae*		
SF200	Apothecium	*Cg. pusilla*	None	None	-	*T .pusillae*		SWEDEN, Öland, Ottenby. Alt. 10 m, 56°15.278' N 016°26.152' E. Stora Alvaret, on wall of calcareous rock exposed to wind. 14-06-2017. Sandra Freire Rallo (S18).
SF201	Thallus		None		-	*T. pusillae*		
SF206	Apothecium	*Cg. decipiens*	None	None	-	-		SWEDEN, Öland, King's Farm. Alt. 3 m, 56°14.172' N 016°24.236' E. On calcareous clift. 14-06-2017. Sandra Freire Rallo (S21).
SF207	Thallus		None		-	-		
SF216	Apothecium	*F. limonia*	None	None	-	*T. caloplacae*		SWEDEN, Öland, King's Farm. Alt. 2 m, 56°14.160' N 016°24.237' E. On calcareous clift. 14-06-2017. Sandra Freire Rallo (S27).
SF217	Thallus		None		-	*T. caloplacae*		
SF220	Apothecium	*Caloplaca* sp.	None	None	-	-		SWEDEN, Öland, King's Farm. Alt. 3 m, 56°14.172' N 016°24.237' E. On calcareous clift. 14-06-2017. Sandra Freire Rallo (S29).
SF221	Thallus		None		-	*T. caloplacae*		
SF226	Apothecium	*Cg. pusilla*	None	None	-	*T. pusillae*		SWEDEN, Öland, Jordhamn. Alt. 3 m, 57°05.845' N 016°53.181' E. On calcareous rock. 15-06-2017. S31. Sandra Freire Rallo.
SF227	Thallus		None		-	*T. candelariellae/ T. caloplacae*		
SF228	Apothecium	*F. oasis*	None	None	-	*T. caloplacae*		SWEDEN, Öland, Jordhamn. Alt. 3 m, 57°05.845' N 016°53.181' E. On calcareous rock. 15-06-2017. S31. Sandra Freire Rallo.
SF229	Thallus		None		-	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF230	Apothecium	*F. dichroa*	None	None	-	*T. caloplacae*		SWEDEN, Öland, Jordhamn. Alt. 3 m, 57°05.845' N 016°53.181' E. On calcareous rock. 15-06-2017. S31. Sandra Freire Rallo.
SF231	Thallus		None		-	*T. caloplacae*		
SF232	Apothecium	*Cg. arnoldii*	None	None	-	-		SWEDEN, Öland, Jordhamn. Alt. 6 m, 57°05.878' N 016°53.211' E. On calcareous clifts. 15-06-2017. S32. Sandra Freire Rallo.
SF233	Thallus		None		-	*T. caloplacae*		
SF235	Apothecium	*Cg. pusilla*	None	*Tremella pusillae* basidiomata	*T.pusillae*	*T.pusillae*		SWEDEN, Öland, Jordhamn. Alt. 6 m, 57°05.878' N 016°53.211' E. On calcareous rock. 15-06-2017. Sandra Freire Rallo S33.
SF236	Thallus		None		*T.pusillae*	*T.pusillae*		
SF240	Thallus	*V. flavescens*	None	*Tremella caloplacae* s. str. basidiomata	-	-		SWEDEN, Öland, Jordhamn. Alt. 7 m, 57°05.876' N 016°53.228' E. On calcareous rock. 15-06-2017. Sandra Freire Rallo S35.
SF241	Apothecium	*V. flavescens*	None	*Tremella caloplacae* s. str. basidiomata	-	-		SWEDEN, Öland, Jordhamn. Alt. 6 m, 57°05.878' N 016°53.211' E. On calcareous rock. 15-06-2017. Sandra Freire Rallo S33.
SF242	Thallus		None		-	*Phaeotremella*		
SF244	Apothecium	*V. flavescens*	None	*Tremella caloplacae* s. str. basidiomata	*T.caloplacae*	*T. caloplacae*		SWEDEN, Öland, Jordhamn. Alt. 8 m, 57°05.850' N 016°53.221' E. On calcareous clift. 15-06-2017. Sandra Freire Rallo S37.
SF359	Apothecium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°21'59.4"N 3°56'43.1"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S99) & Ana Millanes.
SF360	Thallus		None		-	-		
SF361	Apothecium	*X. parietina*	None	None	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S101) & Ana Millanes.
SF362	Thallus		None		*T. parietinae*	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF363	Apothecium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'01.1"N 3°56'43.6"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S103) & Ana Millanes.
SF364	Thallus		None		-	-		
SF365	Apothecium	*X. parietina*	None	*Tremella parietinae* basidiomata	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'02.5"N 3°56'43.5"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S105) & Ana Millanes.
SF366	Thallus		None		*T. parietinae*	-		
SF367	Apothecium	*X. parietina*	None	None	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'02.4"N 3°56'43.8"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S107) & Ana Millanes.
SF368	Thallus		None		-	-		
SF369	Apothecium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'02.4"N 3°56'43.8"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S108) & Ana Millanes.
SF370	Thallus		None		-	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF371	Apothecium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'03.3"N 3°56'44.0"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S109) & Ana Millanes.
SF372	Thallus		None		-	-		
SF373	Apothecium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'03.5"N 3°56'44.4"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S111) & Ana Millanes.
SF374	Thallus		None		*T. parietinae*	-		
SF375	Apothecium	*X. parietina*	None	*Tremella parietinae* basidiomata	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'05.3"N 3°56'45.1"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S113) & Ana Millanes.
SF376	Thallus		None		*T. parietinae*	-		
SF377	Apothecium	*X. parietina*	None	None	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'05.3"N 3°56'45.1"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S115) & Ana Millanes.
SF378	Thallus		None		-	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF379	Apothecium		None		-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Fraxinus angustifolia bark. 40°22'07.6"N 3°56'46.5"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S117) & Ana Millanes.
		X. parietina	None	None				
SF380	Thallus				*T. dendrographae*	-		
SF382	Apothecium		None		*T. candelariellae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with Quercus ilex, Fraxinus angustifolia, Retama sphaerocarpa. On Populus nigra bark. 40°22'09.8"N 3°56'49.2"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S120) & Ana Millanes.
		X. parietina	None	None				
SF383	Thallus				-	-		
SF402 (FISH-XP1)	Apothecium		None		-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°21'59.4"N 3°56'43.1"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S129) & Ana Millanes.
		X. parietina	None	None				
SF403 (FISH-XP1)	Thallus				-	-		
SF404 (FISH-XP2)	Apothecium		None		*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S130) & Ana Millanes.
		X. parietina	None	None				
SF405 (FISH-XP2)	Thallus				-	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF406 (FISH-XP3)	Apothecium	*X. parietina*	None None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'01.1"N 3°56'43.6"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S131) & Ana Millanes.
SF407 (FISH-XP3)	Thallus				-	-		
SF408 (FISH-XT1)	Apothecium	*X. parietina*	None *Tremella parietinae* basidiomata None	*Tremella parietinae* basidiomata	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S132) & Ana Millanes.
SF409 (FISH-XT1)	Apothecium				-	-		
SF410 (FISH-XT1)	Thallus				-	-		
SF411 (FISH-XT2)	Apothecium	*X. parietina*	None *Tremella parietinae* basidiomata None	*Tremella parietinae* basidiomata	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 440°22'01.1"N 3°56'43.6"W. Alt. ca. 573 m. 29/04/2021. Sandra Freire Rallo (S133) & Ana Millanes.
SF412 (FISH-XT2)	Apothecium				*T. parietinae*	-		
SF413 (FISH-XT2)	Thallus				*T. parietinae*	-		
SF423	Hymenium	*X. parietina*	None None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 13/02/2023. Ana Millanes.
SF424	Thallus				-	-		
SF425	Hymenium	*X. parietina*	None None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 13/02/2023. Ana Millanes.
SF426	Thallus				*T. parietinae*	-		

(Continue)

DNA code	Fragment	Species	*Tremella* basidiomata in analysed fragment	*Tremella* basidiomata on specimen	GenBank ID (ITS region)	GenBank ID (LSU region)	GenBank accession number	Specimen data
SF427	Hymenium	*X. parietina*	None	None	-	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 13/02/2023. Ana Millanes.
SF428	Thallus		None		*Pseudotremella/ Naganishia*	-		
SF429	Hymenium	*X. parietina*	None	*Tremella parietinae* basidiomata	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 13/02/2023. Ana Millanes.
SF430	Thallus		None		-	-		
SF431	Hymenium	*X. parietina*	None	*Tremella parietinae* basidiomata	*T. parietinae*	-		SPAIN, Madrid, Villaviciosa de Odón, Área Recreativa Dehesa El Sotillo. Forest with *Quercus ilex*, *Fraxinus angustifolia*, *Retama sphaerocarpa*. On *Fraxinus angustifolia* bark. 40°22'00.4"N 3°56'43.8"W. Alt. ca. 573 m. 13/02/2023. Ana Millanes.
SF432	Thallus		None		-	-		

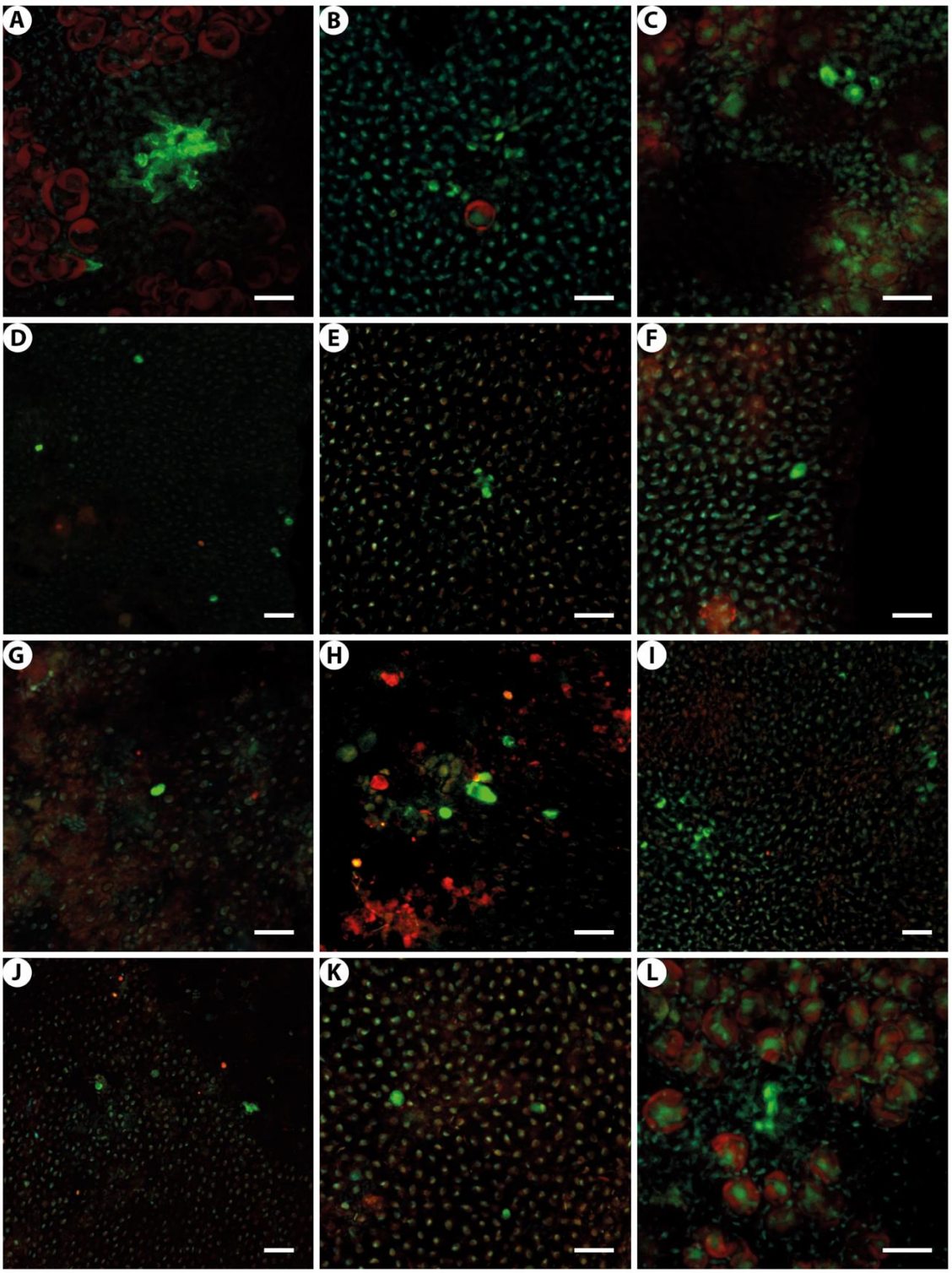

Fig. S1. *Tremella parietinae* yeast stage in thalli of *Xanthoria parietina* specimens without galls, sum slides projection. Green: *T. parietinae*, red: algal autofluorescence and *X. parietina*. A) *X. parietina* thallus lower cortex. *T. parietinae* yeasts hybridized in green intermixed with *X. parietina* hyphae with strong green autofluorescence. B-G, I-K) *X. parietina* thalli upper cortices. H, L) *X. parietina* thalli lower cortices. Scale bars: 5 μm.

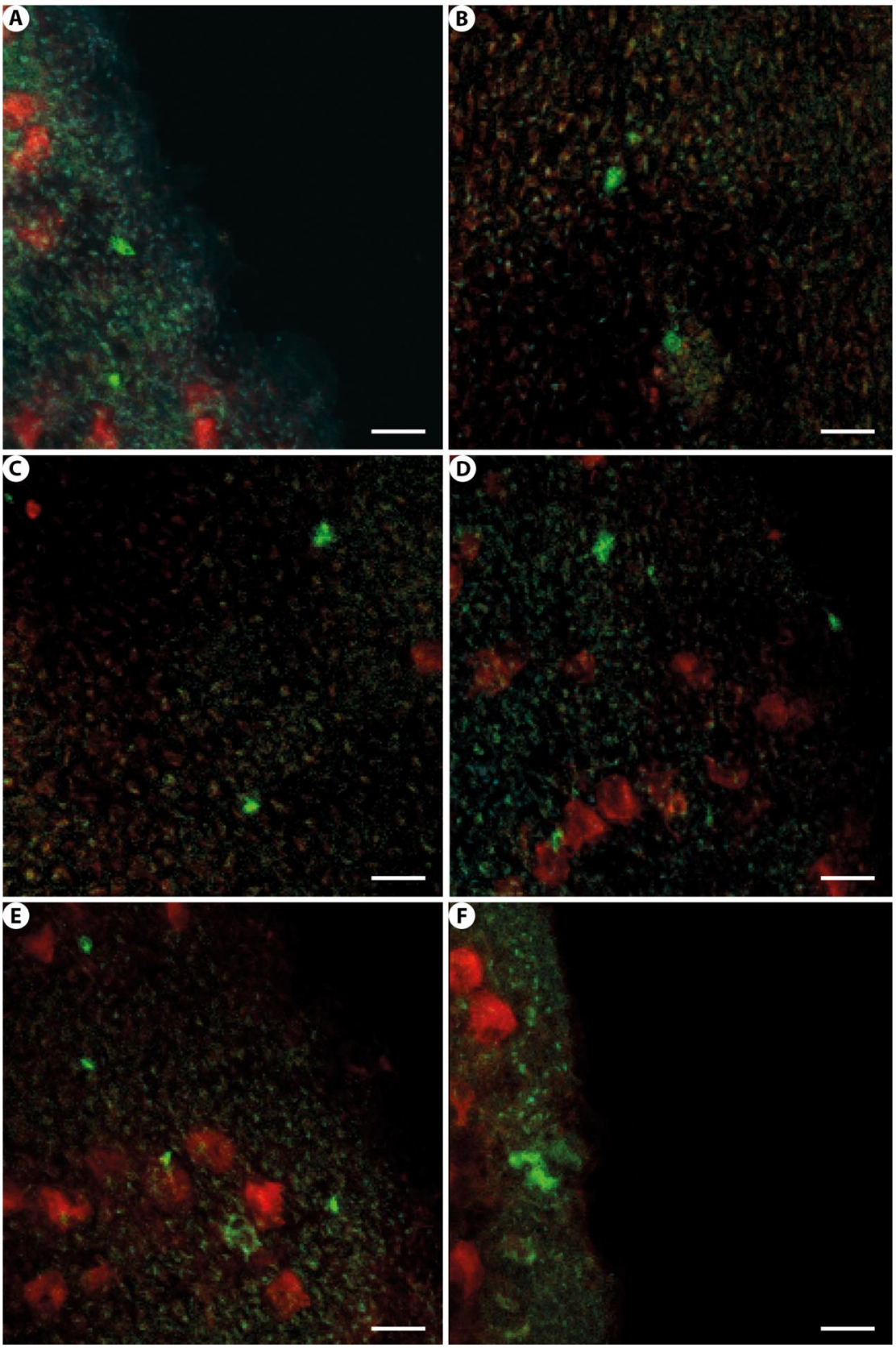

Fig. S2. *Tremella parietinae* yeast stage in apothecia of *Xanthoria parietina* specimens without galls, sum slides projection. Green: *T. parietinae*, red: algal autofluorescence and *X. parietina*. A, D-F) *X. parietina* thalline margin. B, C) Upper part of *X. parietina* hymenium. Scale bars: 5 µm.

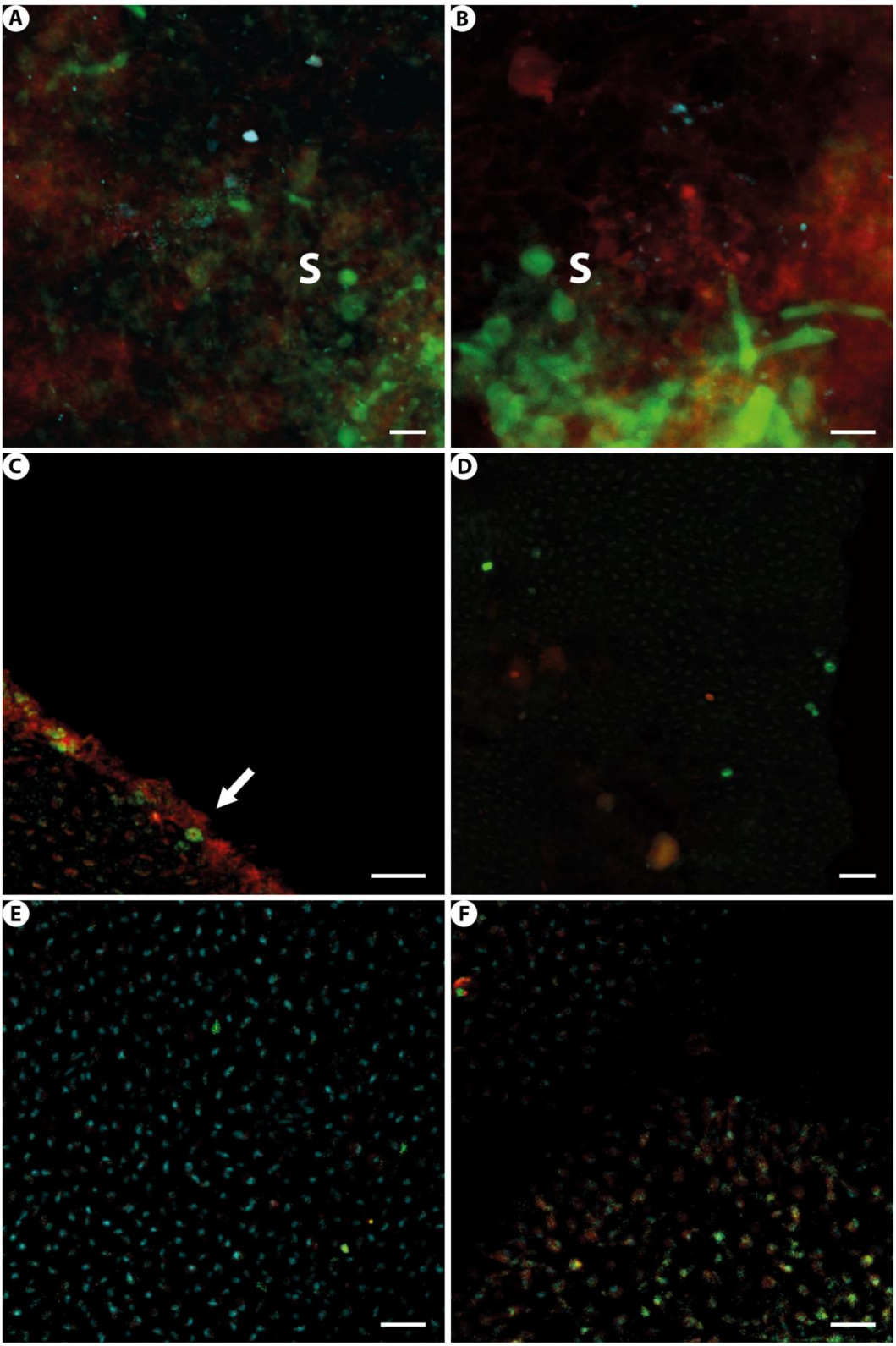

Figure S3. *Tremella parietinae* on *Xanthoria parietina* specimens with galls, sum slides projection. Green: *T. parietinae*, red: algal autofluorescence and *X. parietina*. A, B) Filamentous stage of *T. parietinae* in the hymenium of *X. parietina*. C) Yeast of *T. parietinae* in the upper part of the thalline margine of *X. parietina*. Arrow indicates the position of the yeast. D-F) Yeast of *T. parietinae* in the hymenium of *X. parietina*. Scale bars: 5 μm. S: basidiospore.

General discussion

General discussion

Diversity within the *Tremella caloplacae* complex

Tremella caloplacae (Zahlbr.) Diederich is a species complex. In a first study, at least nine putative species have been delimited within this complex, eight of which correspond to new species and the remaining species to *T. caloplacae* s. str. (Chapter 1, Freire-Rallo et al. 2023a). Each of these putative species grows on a specific host lichen species or genus. In a subsequent study, five species have been formally described within the *T. caloplacae* complex, and *T. caloplacae* s. str. has been circumscribed to exclusively grow on *Variospora* species (Chapter 2, Freire-Rallo et al. 2023b). *Tremella elegantis* develops its basidiomata in the hymenium of *Rusavskia elegantis*, *T. nimisiana* in the hymenium of several *Xanthocarpia* species, *T. parietinae* in the hymenium of *Xanthoria parietina*, *T. pusillae* in the hymenium of *Calogaya pusilla* and *T. sorediatae* in the thallus of *R. sorediata*. In addition, three more species have been left unnamed while waiting to expand the sampling and obtain more data: *Tremella* sp. 13 on *Calogaya biatorina*, *Tremella* sp. 14 on *Calogaya decipiens* and *Tremella* sp. 15 on *Polycauliona* sp.

Tremella lichenicolous fungi are often inconspicuous and therefore difficult to detect and sample. The galls that they induce on their host lichens are the most obvious sign of their presence, but it is known that a very small proportion of the spectrum of host species develop these structures (Freire-Rallo et al. 2023a). As has been verified in other Teloschistaceae lichenicolous species complexes, increasing the sampling provides a better global vision of the existing diversity (Millanes et al. 2015, 2016). Due to the scarcity of specimens found during the surveys carried out, three species have been left unnamed; further sampling would help to elucidate whether *T. nimisiana* and *T. caloplacae* s. str. are actual species complexes and would avoid the limitations in the

analyses due to the poor state of some specimens, such as the *Tremella* found growing in *Leproplaca xantholyta*. The number of species comprised in the *T. caloplacae* complex is likely to be higher than that found to date and further sampling would be critical to unravel the actual diversity within the complex.

Since the first record of *Tremella caloplacae* (then *Lindauopsis caloplacae* Zahlbr.) growing on *Variospora aurantia* (then *Caloplaca callopisma*), its characterization has been problematic. What was initially considered a hyphomycete with conidia was found to be a basidiomycete with two-celled basidia (Zahlbruckner 1906; Diederich 1996; Sérusiaux et al. 2003), which could grow and develop not only in species of the genus *Variospora*, but in other Teloschistaceae lichen hosts (Sérusiaux *et al*. 2003; Diederich 2007; Diederich et al. 2022a; Freire-Rallo et la. 2023a). Lichenicolous Tremellomycete, and particularly lichenicolous *Tremella* species, have scarce diagnostic morphological characters and finding morphological differences that serve to differentiate species within this group of fungi is high (Diederich 1996). Consequently, experts have gathered other evidence, as ecological data, to delimit lichenicolous species (Millanes et al. 2016b; Diederich et al. 2018, 2022a). The exhaustive study of the morphology and ecology within the *T. caloplacae* complex made possible to identify the morphological and ecological differences among each formally described species (Freire-Rallo et al. 2023b). Phylogenetic analyses have also proven to be powerful tools for lichenicolous species recognition (Millanes et al. 2021; Diederich et al. 2022a,b). Millanes et al. (2011) were the first to perform phylogenetic studies on lichenicolous *Tremella* and they suggested that several of the lichenicolous species described at that time could be actual species complexes. The aforementioned scarcity of morphological diagnostic characters, together

with the difficulty to cultivate these fungi, make the phylogenetic approach very suitable for the study of lichenicolous fungi.

The ITS region, known as the fungal barcode (Eberhardt 2010; Schoch et al. 2012), has been used to identify species (Suwannasai et al. 2013). The use of only one or few regions has been proven to lead to imprecise results when there is recombination or hybridization or incomplete lineage sorting in the system (Chambers and Hillis, 2020; Paloi et al. 2022). Thus, studies have been carried out to find other regions, or a combination of them, that can be used as fungal barcode to provide more accurate results (Pino-Bodas et al. 2013; Stielow et al. 2015). For lichenicolous fungi, the combination of the ITS and the nuLSU regions has proven to result in accurate species delimitation (Millanes et al. 2016a; Diederich et al. 2022b; Freire-Rallo et al. 2023a). One of the main drawbacks of phylogenetic species delimitation methods is that most of them are designed to analyze a single region (Rannala & Yang 2020). Nevertheless, region concatenation can lead to accurate results, while increasing the number of regions analyzed does not necessarily result in a noticeable improvement of the species delimitations (Luo et al. 2018). Since they are based on different principles and have diverse assumptions, each phylogenetic method for species delimitation can provide different results (Miralles & Vences 2013; Vitecek et al. 2017; Becchimanzi et al. 2021). Methods based on coalescence such as GMYC (single and multiple approaches), BTT or ABGD, can show results that reflect the structure of the population (Sukumaran & Knowles, 2017) or that are affected by the sample size or the presence of singletons (Puillandre et al. 2012; Pentinsaari et al. 2017). Furthermore, GMYC tends to overestimate the number of species (Fujita et al. 2012; Luo et al. 2018), unlike ABGD, which tends to underestimate it (Ritchie et al. 2016; Pentinsaari et al. 2017; Luo et al. 2018). Considering the multiple limitations that each

species recognition method has, using an integrative approach to combine data from different lines of evidence will result in more precise species delimitations.

Coevolution of *Tremella caloplacae* s. l. with its hosts

The diversity that exists within the *T. caloplacae* complex is closely related to the great diversity in the Teloschistaceae lichens (Chapter 1, Freire-Rallo et al. 2023a). It has been shown that the origin of *T. caloplacae* s. l. is linked to the radiation of the Teloschistaceae, that originated in the Cretaceous about 100 Mya (Gaya et al. 2015). Results show that the diversification of the known diversity within *T. caloplacae* s. l. began around 30 Mya, although the origin of this group might be previous to this age if the real diversity of the *T. caloplacae* complex was known (Chapter 1, Freire-Rallo et al. 2023). Gaya et al. (2015) related diversification in Teloschistaceae with a change of habit towards rocky substrates with high sun exposure, consequently leading to physiological changes in lichens. From the point of view of *T. caloplacae* s. l., the period of change in their host lichens could provide suitable conditions for its own diversification (Freire-Rallo et al. 2023). This, added to the fact that lichenicolous fungi maintain a very close relationship with their hosts, make it a very appropriate system to study coevolution between both organisms.

The coevolutionary studies of the system formed by the *T. caloplacae* complex and its hosts suggest that host switching is the evolutionary event that explains their evolutionary history. This result is in line with the only other coevolutionary study performed on lichenicolous fungi. Millanes et al. (2014a) verified that *Biatoropsis usnearum* s. l. (Tremellaceae) and their host lichens of the genera *Usnea* and *Protousnea* (Parmeliaceae) have undergone host switch events in their joint evolutionary history. Incongruence between the phylogenies of the studied species is one of the main pieces of evidence

against cospeciation as driving evolutionary force (Wang et al. 2019), although congruent phylogenies of species could also be the result of different evolutionary events others than cospeciation (de Vienne et al. 2013). Phylogenies of both *T. caloplacae* s. l. and their Teloschistaceae hosts show incongruences among them (Chapter 1, Figs. 2 and 4). This together with the non-significant results of the Parafit and PACo analyses and the posterior diversification of *Tremella* to that of the Teloschistaceae (Chapter 1, Table 4), support that host switching instead of cospeciation is the main force behind diversification in this system.

The great diversification undergone by the Teloschistaceae after their change towards rocky substrates and higher exposure to the sun, could have entailed for the *Tremella* fungi a great diversity of environments and changing ecological conditions (Freire-Rallo et al. 2023a). The divergence times of the *Tremella* species are generally posterior to those of their Teloschistaceae hosts (Chapter 1, Figs. 4, S1, S2 and Table 4). In this context, the lichenicolous *Tremella* species had the opportunity to switch to potential new hosts, and their adaptation to each particular host could have led to a particular evolutionary event known as sequential or cascade speciation (Feder & Forbes, 2010; Hood et al. 2015). However, in some particular cases such as in *T. elegantis*, *T. parietinae*, *T. pusillae*, *T. sorediatae* and *Tremella* sp. 14 that grow in species of *Calogaya*, *Rusavskia* and *Xanthoria*, the moment of divergence of the *Tremella* species precedes that of their Teloschistaceae hosts. These cases are consistent with events of extinction of the *Tremella* optimal host lichen in which the *Tremella* fungi could have been forced to survive in an alternative lichen host to which it would have eventually adapted (Janz et al. 2006; Forbes et al. 2009). Host switching events in systems with specialist parasites could seem paradoxical, but these events would be explained by the concept of Ecological Fit (Janzen

1985) and the "Stockholm Paradigm" (McLennan & Brooks, 2002; Agosta et al. 2010; Janz 2011; Hober & Brooks, 2015). The host switch events that the species within the *T. caloplacae* complex seem to have undergone and their subsequent adaptation to their Teloschistaceae hosts could be related to the phenotypic flexibility of the parasites. These would have dispersed quickly and colonized new hosts that were less optimal but available in a "sloppy fitness space", becoming generalist parasites that would adapt to their new hosts over time. In other fungi with dimorphic life cycles, rapid dispersion in order to maximize their spreading capacity is associated with the yeast phase of the organism (Boyce & Andrianopoulos, 2015).

***Tremella caloplacae* s. l. life cycle**

Tremella parietinae is a dimorphic species that completes its life cycle on *X. parietina*. It has a filamentous phase that induces gall formation in the hymenium of the host and a yeast phase that can be found in the hymenium and the thallus of *X. parietina* hosts (Chapter 3, Figs. 2, S1, S2 and S3). The presence of both phases has been verified with PCR and FISH-CLSM analyses, in which the filamentous phase was only detected on specimens with galls while the yeast phase was detected on specimens with and without galls. Dimorphism is a typical characteristic of the Tremellomycetes (Prillinger et al. 1997; Weiss et al. 2014) and several fungicolous fungi of the genus *Tremella* (Chen 1998). Recently, Tuovinen et al. (2019, 2021) detected the presence of *T. lethariae*, *T. macrobasidiata and T. variae* yeasts in the thallus of *Letharia vulpina*, *Lecanora chlarotera* and *L. varia* specimens respectively, with and without galls by performing FISH-CLSM and molecular analysis. However, the dimorphic character of lichenicolous *Tremella* fungi has been known for a long time (Diederich 1996; Zamora et al. 2011, 2016). Likewise, *T. pusillae* and *T. caloplacae* s. str. could also be considered dimorphic

species that complete their life cycles on *Calogaya pusilla* and *Variospora* spp. respectively (Chapter 3, Table S1). In the pilot study carried out, a small sampling of Teloschistaceae lichen specimens were analyzed by PCR for the presence of *T. caloplacae* s. l., that was detected in the thalli and apothecia of the Teloschistaceae specimens with and without galls. The identity of the sequences obtained, and the phylogenetic analyses carried out confirm that they correspond to *T. pusillae* and *T. caloplacae* s. str.

The basidiomata in Basidiomycetes lichenicolous fungi are developed in the dikaryotic filamentous phase. The *T. caloplacae* s. l. basidiomata has always been associated with the induction of gall formation on its host lichens, but in the specific case of *T. parietinae*, it has been detected a basidioma in an hymenium without galls of a specimen of *X. parietina* with galls (Chapter 3, Fig. 2D). Other *Tremella* lichenicolous species form basidiomata without actually inducing gall formation in their hosts (Diederich et al. 2022) or inducing them but only when the basidiomata are mature, such as *T. endosporogena* (Diederich et al. 2022). The mechanisms that regulate the development of galls in the hymenium or thallus of host lichens have been related to the production of chemical compounds involved in growth-induction and degradation of the lichen photobiont or mycobiont cell walls (Grube & de los Ríos, 2001), generated as consequence of direct contact between both organisms (Lawrey & Diederich, 2003). In other fungal and lichenicolous parasitic fungi, the contact between the parasite and the host is carried out by means of haustoria (Bushnell 1972; Zugmaier & Oberwinkler, 1995). In the basidioma of *T. parietinae* found without gall development, the presence of haustoria was not detected, which leads to suggest that the development of galls in *T. parietinae* could be

related to the presence of haustoria in the basidioma, which indeed are present in fully developed *T. parietinae* basidiomata (Chapter 2, Fig. 5F-N; Chapter 3, Fig. 2E).

The presence of *T. parietinae* yeast phase in the thalli, hymenia and thalline margins of specimens of *X. parietina* with and without galls has been confirmed by PCR and FISH-CLSM (Chapter 3, Figs. 2, S1, S3 and S3, and Tables 2 and S1). Similar results were obtained in the pilot study carried out on Teloschistaceae lichen specimens, in which the presence of *T. caloplacae* s. l. was detected in fragments of thallus, hymenium and complete apothecium without galls from specimens with and without galls (Chapter 3, Table S1). Although the presence of lichenicolous Basidiomycetes has been long acknowledge by the development of galls in the lichen host (Lawrey & Diederich, 2003), the increase in metabarcoding techniques use has evince that lichenicolous Basidiomycetes are also present in lichens devoid of galls. Spribille et al. (2016) detected the presence of the lichenicolous Cyphobasidium (Tremellomycetes) in several species of Lecanoromycete macrolichens, but not in all the specimens studied. In this study it was also hypothesized that Cyphobasidium could be a member of the lichen symbiosis or play an important role in its phenotypic expression. However, other studies show that Basidiomycete lichenicolous fungi follow the distribution pattern of their hosts and can be considered widespread (He et al. 2022). Nevertheless, they are not ubiquitous and therefore could not be considered a necessary agent for the establishment of lichen symbiosis. The nature of the relationships that lichenicolous Basidiomycetes yeasts have with the lichens in which they live is still unclear. Some authors consider them commensal organisms or symbionts (Spribille et al. 2016). However, to study the role they play in the lichen it might be important to analyze their presence in quantitative terms. Due to the power and precision of metabarcoding techniques, the presence of organisms with a

negligible quantitative representation can be detected (Shelton et al. 2023). In these cases, it would be difficult to discern which of these species would really form part of the lichen mycobiota and which are circumstantially present or could be even considered mere contaminations (Ficetola et al. 2016).

While the yeast phase of lichenicolous fungi has been considered non-pathogenic, this has traditionally not been the case for their filamentous phase. Many species of lichenicolous Basidiomycetes are considered to some extent parasites of lichens on which they develop basidiomata (Lawrey & Diederich, 2003; Diederich et al. 2018; Honegger 2023). The filamentous phase of dimorphic fungi can develop from their monokaryotic unicellular phase when two compatible yeasts mate. Using metabarcoding techniques, many lichenicolous fungi are detected in a great diversity of host lichens (Spribille et al. 2016; Fernández-Mendoza et al. 2017); however, the low number of lichens found with visible signs of development of the filamentous phase of these lichenicolous fungi is noteworthy (Banchi et al. 2018; Freire-Rallo et al. 2023a). The optimal physical or chemical environmental conditions behind the development of the filamentous phases are still unknown.

Host specificity in the *T. caloplacae* complex

Lichenicolous fungi are considered organisms with a very high level of specificity with respect to the host, far surpassing other types of specialist organisms (Lawrey & Diederich, ,2003). Specialist species are very common in lichenicolous fungi, becoming more abundant than generalists (Lawrey & Diederich, ,2003). This high level of host specificity, referred to the filamentous phase, has sometimes been used as another diagnostic character for the recognition of Tremellomycetes lichenicolous fungal species

(Diederich et al. 2022). However, the most recent studies on the yeast phase of lichenicolous Basidiomycetes show that this monokaryotic unicellular phase of the life cycle is less specific than the dikaryotic phase (Fernández-Mendoza et al. 2017; Mark et al. 2020). *Cyphobasidium* yeasts were found on numerous Parmeliacea lichens (Spribille et al. 2016), however, their filamentous phase has been found developing basidiomata and inducing gall formation exclusively on *Hypogymnia* spp. and *Usnea* spp. (Millanes et al. 2016a). The yeast phase of *Tremella* lichenicolous fungi was also found in lichens other than those in which they develop basidiomata, although closely related (Tuovinen et al. 2021). It seems that the specificity shown by the filamentous phase of some lichenicolous fungi is superior to the specificity of their yeast phases.

Some authors have considered the specialist character of the species as a typical feature of biotrophic parasitism (Lawrey & Diederich, 2003). Biotrophic organisms base their own survival on the survival of their host, maintaining a low level of parasitism that allows them to extend the life of the host over time (Jeffries & Young, 1994). In lichenicolous fungi, gall induction has been considered a type of virulence in parasitic lichenicolous fungi (Hawksworth 1982a). Some authors have pointed out that *Tremella* parasitic fungi cannot be considered obligate parasites since it is possible to cultivate their yeast phase in the absence of the host (Pippola & Kytöviita, 2009). Following this same reasoning, the *Tremella* lichenicolous fungi of which axenic cultures have been achieved (J. C. Zamora pers. com.) could also be non-obligate parasites if considered parasites. However, the parasitic character of lichenicolous fungi is a controversial subject with conflicting opinions, whose unraveling needs further research.

General conclusions

General conclusions

1. *Tremella caloplacae* s. l. is a species complex that comprises at least 9 putative species, each one of them growing and developing in a specific Teloschistaceae lichen host genus or species.
2. The combination of ITS and nuLSU regions is confirmed as a good barcode for lichenicolous Tremellales.
3. The known diversity within *T. caloplacae* s. l. would have diversified approximately 30 Mya in the Oligocene; even though, if considering the potential unknown diversity, *T. caloplacae* could have originated earlier. Diversification in the *T. caloplacae* complex could be, in turn, a consequence of the rapid diversification of the Teloschistaceae in the late Cretaceous. *Tremella* species would have specialized on specific lichen hosts among the great diversity of new ecological niches offered by the adaptive radiation of the Teloschistaceae.
4. Host switch is the main event that could explain the diversification and the evolutionary history between the *T. caloplacae* complex and the Teloschistaceae lichens they inhabit.
5. Five new species have been formally described in the *T. caloplacae* complex and *T. caloplacae* s. str. have been re-circumscribed to specimens growing on *Variospora* spp. Another three species of *Tremella* have been left unnamed due to the low number of specimens known.
6. Despite the scarcity of morphological characters that are taxonomically useful in lichenicolous *Tremella* species, species within the *Tremella caloplacae* complex can be morphologically characterized. The combination of morphological, molecular and ecological data, following and integrative approach, results in accurate species descriptions.
7. *Tremella parietinae* is a dimorphic species that alternate between a unicellular monokaryotic yeast phase and a dikaryotic filamentous phase. The second develops the sexual reproductive structures or basidiomata. This filamentous phase is restricted to grow in the hymenium of its *Xanthoria parietina* hosts, while the yeast phase can be found also in the thallus and thalline margin of *X. parietina* specimens with and without galls.

8. Basidiomata in *T. parietinae* can be developed without inducing the formation of galls in the hymenium of its *X. parietina* hosts.
9. DNA from *Tremella caloplacae* s. str., *T. candelariellae*, *T. dendrographae* and *T. pusillae* has been amplified from asymptomatic thalli or asymptomatic areas of the thallus, in different lichen species of the Teloschistaceae with and without visible *Tremella* galls. This occurs in a in wider range of lichen hosts compared to the confirmed filamentous phase, which suggests that the yeast phase is less specific towards the lichen host.

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