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Complexity of lichen symbiosis

Komplexita lišejníkové symbiózy

Ph.D. Thesis

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Author's declaration

I hereby declare that I have written thesis independently using listed references. I have submitted neither this thesis nor its parts to acquire any other academic degree.

Prohlášení autorky

Čestně prohlašuji, že jsem nepředložila práci ani její části k získání jiného nebo stejného akademického titulu a že jsem práci zpracovala samostatně za použití citované literatury.

In Prague, January 2023 Ivana Černajová

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List of papers included in the thesis

Paper 1

Černajová I. and Škaloud P. (2019) The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. nov. Fungal Biology 123: 625-637. https://doi.org/10.1016/j.funbio.2019.05.006

Paper 2

Černajová I. and Škaloud P. (2020) Lessons from culturing lichen soredia. Symbiosis 82: 109-122. https://doi.org/10.1007/s13199-020-00718-4/

Paper 3

Černajová I., Schiefelbein U. and Škaloud P. (2022) Lichens from the littoral zone host diverse Ulvophycean photobionts. Journal of Phycology 58: 267-280. https://doi.org/10.1111/jpy.13234

Paper 4

Černajová I., Steinová J, Škvorová Z. and Škaloud P. (2022) The curious case of *Cladonia luteoalba*: no support for its distinction. Lichenologist 54: 345–354. https://www.doi.org/10.1017/S002428292200024X

Paper 5

Černajová I., Schmidtová J., Schiefelbein U., Dal Grande F. and Škaloud P. (2023) Niche expansion through photobiont switch: contrary evidence from highly-selective seashore lichen communities, manuscript.

Authors' contributions

Paper 1: IČ an PŠ designed the study, IČ peformed the molecular laboratory work and analysed the data, PŠ co-analyzed the data, IČ wrote the manuscript and PŠ reviewed and edited the manuscript

Paper 2: IČ an PŠ designed the study, IČ peformed the in-vitro experiments and molecular laboratory work and analysed the data, PŠ reviewed the data, IČ wrote the manuscript and PŠ reviewed and edited the manuscript

Paper 3: IČ: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (equal). US: Investigation (equal), PŠ. Conceptualization (equal); Formal analysis (supporting); Funding acquisition (lead); Methodology (supporting).

Paper 4: IČ, JS and PŠ designed the study, IČ and JS collected the material, IČ performed the culturing and laboratory work, ZŠ participated in the molecular laboratory work, IČ analysed the data, JS and PŠ reviewed the data, IČ wrote the manuscript and the co-authors edited the manuscript

Paper 5: IČ and PŠ designed the study, US, JS, IČ and PS collected the material, JS performed the laboratory work, IČ participated in the laboratory work, JS, IČ and PŠ analysed the data, FDG lead the creation of metabarcoding data analysis pipeline, IČ wrote the manuscript and the co-authors edited the manuscript

On behalf of all the co-authors, I declare the keynote participation of Ivana Černajová in completing the research and writing the papers, as described above.

In Prague Pavel Škaloud

Abstract

Lichens are currently viewed as complex symbiotic systems. In addition to the main mycobiont and photobiont, a variety of associated fungi, bacteria and algae/cyanobacteria (minor/accessory photobionts) have been recognized. Their diversity has been intensively studied, but is still far from being fully apprehended. Likewise, recognition of the significance of the associated organisms to the whole system is still at the beginning but various crucial roles, from constitutive morphogenetic through physiological to various means of increasing the lichen's fitness, have already been suggested.

The present thesis attempts to approach lichens in their full complexity. Focusing on two model systems; the *Cladonia-Asterochloris* association and ecologically delimited communities of Verrucariaceae; it aims: i) to examine patterns in photobiont choice and their relationship to lichen ecology; ii) to set a framework for in-vitro mycobiont-photobiont compatibility testing; iii) to explore the diversity of selected associated fungi and their possible relationships with the lichen host.

We have shown that Verrucariaceae in the intertidal zone associate with largely understudied Ulvophycean photobionts. They mainly belong to Kornmanniaceae, Ulvales, and include a variety of novel lineages, one of which was circumscribed as *Undulifilum symbioticum* gen. et sp. nov. Also, *Urospora* sp., Ulotrichales, an order previously not known to include lichen symbionts, has been recognized and confirmed as a photobiont. *Hydropunctaria maura*, a common wide-spread seashore lichen, was highly selective in its photobiont choice. It generally maintained the association with *Pseudendoclonium submarinum*, regardless of its abundance in the pool of free-living algae and regardless of the seawater salinity level.

In-vitro development of *Cladonia fimbriata* soredia generally exhibited the previously published stages. However, no thalline structures were achieved in the experiments. This cannot be evaluated as a sign of incompatibility as, obviously, only compatible partners are spread by soredia. Importantly, the soredium disintegrates at the beginning of its development (both in-vitro and in-situ) and the symbionts need to recognize each other anew. Thus, these observations establish a suitable reference frame for future compatibility testing.

Species of *Cladonia* commonly associated with diverse Cystobasidiomycete yeasts, previously hypothesized to represent a third constituent of the symbiosis. The association was neither constant nor linked to the lichen morphology, i.e., presence of the cortex layer or the specific phenotype of *C. luteoalba*, as suggested by previous studies. Some of the yeasts were isolated into culture for the first time and *Lichenozyma pisutiana* gen. et sp. nov, Microsporomycetaceae, was circumscribed. We also showed that Cystobasidiomycete yeasts, as well as other diverse associated fungi, are spread with lichen soredia.

Thus, the present results contribute to our knowledge of the diversity of lichen symbionts and the patterns in their associations. Yet, they highlight the need for further studies and open more questions for future research.

Abstrakt

Lišejníky v současnosti považujeme za komplexní symbiotické systémy. Kromě hlavního mykobionta a fotobionta se v jejich stélkách nachází množství sekundárních hub, bakterií a řas nebo sinic. Diverzita těchto asociovaných organismů je intenzivně studována, avšak k jejímu celkovému zachycení máme stále daleko, stejně tak k porozumění jejich významu pro fungování lišejníku jako celku. K zásadním předpokládaným funkcím asociovaných organismů patří vliv na morfogenetické a fyziologické procesy, případně i různé způsoby zvyšováni fitness lišejníku.

Tato dizertační práce se snaží nahlížet na lišejníky v jejich celkové komplexitě. Soustřeďuje se na dva modelové systémy – na symbiózu mezi rody *Cladonia* (dutohlávka) a *Asterochloris* a na ekologicky vymezená společenstva lišejníků čeledi Verrucariaceae. Klade si za cíl: i) prozkoumat vztah mezi výběrem fotobionta a ekologií lišejníků, ii) definovat referenční rámec pro testování kompatibility mykobiontů a fotobiontů in vitro, iii) prozkoumat diverzitu vybraných sekundárních hub a jejich možné vztahy k hostitelským lišejníkům.

Z našich studií vyplývá, že fotobionti obojživelných lišejníků čeledi Verrucariaceae z přílivové zóny mořského pobřeží jsou málo známé ulvofytní řasy. Většina z nich patřila do čeledi Kornmanniaceae (Ulvales) a mnohé představují nové vývojové linie. Jednu z nich jsme popsali jako *Undulifilum symbioticum* gen. et sp. nov. Dalším fotobiontem byla *Urospora* sp. (Ulotrichales), zaznamenána v lišejnících vůbec poprvé, a navíc jako jediný symbiotický zástupce z celého řádu. Běžný a široce rozšířený lišejník *Hydropunctaria maura* vykazoval vysokou míru selektivity. Nejčastěji tvořil symbiózu s řasou *Pseudendoclonium submarinum*, a to bez ohledu na jeho abundanci ve společenstvech dostupných volně žijících řas a nezávisle na salinitě mořské vody.

Sorédie lišejníku *Cladonia fimbriata* se v in vitro experimentech vyvíjely srovnatelně s doposud publikovanými údaji. Avšak struktury stélkového charakteru nedosáhly. V sorédiích se zjevně šíří jenom kompatibilní symbionti, proto nepřítomnost pokročilejších struktur v experimentech nelze považovat za znak nekompatibility partnerů. Sorédie se na počátku vývoje (jak in vitro tak in situ) rozpadají a symbionti se tak musí umět nově rozpoznat, tudíž počáteční stádia lze srovnávat s vývojem lišejníku de novo. Pozorování in vitro experimentů tak definuje optimální referenční rámec pro budoucí testování kompatibility jednotlivých partnerů.

Kvasinky třídy Cystobasidiomycetes, které byly navrženy jako třetí obligátní symbiont lišejníků, se běžně vyskytovaly v různých druzích lišejníků rodu *Cladonia*. Tento vztah však nebyl stálý a nesouvisel s morfologií lišejníku, tj. s přítomností svrchní kůry a tvorby specifického fenotypu *C. luteoalba*, jak naznačovaly předchozí studie. Některé z kvasinek se mi vůbec poprvé povedlo izolovat do kultury a díky tomu jsme popsali *Lichenozyma pisutiana* gen. et sp. nov. (Microsporomycetaceae). Ukázali jsme, že kvasinky třídy Cystobasidiomycetes se spolu s množstvím dalších hub šíří pomocí lišejníkových sorédií.

Tyto dílčí výsledky zásadním způsobem přispívají k znalosti diverzity lišejníkových symbiontů a vztahů mezi nimi. Zároveň otvírají nové otázky a zdůrazňují potřebu navazujících studií.

Contents

1. Introduction

Ever since the symbiotic nature of lichens was proposed (Schwenderer 1867), there have been recurring attempts to define and redefine them. One of the elementary problems lied in finding the boundary between lichens of all morphologies and all the other associations between fungi and autotrophic organisms (Hawksworth 1988), such as mycorrhizae, fungi parasitising macroscopic algae, *Geosiphon pyriforme* (Glomeromycota) in stable association with *Nostoc* (Cyanobacteria) or unstable fungal-algal association, such as *Schizoxylon albescens* with *Coccomyxa/Pseudococcomyxa* (Muggia et al. 2011). Some of the attempts were quite inventive, e. g., "a fungus … living with, but not apparently harming, an alga, which is studied by lichenologists" (Hawksworth 1978). All the currently accepted definitions are derived from the definitions by Ahmadjian (1982) and Hawksworth (1983), combining into *a stable self-supporting fungal-algal/cyanobacterial association which forms a thallus different from either symbiont in the free-living (= unlichenized) state*.

The morphogenetic effect of lichenization was also emphasized in the latest classification of lichenized fungi (Lücking et al. 2017), pointing out to the filamentous algal-like *Coenogonium* species, which differ in their appearance from their aposymbiotic photobionts. By this logic, mycophycobioses, the associations of Verrucariales fungi with macroscopic algae, which form the outer structure (Hawksworth 2000), also fulfil the definition of lichens. At least in two cases, *Mastodia tesselata* and *Turgidosculum ulvae*, the fungal symbionts induce anatomical and morphological changes in their associated algae (*Prasiola* sp. and *Blidingia* sp., respectively; Pérez-Ortega et al. 2010, 2018). In other cases, such changes are not known yet, but the mycobiont enhances the desiccation tolerance of the photobiont, thus changing its ecology (briefly summarized by, e.g., Honegger 2012). However, this view is not generally shared, as some authors insist on the exhabitant nature of the lichen mycobiont (e.g., Hawksworth and Grube 2020).

Another issue in fitting a definition rose from accepting the fact that multiple organisms participate in lichen symbiosis. Poelt´s recognition of multiple-membered symbioses (Poelt 1977) was adopted by Hawksworth (1988), who used the terms two-/three-/four-biont symbioses to include cephalodiate, algicolous or bryophilous lichens, lichens parasitised by algicolous fungi, lichenicolous fungi or lichenicolous lichens as well as supposed mechanical hybrids of lichens. Later, asymptomatic endolichenic fungi, analogous to plant endophytes, were evidenced within lichen thalli (Petrini et al. 1990) and lichens were regarded as "consortia with unknown numbers of participants" (Honegger 1992).

Simultaneously, throughout the time, lichens had also been perceived as miniature self-contained ecosystems, taking into account all the associated microfungi, bacteria (e.g., decomposers) and invertebrates (Nash III. 2008 and references therein). Since the change of the millennium, this view gained more weight as the presence of countless microscopic fungi, bacteria and algae on and within lichen thalli became clear, thanks to the rapid development and wide accessibility of molecular methods. Subsequently, some of the key roles in nutrient provision, degradation of old tissues, synthesis of vitamins or hormones, detoxification processes and protection against various stresses were attributed to the associated bacteria (reviewed by Aschenbrenner et al. 2016).

In 2016, the ground-breaking paper by Spribille et al. (see below for more detail) introduced Cyphobasidiomycete yeasts as possible third obligatory partners of certain macrolichens and intensified the debate on what role the associated microorganisms play in the symbiosis itself and

what their influence on the resulting lichen phenotype is (Spribille et al. 2016, Spribille 2018, Hawksworth and Grube 2020). And thus, more than 150 years after the recognition of the symbiotic nature of lichens, we again struggle with the delimitation of what we understand under the word "lichen" and face new calls for their redefinitions (Hawksworth and Grube 2020, Allen and Lendemer 2022).

For now, the status given to all the associated microorganisms appears to be more a question of interpretation (and personal taste) than of evidence and gives the impression of an ongoing paradigmatic shift (Komárek 2017). It became clear that lichen symbiosis is very complex and dynamic and should be approached as such. Witnessing these (possible) shifts has been an exciting and stimulating experience and I am lucky to have worked on my doctorate during this time. The present thesis represents an attempt to contribute to understanding the enormous complexity of lichens.

1.1 Photobiont choice

Symbiotic interactions between the main partners, understood through the lichen´s photobiont choice, are controlled at various levels. At the evolutionary level, taxonomic specificity, cospeciation and recognition capability may result from adaptive coevolution (Thompson 1994). At the environmental level, association patterns may be shaped by overlaps in ecological requirements of both symbionts or by their local availability (Beck et al. 2002). At the level of biological characteristics of individual lichen species, association patterns may depend on lichen functional traits, such as reproduction type. And finally, stochastic processes, like drift or dispersal, may also play their roles (Yahr et al. 2004).

Coevolution between lichen symbionts is generally low, a fact already implied by the taxa counts alone; there are currently almost a thousand mycobiont and less than 50 photobiont genera recognized, comprising almost 20 000 and 100-200 species, respectively (Škaloud and Peksa 2010, Lücking et al. 2017, Nelsen 2021, Sanders and Masumoto 2021). Thus, photobionts are necessarily shared by a number of mycobionts. Although the estimated proportion of yet-undescribed species is much higher in photobionts, the imbalance is likely to be substantive (Sanders and Masumoto 2021). The lack of strict coevolution does not mean that mycobiont-photobiont associations are not structured.

From a macroevolutionary perspective, histories of the main lichen symbionts, Lecanoromycetes and Trebouxiales are interconnected, as it is estimated that Trebouxiales diversified coincidentally with or prior to Lecanoromycetes, thus plausibly facilitating their early diversification (Nelsen et al. 2020). At the same time, a broadly defined photobiont type (Cyanobacteria/Trebouxiophyceae/ Trentepohliales) does not influence the rate of speciation, extinction or diversification within Lecanoromycetes and transitions between these types were generally rare in evolutionary time (Nelsen et al. 2020).

Consequently, large monophyletic mycobiont groups (families, orders, subclasses or even classes) are conservative in their choice of photobiont type or even photobiont genera (e.g., *Trebouxia* in Umbilicariomycetidae, *Nostoc* in Collematineae, *Trentepohlia* in Graphidaceae; Rambold et al. 1998, Miadlikowska et al. 2006). Switches are relatively rare and usually subsequently conserved

(Miadlikowska et al. 2006), e.g., within Lecanorales from *Trebouxia* to *Myrmecia* and *Asterochloris* in Psoraceae and Leprariaceae, respectively.

On the contrary, photobiont switch is a common phenomenon at a lower taxonomic level. This was initially demonstrated in Physciaceae and Cladoniaceae, whose phylogenies were highly incongruent with the phylogenies of their respective photobionts, possible cospeciation events were rare, and instead, the associations could be presented as interaction networks between mycobiont species and algal lineages/genotypes (Helms et al. 2001, Piercey-Normore and DePriest 2001, Helms 2003). In both cases, specific algal genotypes were shared by variously related mycobiont species and the latter differed in the variety of photobionts they were capable of associating with, i.e., specificity. Similar patterns have now been observed for a number of lichens (e.g., O'Brien et al. 2013, Sing et al. 2016, Magain et al. 2017, Vančurová et al. 2018, Lindgren et al. 2020, Moya et al. 2021). At the species/lineage level, the specific associations are thus delimited by the species specificity resulting from evolutionarily derived capabilities of partner recognition (Schaper & Ott 2003, treated in more detail in the next chapter).

Environmental drivers of the photobiont choice are complementary, rather than alternative, to the above. Partner selection may be due to similar ecological demands of both symbionts (Beck et al. 2002). As pointed out by Beck et al. (2002), the resulting association patterns may be similar to the patterns caused by cospeciation because phylogenetically related species might share similar environmental requirements. However, in the former case, the underlying phylogenies would be incongruent (Beck et al. 2002), as is the case of many lichens.

In agreement with the environmentally driven associating patterns, photobionts are commonly shared within lichen communities (Rikkinen 1995, Beck et al. 1998, Beck 1999, Yahr et al. 2004, Kaasalainen et al. 2021, Peksa et al. 2022). Consequently, the concept of photobiont-mediated guilds was proposed (Rikkinen et al. 2002) and accepted for both chloro- and cyanolichens. A similar pattern was also observed in other symbiotic systems, such as corals – dinoflagellates (van Oppen et al. 2001) or grasses – endophytic fungi (Rodriguez et al. 2008). For the latter, it was shown that specific endophytes enable tolerating specific stress conditions and the phenomenon was termed habitat-adapted symbiosis (Rodriguez et al. 2008).

A photobiont-mediated guild is a set of mycobiont species associated with the same photobiont, or a set of photobionts, with similar eco-physiological characteristics presumably, best adapted for the given environment, maximizing the lichen holobiont's fitness (Rikkinen 2003). Guilds are the building blocks of communities. Individual mycobiont species are usually restricted to a single guild and they can only switch between algal partners within their respective guild (Rikkinen 2003, Škvorová et al. 2022). Few species are able to cross the guild borders (Kaasalainen et al. 2021, Peksa et al. 2022, Škvorová et al. 2022). This phenomenon has not been particularly explored yet, and thus its consequences are not fully understood. Studies of *Lasallia pustulata* and *Stereocaulon* spp. suggest that switching between photobionts of different environmental requirements widens the ecological niche of the lichens (Rolshausen et al. 2018, Vančurová et al. 2018). However, such a low specificity seems to be exceptional for now.

Environmental availability is another factor strongly influencing the photobiont choice at the population level (Beck et al. 2002). The photobionts can be acquired from the pool of free-living algae, adopted from already established lichen thalli or their vegetative propagules (Hawksworth et

al. 1979, Bubrick et al. 1984, Rikkinen et al. 2002, Sanders and Lücking 2002, Sanders 2014). Available photobiont pools have mostly been studied as inventories of photobionts in given lichen communities (e.g., Paulsrud et al. 2000, Yahr et al. 2004, Vančurová et al. 2018, Kaasalainen et al. 2021). A single study focusing on free-living photobiont pools has been published so far (Vančurová et al. 2020). Mycobiont species in these studies seem to be selective towards their photobionts, i.e., they have preferences for certain algal lineages and do not choose the most available photobionts. This selectivity may be, however, environmentally conditioned (Yahr et al. 2006).

And finally, links between lichen functional traits and photobiont choice have also been documented. For instance, vegetatively reproducing species are usually more specific towards their photobionts than sexually reproducing species, which have to establish a new partnership after each reproduction (Steinová et al. 2019); crustose lichens tend to be less specific than foliose and fruticose lichens (Helms et al. 2001); and lichens with larger distributional ranges are less specific (Muggia et al. 2014) while habitat-specialists are more specific (Fernández-Mendoza et al. 2011). However, these links have only been studied for few traits and only in few lichen groups so far.

All the above mechanisms are not exclusive, they interact and jointly shape the association patterns in all lichen groups; chlorolichens and cyanolichens, ascomycetes and basidiomycetes. The underlying processes may be universal, but their particular importance varies among taxa and scales in focus. Understanding the driving forces that balance this interplay is fundamental to our understanding of the nature of the lichen symbiosis itself. In specific communities or taxa, it is also crucial for identifying threads resulting from past, ongoing and future environmental changes, making educated predictions about their development and, given their importance in many ecosystems (summarized by Seaward 2008), suggesting appropriate conservation measures (see, e.g., Allen and Scheidegger 2022).

1.2 Symbionts compatibility and recognition

Compatibility in lichens is understood indirectly via the range of realized mycobiont-photobiont associations; only the symbiont combinations known in nature are considered compatible. However, not all compatible partners are equal; many of the studied mycobionts have more or less strong preferences (i.e., selectivity) for certain algae (see previous chapter), and these are often environmentally dependent (e.g., Yahr et al. 2006, Rolshausen et al. 2020). In exceptional cases, some lichens are able to switch to what would be considered an incompatible photobiont under standard circumstances. This has been documented in *Cladonia, Diploschistes* and *Lepraria*, which normally only associate with *Asterochloris* spp., but are capable of associating with *Trebouxia* spp. under extreme conditions, such as heavy-metal polluted substrates in post-industrial habitats (Osyczka et al. 2021) or in Antarctica (Engelen et al. 2010). Mechanisms underlying the compatibility, its flexibility or rigidness are poorly studied and remain largely unrecognized. Generally, four developmental stages in initial lichen thallus formation are distinguished; pre-contact, contact, balanced growth and thallus differentiation stage; and the recognition barriers may occur during each of them (Galun 1988, Honegger 1993).

Reciprocal communication of the potential symbionts is started even before physical contact is made (the pre-contact stage; Galun 1988, Joneson and Lutzoni 2009, Meeβen and Ott 2013, Athukorala et al. 2014). Specific molecules involved in the signalling have not yet been recognized (Meeβen et al.

2013, Piercey-Normore & Athoukorala 2017), but, for example, fungal lectins were suggested to play a role (Kardish et al. 1991). The pre-contact signalling leads to release of specific polyols by the photobiont, specifically ribitol in trebouxioid algae (Richardson et al. 1968). These polyols induce morphological and metabolic changes in the mycobiont leading to envelopment of the photobiont cells by the mycobiont hyphae and/or a gelatinous matrix during the next, the contact stage (Joneson and Lutzoni 2009, Guzow-Krzemińska and Stocker-Wörgötter 2013, Meeβen and Ott 2013, Athukorala et al. 2014). These changes are accompanied by both up- and downregulation of whole sets of genes in both symbionts (Trembley et al. 2002, Joneson and Lutzoni 2011, Athukorala and Piercey-Normore 2015), but further studies are necessary to understand them.

The specificity of the morphological changes, and thus their value for the partner compatibility evaluation, has been questioned because the response has also been observed in incompatible symbiont combinations (not known in nature; Ahmadjian and Jacobs 1981, Guzow-Krzemińska and Stocker-Wörgötter 2013, Meeβen and Ott 2013). Low specificity at early developmental stages could be a beneficial strategy to survive until a more suitable partner is acquired (Ott 1987, Trembley et al. 2002, Piercey-Normore 2006). Additionally, the morphological changes in both partners at the contact stage are temperature- and pH-dependent (Athoukorala & Piercey-Normore 2014) which might be the mechanism behind the environmentally conditioned photobiont selection.

Further developmental stages, the balanced growth and thallus differentiation, are presumably only completed in compatible symbiont pairs; otherwise, one symbiont is overgrown by the other or the undifferentiated mass never develops into a stratified lichen thallus (reviewed by Stocker-Wörgötter 2001, Piercey-Normore and Athoukorala 2017). However, even compatible symbionts often fail to accomplish these stages. Thus, the failure is often rather a result of cultivation conditions than evidence of incompatibility (e.g., Ahmadjian 1962, Guzow-Krzemińska and Stocker-Wörgötter 2013).

Cultivation difficulties are the main obstacle for better understanding of the processes characterizing early lichenization (Galun 1988, Honegger 1996). First, the isolation and long-term cultivation of mycobionts are generally problematic (Crittenden et al. 1995). And then, full lichen thallus resynthesis in-vitro has only infrequently been achieved during the ca. 150 years of attempts (summarized by Stocker-Wörgötter 2001). Resynthesis experiments require specific conditions, such as appropriate medium composition and alternation of drying and re-wetting (Ahmadjian 1962, 1966, Stocker-Wörgötter 1995, Zorer et al. 1997). These conditions are not only species- but probably also laboratory-specific and optimization is needed for each study (Piercey-Normore & Athoukorala 2017).

1.3 Associated fungi

Lichen thalli, as any other multicellular organisms, are inhabited by numerous fungi, jointly termed lichen-associated. They include lichenicolous and endolichenic fungi (e.g., Beck et al. 2014). The former; i.e., parasites inducing visible, although often inconspicuous, modifications on the thalli (Lawrey and Diederich 2003), had been known even before the symbiotic nature of lichens was recognized (e.g., Dillenius 1741, Acharius 1795) and later caused substantial problems in formulation of lichen definition (reviewed by Hawksworth 1988). On the other hand, the presence of endolichenic fungi, i.e., living within living lichen tissues without causing visible symptoms, analogous to plant endophytes (Arnold et al. 2009); was sensed throughout the 20th century due to their

common growth whenever mycobiont isolation into culture was attempted (Hawksworth 1988, Honegger 2012) but has been fully acknowledged only since the 1990s (Petrini et al. 1990, Girlanda et al. 1997). They have been referred to as endolichenic (Arnold et al. 2009) or endothallic (Oberwinkler 2017).

However, these terms are not really adequate because the border between the interior and the exterior of a lichen thallus is not sharp (Fernández-Mendoza et al. 2017) and, additionally, some of the fungi are rather linked to the lichen surface (Spribille et al. 2016, Tuovinen et al. 2019). Therefore, a more general term, asymptomatic lichen-associated fungi (Beck et al. 2014), is more suitable. This term also encompasses the asymptomatic life cycle phases of lichenicolous fungi (e.g., Diederich 2011, Tuovinen et al. 2021) or fungi constituting superficial biofilms (Spribille 2018). These fungi have been intensively studied during the last decades, both based on culture-dependent and DNA metabarcoding approaches (see references below). However, major attention was brought to them since the work of Spribille et al. (2016) who suggested that they play a constructive role in the lichen phenotype.

The asymptomatic lichen-associated fungi have been detected in all screened thalli of all main lichen growth forms (Beck et al. 2014, Muggia et al. 2016, Zhang et al. 2016, Suryanarayanan et al. 2017). They are hyperdiverse, often belonging to yet unknown lineages found in all main classes of Ascomycetes, more rarely in Basidiomycetes (Li et al. 2007, Arnold et al. 2009, Peršoh and Rambold 2012, U'Ren et al. 2012, Zhang et al. 2015, 2016, Muggia et al. 2016, Banchi et al. 2018). However, this imbalance might be at least partly caused by methodological issues, such as sample processing procedures, cultivation media or PCR biases (U'Ren et al. 2014, Muggia et al. 2017, Banchi et al. 2018).

They differ from endophytic, endobryophytic, rock-inhabiting, corticolous, leaf-litter decaying or lichenicolous fungal communities, on scales from plot to continental, although with various degrees of overlaps (Suryanarayanan et al. 2005, Peršoh and Rambold 2012, U'Ren et al. 2010, 2012, Beck et al. 2014, Fleischhacker et al. 2015). Thus, lichens represent an important substrate for fungal diversity. Based on phylogenetic analyses and ancestral state reconstruction, Arnold et al. (2009) even suggested that lichens as a substrate played an indispensable role in the diversification of Ascomycota, serving as an incubator to endophytism, which is evolutionarily unstable, giving way to trophic transitions to pathogenicity or saprotrophism. The ecosystem role of lichens as a substrate, harbouring a complex fungal pool, has also been highlighted; only a part of the hosted community is strictly lichen-associated; the rest (whether trapped diaspores or metabolically active) are either generalists or species with multiple ecological niches, i.e., capable of trophic transitions (U'Ren et al. 2010, Honegger 2012, Fernández-Mendoza et al. 2017, Selosse et al. 2018, Hawksworth and Grube 2020). Lichens thus represent an important inoculum source.

Compared to lichenicolous fungi, which are often strictly species-specific (Diederich et al. 2018), asymptomatic lichen-associated fungi are more generalist (Fernández-Mendoza et al. 2017), although, the lichen identity is among the main drivers of the composition of the associated fungal community (Girlanda et al. 1997, Li et al. 2007, U'Ren et al. 2010, Beck et al. 2014). Interestingly, low host specificity is also found in asymptomatic yeast stages of host-specific symptomatic lichen parasites (Tuovinen et al. 2021). It has been suggested that they physically associate with the photobiont (Arnold et al. 2009). This has only been evidenced for *Tremella* sp. in *Letharia vulpina* (Tuovinen et al. 2019) so far and requires further studies. However, the common growth of fungi

from photobiont-free tissues in attempts at mycobiont cultivation suggests that it is not the case for many of them.

The effect of most of the asymptomatic fungi on their lichen hosts is not known. It can only be guessed from analogies with plant endophytes (Honegger 2012); from neutral mutualism to providing herbivore and parasite protection or drought resistance (reviewed by Rodriguez et al. 2009).

A major significance was attributed to Cyphobasidiomycetes yeasts by Spribille et al. (2016). Studying metatranscriptomes of two phenotypically distinct *Bryoria* species indistinguishable based on DNA sequence data, they found that the species differed by the abundance of specific yeasts in the cortex. They further found the yeasts in 52 lichen genera, including 42 of 56 sampled genera of the family Parmeliaceae, hypothesizing that they are ubiquitous associates of these macrolichens and obligate constituents of the cortical layer. They further hypothesized that the missing yeast, as the third symbiotic partner, may be the reason why advanced lichen structures are not formed in in-vitro resynthesis experiments.

Although not yet properly tested, the hypotheses were immediately adopted by many authors (e.g., Palmqvist et al. 2017, Suryanarayanan and Thirunavukkarasu 2017, Zúñiga et al. 2017) with the potential to revolutionize the way we think about and understand the lichen symbiosis, reflected in emerging calls for redefinitions (Spribille 2018, Hawksworth and Grube 2020, Allen and Lendemer 2022).

1.4 Model systems

Two contrasting model systems were selected for my studies. Members of the family Verrucariaceae associate with an exceptionally wide range of photobionts (Thüs et al. 2011), unfortunately, our knowledge of their diversity is still limited. We focused on Verrucariaceae from the intertidal zone, a very specific habitat, expecting to find a specific, strictly defined set of photobionts. On the other hand, the genus *Cladonia* associates with *Asterochloris* spp. Both genera have been intensively studied (see below) and we have quite a good understanding of the ecology of the individual species as well as the patterns in their associations which can be built on, e.g., in compatibility studies.

Verrucariaceae in the intertidal zone

Verrucariaceae (Eurotiomycetes, Chaetothyriomycetidae) is the third largest lichen family with about a thousand described species (Lücking et al. 2017). Data on their photobionts are quite scarce. Tschermak-Woess in her iconic work (Tschermak-Woess 1989) summarized the contemporaneous knowledge based on cultivations or direct microscopical identifications. Since then, there have been a couple of isolated records (e.g., in Watanabe 1997, Voytsekhovich & Beck 2016) or studies of specific taxa (e.g., Nyati et al. 2007, Gueidan et al. 2011, Gasulla et al. 2019). A single comprehensive inventory using molecular data has been published so far (Thüs et al. 2011). Thus, the associated photobionts are known only for a small portion of Verrucariaceae species (less than 10 % to my knowledge), yet their range is extremely wide.

Verrucariaceae photobionts belong to two non-related Eukaryote supergroups; Archaeplastida (Chloroplastida: Trebouxiophyceae and Ulvophyceae) and TSAR (Stramenopiles: Phaeophyceae and Xanthophyceae), the latter not known from any other lichens (Tschermak-Woess 1989, Gueidan et al.

2011, Thüs et al. 2011). It is expected that the family diversified after a single lichenization event (Lücking et al. 2017) and thus the photobionts must have been switched many times during the evolution. The currently recognized diversity can be briefly summarized as follows:

Among Trebouxiphyceae, the genus *Diplosphaera* (Prasiolales) appears to be the most common. Algal species historically placed under *Diplosphaera, Stichococcus* and *Protococcus* are all intermixed based on molecular data (Thüs et al. 2011). Proschold and Darienko (2020) made an attempt to resolve the complex and segregated various new genera. The photobiont lineages are now placed within the genera *Deuterostichococcus, Pseudostichococcus* and *Diplosphaera*. However, the *Diploshpaera* photobionts isolated by Thüs et al. (2011) cannot be assigned a name and there still remains an undescribed diversity. For the list of mycobiont species associated with *Diplosphaera* sensu lato see Thüs et al. (2011) and Sanders and Masumoto (2021).

More rarely, other trebouxiophycean genera were also reported as photobionts of Verrucariaceae (Tschermak-Woess 1989, Watanabe 1997, Nyati et al. 2007, Thüs et al. 2011, Voytsekhovich & Beck 2016): *Asterochoris* sp. (from *Bagliettoa cazzae* and *Heteroplacidium contumescens*); *Auxenochlorella* sp. (from *Psoroglaena stigonemoides*); *Chloroidium* sp. (from *Verrucaria nigrescens*, based on light microscopy (LM) only); *Elliptochloris bilobata* (from *Verrucaria sublobulata*); *Myrmecia* spp. (from *Placidium* and *Heteroplacidium* species, and from *Catapyrenium rufescens*, *Dermatocarpon* spp. and *V. submersela*, based on LM only); *Trebouxia* spp. (from *Bagliettoa marmorea*, *Polyblastia* sp., *Staurothele* sp., *V. coerulea* and *Verrucaria* sp., based on LM); and an unknown trebouxiophycean lineage (near *Heterochlorella*, from *Psoroglaena epiphylla*).

Among Ulvophyceae, the former genus *Dilabifilum* is the most common Verrucariaceae photobiont (Tschermak-Woess 1989, Thüs et al. 2011). It was divided into the genera *Halofilum*, *Lithotrichon*, *Paulbroadya* and *Pseudendoclonium* (Darienko and Proschold 2017) within the family Kornmanniaceae (Škaloud et al. 2018). They all include photobionts of mainly aquatic/littoral freshwater or marine *Verrucaria* and *Hydropunctaria* species.

The Xanthophycean *Heterococcus* sp. has been found in freshwater (temporarily or permanently submersed) *Hydropunctaria* and *Verrucaria* species (Thüs et al. 2011, Rodriguez-Flakus and Flakus 2021). Historically, *Heterococcus caespitosus* (common in soil) was identified based on LM from both freshwater and marine Verrucariaceae (reviewed by Tschermak-Woess 1989). However, based on molecular data, the *Heterococcus* sp. found by Thüs et al. (2011) represents a distinct undescribed species. Also, its presence in marine lichens requires revision.

The only known Phaeophycean lichen photobiont, *Petroderma maculiforme* is a well-studied photobiont of coastal Californian *Wahlenbergiella tavaresiae* (Wynne 1969, Moe 1997, Peters & Moe 2001, Sanders et al. 2004, 2005, Gueidan et al. 2011). Interestingly, it also forms aposymbiotic macroscopic thalli with a much wider distribution but narrower ecological niche (Sanders et al. 2004).

Finally, marine Verrucariaceae, also form the curious borderline lichens, or mycophycobioses, where the macroscopic thallus is formed by the photobiont and the mycobiont is the inhabitant (Kohlmeyer et al. 2004). *Mastodia tesselata* with *Prasiola* spp. (Trebouxiophyceae, Garrido-Benavett et al. 2017) and *Turgidosculum ulvae* with *Blidingia minima* (Ulvophyeae, Pérez-Ortega et al. 2018) are among the best known.

As evident from the above, there is an extraordinary photobiont choice plasticity not only at the family level, but also within some genera, especially in aquatic and amphibious *Hydropunctaria* and *Verrucaria* lichens. This fact directed our attention to the so-called black belt on the seashore.

Figure 1 Littoral fringe, the black belt, formed mostly by *Hydropunctaria maura*. Kullaberg, Sweden.

On seashore rocks, lichens commonly form a well-recognizable zonation (for more details see e.g., Dobson 2014). The upper part of the littoral zone, the littoral fringe, forms the transition between aquatic and terrestrial environments. Below the littoral fringe, there is the eulittoral zone frequently submersed by the tide, often occupied by barnacles and only few lichen species, e.g., *Collemopsidium foveolatum* (Collemopsidiomycetes, Xanthopyreniaceae) and *Wahlenbergiella mucosa* (Verrucariaceae, Dobson 2014). Above, there is the supralittoral zone, only little affected by sea spray. The littoral fringe, however, is occasionally submersed by the tide, washed by waves frequently and sprayed by seawater heavily. It is biologically defined as the upper limit of periwinkles (*Littorina*) and by the occurrence of *Hydropunctaria maura* (Lewis 1961). *H. maura* commonly forms conspicuous extensive continuous black crusts (Fig. 1), giving the zone also the name black belt/zone. Together with *H. maura*, *Wahlenbergiella striatula*, other *Hydropunctaria* and *Verrucaria* species, as well as the fruticose cyanolichen *Lichina pygmaea* may grow (e. g., Fletcher 1975). In this environment, lichen thalli need to cope with extreme changes in osmotic pressure as a result of constant changes in both salinity and water content, due to the effects of seawater, exposure and drying, as well as absorption of freshwater from rainfall (Dobson 2014). It might be expected that thriving in such conditions is enabled (at least partly) by a specific set of photobionts and the small number of available studies suggested that it would be Ulvophycean algae (Tschermak-Woess 1989, Thüs et al. 2011, Darienko and Proschold 2017, Gasulla et al. 2019).

Cladonia-Asterochloris

Cladonia is the fourth most speciose lichen genus, with ca. 500 species worldwide (Lücking et al. 2017). Many of the species are morphologically extremely variable, and for many species complexes it is difficult to set species boundaries (e.g., Pino-Bodas et al. 2013). The involvement of molecular methods has produced mixed outcomes. Some closely related and difficult-to-distinguish species were supported (e.g., Pino-Bodas et al. 2010, Stenroos et al. 2015); in other cases, morphologically distinguishable entities were synonymized (Kotelko & Piercey-Normore 2010, Pino-Bodas et al. 2010); and finally, ambivalent results with no apparent taxonomic consequences were often obtained (e.g., Piercey-Normore et al. 2010, Steinová et al. 2013, Pino-Bodas et al. 2015). In such cases, the authors often discuss the processes underlying low phylogenetic resolution and discrepancies in the molecular data, such as incomplete lineage sorting, unrecognized paralogs, introgression, homoplasy or horizontal gene transfer. Despite these difficulties, thanks to the long-lasting tradition (some *Cladonia* species have been known since pre-Linnean times (Burgaz and Ahti

2009 and references therein)) and intensive interest in the genus until the present (e.g., Osyczka 2006, 2011, Burgaz and Ahti 2009, Ahti et al. 2013, Pino-Bodas et al. 2021), there is generally a very good understanding of the distribution and ecology of majority or the European species or accepted recognizable entities.

Cladonia lichens always associate with the green alga *Asterochloris*, with a single known exception in extreme habitats (Osyzcka et al. 2021). The exclusivity is not mutual; *Asterochloris* is one of the most common lichen photobionts (Miadlikowska et al. 2006), associating also with, e.g., *Diploschistes, Hymenelia, Ionaspis, Lepraria, Pilophorus, Pycnothelia, Squamarina* or *Stereocaulon.* There are currently 18 *Asterochloris* species described, with a number of formally yet undescribed species-level lineages (Piercey-Normore and DePriest 2001, Nelsen and Gargas 2006, Škaloud and Peksa 2010, Kim et al. 2017, 2020, Vančurová et al. 2020, Pino-Bodas and Stenroos 2020, Kosecka et al. 2021). The numbers will probably rise when the poorly studied subtropical and tropical regions have been better explored (Pino-Bodas and Stenroos 2020). However, for European species and lineages, high-quality data on the distribution and ecological requirements are already available (Škaloud and Peksa 2010, Peksa and Škaloud 2011, Škaloud et al. 2015, Moya et al. 2015, Vančurová et al. 2018, Steinová et al. 2019, Vančurová et al. 2020, Vančurová et al. 2021, Škvorová et al. 2022).

Cladonia mycobionts have repeatedly been successfully isolated into culture and also used in resynthesis experiments (e.g., Ahmadjian 1966, Stocker-Wörgötter 1995, Zorer et al. 1997, Bačkor and Fahselt 2003, McDonald et al. 2013, Athukorala and Piercey-Normore 2015). First genomes of both symbiotic partners were obtained by Armaleo et al. (2009) from cultures of *C. grayi – A. glomerata*. Pilot studies on gene expression during the first stages of thallus development have also been conducted (Joneson and Lutzoni 2011, Athukorala and Piercey-Normore 2015).

The peculiar *C. luteoalba* (see Fig. 1 in Paper 4) is a specific case. It is morphologically well-recognizable and often grows in association with other *Cladonia* species (specifically from the *C. coccifera* agg., Stenroos et al. 2019). Remarkably, its chemotype corresponds to the chemotype of the associated *Cladonia*. Due to this pattern, it was called enigmatic by Stenroos (1990) and she proposed several possible explanations. In one of them, the initially lichenicolous *C. luteoalba* parasitizes an existing *Cladonia* thallus, then acquires its photobiont and forms a symbiotic thallus of its own. Subsequently, *C. luteoalba* has been used as an example of a lichen that obtains its photobiont through theft (Nelsen & Gargas 2009, Dal Grande et al. 2012, Williams et al. 2017), although no *C. luteoalba* photobiont sequence had ever been published. Another possible explanation, that the *C. luteoalba* morphotype is induced by an infection, was ruled out by the author due to occasional production of reproductive structures (Stenroos 1990). However, attempts to obtain sequences of the mycobiont always resulted in sequences of the associated *C. coccifera* agg. sp. (J. Steinová, unpubl.) and so, the status of *C. luteoalba* remains unknown and the pattern in its chemotype unexplained.

Taken together, the robust data on the ecology and distribution of both partners, their association patterns and the feasibility of cultivation experiments make *Cladonia-Asterochloris* an ideal system for studying compatibility of the partners as well as possible effects of associated fungi on the symbiotic outcome.

2. Aims of the study

The general purpose of my studies was to contribute to understanding the diversity and association patterns of lichen symbionts and their possible roles in the symbiotic system. The specific aims were:

1. To examine the photobiont diversity and the relationship between lichen ecology and its photobiont choice in:
    a. An ecologically strictly defined community ("the black belt")
    b. *Cladonia luteoalba*
2. To set a framework for mycobiont-photobiont compatibility testing in *Cladonia*.
3. To explore the relationship between a lichen and its associated fungi, particularly:
    a. The conection between specific associated fungi and the lichen phenotype
    b. The role of lichen vegetative propagules in spreading the associated fungi

3. Key results and discussion

3.1 Photobionts

Papers 3 and 5 represent the first inventories focused specifically on intertidal lichen photobionts. We found complete dominance of Ulvophytes. The vast majority of the photobionts belonged to the family Kornamnniaceae (Ulvales); *Pseudendoclonium submarinum* was by far the most common. Five other species-level lineages within the genus *Pseudendoclonium* were detected. Only one of them had previously been known (*P. commune*) and two were found both in Europe and Chile (*Pseudendoclonium* sp. P3 and *P.* aff. *arthropyreniae*). Additionally, another unknown lineage, closely related to *Kornmannia leptoderma* (labelled *Kornmannia*2), was found as a photobiont of the mycobiont lineage V2. And finally, we detected, cultivated and circumscribed *Undulifilum symbioticum* gen. et sp. nov. in Paper 3.

Based on phylogenetic analyses (Škaloud et al. 2018), the family Kornmanniaceae consists of ten genera of morphologically and ultrastructurally dissimilar algae, half of which contain photobionts of amphibious lichens. The family is predominantly marine but there are several transitions to brackish, freshwater or aerophytic habitats (see Fig. 4 in Paper 3). Such flexibility has been documented even at the intraspecific level. For example, *Halofilum ramosum*, has been isolated from the green biofilm on a wall of ruins as well as from intertidal lichens, its identity in both cases verified by DNA sequence data (Darienko and Proschold 2017). Additionally, physiological experiments found distinct osmoregulatory responses between strains of *H. ramosum* isolated from lichens from different vertical zones on the seashore and the hypervariable chloroplast RPL10A region sequence data suggested that the eco-forms might actually represent young sister species (Gasulla et al. 2019). We hypothesized that this evolutionary flexibility, observed at various levels of the family, is connected with the capacity for dynamic osmoregulatory changes that are inevitable in the intertidal zone and makes the members of Kornmanniaceae the most successful intertidal lichen photobionts.

A minor part of the intertidal photobionts was identified as *Urospora* sp. (Acrosiphoniaceae, Ulotrichales) based on DNA sequence data. A single lineage was found in four Baltic and three Chilean specimens. These are the first-ever records of *Urospora* as lichen photobionts. Based on 18S and ITS rDNA, they are intermixed with *U. wormskioldii*/*penicilliformis*, which are macroscopic filamentous algae of the intertidal zones of cold seas (Lindstrom and Hanic 2005). The finding was surprising not only because of the macroscopic nature of the closest relatives, but also because no other lichen photobiont is known in the whole order Ulotrichales. For verification, DNA was isolated again from ethanol-surface sterilized pieces of thalli of the Chilean samples, and amplification and sequencing were repeated. Each time, chromatograms with single distinct peaks were obtained. The finding was supported by microscopical observations of the lichen thalli; the photobiont cells contained several pyrenoids (Fig. 8 in Paper 5), a feature typical of the genus (Leliaert et al. 2009). At both study sites, *Urospora* sp. was found in association with mycobionts of the *Wahlenbergiella* group, which is understudied and includes a number of deep undescribed lineages (Pérez-Ortega et al. 2010, also Fig. 2 in Paper 3). Possibly, *Urospora* will turn out to be not an uncommon lichen photobiont when the lichen group will have been more closely studied.

The composition of the mycobionts, which is generally the strongest predictor of the photobiont diversity (e.g., Vančurová et al. 2018), differed completely between the study sites. On the

Patagonian shore, 13 mycobiont genotypes, representing ten phylogenetic lineages, were found. Only one of them could be given an existing name (*Mastodia tessellatula*), while the rest represents an undescribed diversity (within the genus *Hydropunctaria* and the *Wahlenbergiella* group). At the shore of the Baltic Sea, Kattegat and the North Sea, the wide-spread *Hydropunctaria maura*, two other *Hydropunctaria* species (*H. oceanica* and *H. aractina*, previously only known from Great Britain and northern Norway, respectively), a rare *Verrucaria ceuthocarpa* and a common *V. ditmarsica*, which comprises four cryptic lineages in our dataset, were found.

The photobionts were shared between the sites at the level of algal families; however, specificity and selectivity were recognized at the genus and species level (Fig. 7 in Paper 3 and Fig. 9 in Paper 5). At both study sites, the genus *Hydropunctaria* was the most specific, associating only with Kornmanniaceae photobionts. *V. ditmarisca* also associated only with Kornmanniaceae in our study, no other photobiont data from the species are available. However, within Verrucariaceae, it forms a long lineage together with the borderline lichen *Turgidosculum ulvae* associating with *Blidingia minima* (Pérez-Ortega et al. 2018). *Wahlenbergiella* group also associated with Kornmanniaceae as well as with *Urospora* sp. This group also includes the borderline lichen *Mastodia tessellata,* which associates with *Prasiola* sp. (Pérez-Ortega et al. 2010, Garrido-Benavent et al. 2017).

Specificity and selectivity of individual mycobiont species could only be evaluated for the three best sampled lineages in Europe. *V. ceuthocarpa* (5 samples) and *V. ditmarsica*1 (11 samples) always associated with *Urospora* sp. and *P. commune*, respectively. However, in four specimens of *V. ceuthocarpa* and two specimens of *V. ditmarsica*1, an additional photobiont was detected (*P.* aff. *arthropyreniae* and *P. submarinum*, respectively). *H. maura* exhibited the lowest specificity, as it associated with four species of *Pseudendoclonium* – *P. submarinum* (70 samples), *P. commune* (2 samples), P1 (1 sample) and P3 (1 sample).

Clearly, *H. maura* was strongly selective for *P. submarinum* and was consistent in this preference across different salinity zones (Figs. 1 and 9 in Paper 5). Thus, the wide ecological amplitude of *H. maura* is not facilitated by photobiont switch as documented in other lichens (Rolshausen et al. 2018, 2020, Oszycka et al. 2020, Vančurová et al. 2020). He hypothesized that the success of the holobiont is given by a more or less stable association of two generalists (both the mycobiont and the photobiont) but a more detailed study will be necessary to support this.

The selectivity of *H. maura* was further supported by the composition of the pool of available free-living algae in the lichen's surroundings which was sampled at seven sites. At each of them *H. maura* associated with *P. submarinum* although the choice of its compatible partners (algae it associated with at other sites) was wider and *P. submarinum* was the most abundant of them at only three sites (Fig. 10 in Paper 5). *Urospora* was also available at six of the seven sites but was only selected by *V. ceuthocarpa* which occurred at one site only. This suggests incompatibility of *Urospora* with the other mycobiont species studied on one hand, and high specificity of *V. ceuthocarpa* towards *Urospora* on the other, as various *Pseudendoclonium* species were also available at the site. These data also imply that the distribution of the rare *V. ceuthocarpa* is not limited by the availability of its photobionts.

The case study of *Cladonia luteoalba* and its *Asterochloris* photobionts in Paper 4 gives a different picture. Given that the *C. luteoalba* morphotype does not represent any coherent phylogenetic entity

(for details see Paper 4) any patterns in its photobiont specificity and photobiont choice should be interpreted very cautiously. Still, there are two facts worth noting.

First, the specificity of the association appears to be stronger than environmentally driven choice. *C. straminea* lineage is specific to *A. glomerata* and *A. irregularis*, which are the most common *Asterochloris* species in cold climates (Škvorová et al. 2022). However, it maintains these photobionts even in milder climates where other *Asterochloris* species are available (and *C. coccifera* agg. representatives associate with those there).

Second, finer-scale collection data might clarify a possible environmentally driven selection. In *C. coccifera* agg. it is currently impossible to delimit species based on molecular data probably due to incomplete lineage sorting and ongoing speciation (Steinová et al. 2013, 2019). The aggregate as a whole had been shown to associate with an unusually high number of *Asterochloris* photobionts (Steinová et al. 2019) and we found additional species/lineages in *C. luteoalba* belonging to this group (Fig. 4 in Paper 4). Where more specimens were collected at a site, they usually shared their photobionts. If not, a reason could usually be found, either obvious - such as different substrate (rock vs. pine tree bark - *Asterochloris* sp. StA3 and *A. italiana*, respectively), or minor – such as a specific position on a boulder (top vs. sides – *A. stereocaulonicola* and *A. italiana*, respectively). Although the sampling was not robust enough, the data suggest that even microhabitat or microclimatic data could influence the photobiont choice and should be given more attention.

3.2 In-vitro compatibility

Initially, I started co-culturing various combinations of *Cladonia* – *Asterochloris* species with the aim to distinguish compatible partners from incompatible and test changes in the compatibility under different conditions. However, the evaluation of the experiments was not straightforward. First, no thallus-like structures were achieved. Second, the development of the co-cultures showed a relatively high degree of randomness, i.e., if co-cultures were inoculated in replicates, these often developed differently, at least considering the timing. And third, the sequence of the developmental stages was not rigid and different structures, that could be considered signs of compatibility according to the literature, occurred in different combinations following the contact stage (Fig. 2). Thus, a reliable reference frame was needed and studying the development of soredia was selected as a suitable option.

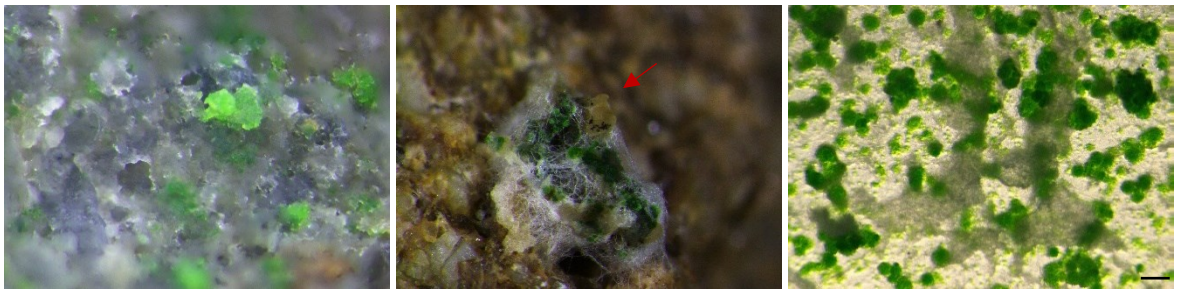

Figure 2 Examples of in-vitro symbiont interactions. **A** Mucilaginous matrix sticking the partners together. **B** Loose arachnoid structure growing from compact mycobiont clusters (arrow) and enclosing groups of photobiont cells. **C** Detail of the loose arachnoid medullary structure on agar. Scale bars represent 200 µm.

The development of the soredia of *Cladonia fimbriata* in-vitro, summarized in Paper 2, followed the previously reported scheme, which was comparable for both in-vitro and in-situ observations of soredia development (Schuster 1985, Stocker-Wörgötter and Türk 1988, 1989, Stocker-Wörgötter 1991) as well as de-novo lichen resynthesis from spores (Galun and Garthy 1988, Zorer et al. 1997). An important feature is that the symbionts fall apart at the beginning of the soredium development; the mycobiont germinates into a loose arachnoid mycelium spreading at the cultivation medium surface and the photobiont divides asexually forming a cluster of cells at the place of the original soredium (Fig. 2a in Paper 2). This implies that the partners need to recognize each other anew before further development and the subsequent processes are analogous to the reestablishment of the symbiosis de novo from mycobiont spores and photobiont cells (Athukorala et al. 2014). At the end of the soredium development a thallus-like structure should be formed (Ahmadjian 1966, Stocker-Wörgötter and Türk 1988, Stocker-Wörgötter 1995). No such structure developed in our experiments, the most advanced stage reached was the primordium (Fig. 2h, 3d and 3e in Paper 2). A primordium exhibits certain stratification; a layer of dense fungal network is formed on its surface enclosing the photobiont inside. In our experiments, it did not have the anatomy of a cortex. Instead, it was composed of aerial hyphae (Fig. 3a in Paper 2) that expanded after each re-wetting cycle, enlarging the primordium and colonizing more substrate (Fig 3f in Paper2). These observations show that the compatibility of the partners is not disproved by the lack of formation of advanced morphological structures, as long as the primordium stage is formed. We hypothesized that further development in-vitro probably depends on finding the right conditions, which, in addition to pH, humidity and temperature, might involve stresses other than drying, for example night temperature drops, or air movement as a mechanical stimulus. The results of this study represent a convenient reference-frame for our future studies of compatibility of *Cladonia* mycobionts with diverse photobionts.

As a secondary outcome, this study also has an important methodological implication. Mycobiont culturing is generally problematic (Crittenden et al. 1995). It is usually accomplished by in-vitro spore germination or by thallus fragments cultivation. Single-spore isolates are obviously preferred if molecular and genomic studies are targeted, however in-vitro maturation, discharge and germination of spores might be difficult to achieve in many lichen species. On the other hand, the thallus fragment method (Yamamoto et al. 1985) is theoretically convenient for any lichen species. But, it is often hindered by the reluctance of the mycobionts to grow (Crittenden et al. 1995). Additionally, a high contamination rate is a problem in both methods, despite careful and elaborate thallus washing steps and clean benchwork (Crittenden et al. 1995). The contamination rate for inoculating soredia in our study varied depending on the media used (Table 3 in Paper 2) and was comparable to other isolation methods (Crittenden et al. 1995), but the process was much less time-consuming and required incomparably less effort – soredia are simply transferred from the intact thalli onto the cultivation media with a sterile needle. The mycobiont isolation success was also very promising – 55-91 % of the uncontaminated plates. The figure is difficult to compare with other studies, as unsuccessful efforts are hardly ever reported. Zakeri et al. (2022) reported isolation success rates of 63-65 % for the spore discharge method, 0-18 % for thallus fragments and 52-64 % for soredia. Crittenden et al. (1995) attempted to culture a wide spectrum of lichen mycobionts (1183 species from 14 orders) and accounted 59 % of the isolation failures (however in terms of species numbers) with fragments to the lack of growth. In my experience with *Cladonia* species

(unpublished), the isolation success using the thallus fragment method is many times lower and requires much higher effort than isolating soredia. Isolation success using the spore discharge method may be comparable to soredia isolation and is much faster; spores usually discharge and germinate within a couple of days. But the workflow is more elaborate. So, in my opinion, soredia isolation is the most straightforward method for the acquisition of mycobiont cultures from sorediate lichen species.

3.3 Associated fungi

In Paper 1 we explored the occurrence and diversity of Cystobasidiomycete yeasts in *Cladonia* throughout Europe. We detected their presence in 99 out of 104 screened thalli and obtained sequences from 56 of them (corresponding to 27 *Cladonia* species). I also isolated seven cultures and those were the first lichen-associated Cystobasidiomycete yeast cultures ever published.

The diversity we obtained was quite different from that reported by Spribille et al. (2016). Most of the yeasts we detected belonged to Microsporomycetaceae, compared to only four sequences in Spribille et al. (2016). Thanks to the culturing success, we even described *Lichenozyma pisutiana* gen. et. sp. nov. within the family. A new unknown lineage related to *Symmetrospora* was the second most abundant. And finally, we only detected two representatives of Cyphobasidiales, an order described by Spribille et al. (2016), where the vast majority of the yeasts they found in Parmeliaceae lichens belonged.

So, we hypothesized that the yeast – lichen association might be specific at a higher taxonomic level; Microsporomycetaceae are mostly specific to *Cladonia* and Cyphobasidiales mostly to Parmeliaceae. This assumption has been disproved since. First, the range of lichen hosts of specific cystobasidiomycete lineages has widened; *L. pisutiana* has been found in *Lecanora* spp. and *Rhizoplaca melanophthalma* (Mark et al. 2020, Cometto et al. 2022, both Lecanoraceae), various lineages within Microsporomycetaceae as well as Cyphobasidiales also in Parmeliaceae, Physciaceae and Lecanoraceae (Mark et al. 2020) and our *Symmetrospora*-related lineage also in *Tephromela atra* (Tephromelataceae, Cometto et al. 2022). Second, some lichens were shown to associate with multiple cystobasidiomycete lineages; in Paper 2 we found that isolates of three distantly related lineages (*Cystobasidum* sp., *Microsporomyces* sp. and an unknown lineage; Fig. 1 in Paper 2) associated with soredia of *Cladonia,* and Mark et al. (2020) reported a similar situation in *Parmelia sulcata, Pseudevernia furfuracea, Physcia adscendens/tenella* and *Lecanora* spp. They concluded that the lichen-associated Cysobasidiomycete yeasts are much less mycobiont-specific than the photobionts (Mark et al. 2020).

Our data also challenged the hypothesis of Spribille et al. (2016) that these yeasts are cortex-associated in macrolichens. We found them in corticate (e.g., *Cladonia furcata*), partly corticate (e.g., *C. pocillum*) and ecorticate (e.g., *C. rangiferina*) species. It is thus likely that in *Cladonia* these fungi are rather either constituents of a superficial biofilm (as suggested by Spribille, 2018) or live within the thallus without association to the cortex as many other fungi.

The ubiquity of the association between Cystobasidiomycete yeasts and macrolichens remains questionable. Spribille et al. (2016) reported it from 42 of 56 (75 %) sampled Parmeliaceae genera, and in 52 lichen genera in total. We found cystobasidomycetes in 27 *Cladonia* species (95.2 % of the studied specimens; Table1 in Paper 1) and also in association with *Cladonia* soredia (Paper 2). Mark

et al. (2020) found them in 59.8 % of their 838 samples (representing 10 lichen species) and Cometto et al. (2022) isolated five cultures representing three distinct Cystobasidiomycete lineages from two lichen species. On the other hand, Lendemer et al. (2019) found them in only nine out of 413 (i.e., 2.2 %) lichen samples (representing 339 species and 57 families) and Smith et al. (2020) only in five out of 35 (14 %) samples, both using metagenomic data. Smith et al. (2020) discuss exhaustively the potential biases of their results, such as the expected dominance of the main symbionts in lichen metagenomic reads (Pizarro 2019, Tagirdzhanova et al. 2021) and a potential bias from the bioinformatics pipeline. On the other hand, both studies present convincing evidence of their capability to detect Cystobasidiomycetes; Lendemer et al. (2019) stated that in several cases, the coverage of the cystobasiomycete rDNA was comparable to, or higher than, that of the lichen mycobiont; and Smith et al. (2020) detected a relatively higher abundance of Cystobasidiomycete sequences in all three studied samples of *Bryoria fremontii*, which is the species previously shown to contain a whole superficial layer of the yeasts and with which the whole story began (Spribille et al. 2016).

Meanwhile, it has been shown that other basidiomycetous yeasts are also common in lichens. As anticipated by Oberwinkler (2017), they are the haploid phase of dimorphic lichenicolous fungi. *Tremella* species are well-known lichen parasites, forming visible galls on the thalli (e.g., Millanes et al. 2012, 2014, 2015). Interestingly, their yeast stage has now been largely found also in asymptomatic thalli, sometimes even widening the host range (Tuovinen et al. 2019, 2021). The yeast stage of *T. lethariae* was restricted to the lichen cortex, while the filamentous stage formed galls on the surface and extended inwards, making contact with the photobiont cells (Tuovinen et al. 2019). On the other hand, the yeast stages of *T. macrobasidiata* and *T. variae* were distributed across *Lecanora* spp. thalli and hymenia (Tuovinen et al. 2021). Recently, 76 basidiomycetous yeast strains were isolated from lichen thalli, representing Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes (Cometto et al. 2022).

Although the hypothesis of Spribille et al. (2016) that a basidiomycete yeast might be a third obligate lichen symbiont was regarded very critically by some authors (e.g., Oberwinkler 2017) and has not received much factual support for now, it undoubtedly had a very important consequence; it inspired and provoked further studies of lichen-associated fungi (see the references above), bringing us closer to understanding the enormous complexity of the lichen symbiosis. Still, a functional aspect of the association might be discovered one day. Analysing metagenome-assembled genomes, Tagirdzhanova et al. (2021) already suggested that cortex-associated basidiomycetes may participate in the production of the extracellular matrix, crucial in the constitution of the symbiosis, as well as in nutrition acquisition.

The relationship between lichens and their associated fungi can also be seen from another perspective; lichens represent an important substrate for a diversity of these fungi (Arnold et al. 2009). In theory, they can be classified into four ecological groups: i) lichenicolous/strictly lichen-associated (symptomatic or symptomless), ii) generalists shared with the environment, iii) fungi with multiple ecological niches, and iv) transient fungi, i.e., accidentally trapped inactive diaspores (Honegger 2012, Fernández-Mendoza et al. 2017, Oberwinkler 2017, Selosse et al. 2018, Hawksworth and Grube 2020). For any of them, lichen vegetative propagules are one of the few means of leaving the thallus and proceeding with their life cycle. In Paper 2, we showed that diverse fungi co-disperse with lichen soredia, as previously documented for bacteria (Aschenbrenner et al. 2014). Among our

isolates (Table 1 in Paper 2), there were apparently fungi present on/in other substrates at the collection sites, i.e., fungi previously known from various parts of pine trees (e.g., *Cystobasidium pinicola, Pseudocamaropycnis pini*) or from soil (undescribed species, but with high similarity matches in GenBank). Whether any of them represents transient species, species with multiple niches, or simply diaspores stuck at the lichen surface, can only be guessed with the current state of knowledge. We also recovered a couple of isolates of unknown fungi, with BLAST search matches lower than 90 %. If these represent unknown strictly lichen-associated fungi, or any other of the above groups, can again only be speculated. In any case, we have shown that lichen soredia serve as a dispersal vector for multiple fungi. This fact implies yet another possible role of lichens in enhancing ecosystem diversity.

Finally, in Paper 4 we were not able to match the phenotype of *Cladonia luteoalba* with any associated fungi. The three hypotheses of Stenroos (1990) to explain the phenotype were: mechanical hybridization, a commensalistic symbiosis system of two mycobionts with one photobiont and a disease that induces morphological changes to other *Cladonia* species. She considered the second option to be the most plausible. Our data did not support any of them. The first two options would involve two distinct *Cladonia* entities in the thallus. No such case was found, neither by culturing nor by metabarcoding – there was always a single dominant mycobiont present (Fig. 3 in Paper 4). The third option was not supported either, but its resolution suffered from methodological shortcomings.

We expected that if a fungus was causing the phenotype change, metabarcoding would reveal a specific genotype in all the *C. luteoalba* samples but in none of the control thalli. It did not. However, the primers we used were originally designed to favour *Cladonia* sequences in soil samples. During the testing, their performance was poor; they were not specific, amplifying a whole range of soil fungi. However, when used for lichen thalli, they probably performed differently and the fungal spectrum we obtained cannot be considered representative. The bias is obvious; Lecanoromycetes are highly represented in the dataset while Basidiomycota are almost absent (Table S4 in Paper4). Consequently, we believe that the third hypothesis of Stenroos (1990), an external cause of the *C. luteoalba* phenotype, is the most probable. Although, our data were not sufficient to either prove or disprove it. The next step in "cracking the enigma" should be to explore *C. luteoalba*-associated fungi in more detail, ideally together with bacteria and viruses.

4. Conclusions

The studies within this thesis were done during times of calls for redefinitions of lichen symbiosis which resulted from acknowledging the diversity of organisms associated with lichen thalli and from the significance recently attributed to them (Spribille et al. 2016, 2022, Spribille 2018, Tuovinen et al. 2019, Hawksworth and Grube 2020, Allen and Lendemer 2022). There are various options (indirect and direct) to explore possible functional aspects of an association; e.g., evaluating the frequency of the association, reading genomes of the symbionts or via experiments. In any case, recognizing the identity and diversity of the participating symbionts is a fundamental first step.

There had only been isolated records of photobionts of intertidal lichens. We showed that they mainly include closely related genera within Kornmanniaceae, Ulvales (Papers 3 and 5) and that a significant diversity within the family is still not known. *Urospora*, Ulotrichales, adds to the list of peculiar photobionts of intertidal Verrucariaceae lichens (e.g., in Hawksworth 2000, Honegger 2012). In communities of intertidal lichens, the specificity may be low but the selectivity is high (Paper 5), contradicting the principle of ecological niche widening through photobiont switch (Rolshausen et al. 2018, 2020). The ubiquitous *Hydropunctaria maura* represents a great model for further studies. Importantly, we have shown that mycobionts choose from algae that are rare, but certainly present among the free-living algal communities. Our data suggest that even the ecologically strictly defined intertidal zone consists of several photobiont-mediated guilds. Further studies will be needed to address the intertidal photobiont diversity and ecology, in order to subsequently explore these phenomena. Additionally, photobiont plurality, detected in several thalli here (Paper 5), deserves more attention; its advantages might be more pronounced in the intertidal zone compared to other habitats, as already suggested by Chrismas et al. (2021). Likewise, *Urospora*-associated lichens represent a promising study system as they offer an analogy to the association of *Wahlenbergiella tavaresiae* with *Petroderma maculiforme*, which is macroscopic, but ecologically more restricted, when free-living (Sanders et al. 2004). However, the identity, phylogenetic relationships and distribution of the *Urospora* photobionts need to be uncovered first.

On the contrary, the association patterns of *Cladonia* – *Asterochloris* species are relatively well-known (e.g., Peksa and Škaloud 2011, Pino-Bodas et al. 2020). However, they might change under changing environmental conditions (Yahr et al. 2006). The mechanisms underlying this flexibility are not well understood. Altered selectivity, achieved via altered recognition, may be one of them (Athoukorala & Piercey-Normore 2014). This hypothesis can be tested in in-vitro experiments. Culturing soredia in Paper 2, we have established a reference frame for the in-vitro development of a compatible symbiont combination relevant to our laboratory. This was a necessary first step that will enable further experiments. In addition to compatibility of the main symbionts, the effect of the presence of associated fungi on the initial development can also be tested now.

Morphogenetic effects of the associated Cystobasidiomycete yeasts on some macrolichens were suggested by Spribille et al. (2016) and have been discussed since then. In Paper 1, we explored their diversity in the genus *Cladonia* and found a variety of additional lichen-associated fungal taxa in Papers 2 and 4. No relation was found between their diversity and lichen morphology but future research may bring new hints (as in Tagirdzhanova et al. 2021). On the other hand, the importance of lichen thalli as a substrate and its possible ecological consequences should be highlighted. Further diversity studies (including substrates other than lichen from the same habitats) are necessary to

understand the ecology, specificity and association patterns of fungal communities with their lichen hosts.

On the whole, the present thesis emphasizes the benefits of applying multiple approaches to lichen symbiosis because due to its complexity it cannot be fully understood from separate perspectives alone. First, the interactions between the main partners or with accessory symbionts cannot be understood without high-quality diversity data. And second, the interaction patterns need to be kept in mind when interpreting issues in lichen biology, such as distribution data, ecological requirements, recognition mechanisms or integral roles of individual partners.

5. References

Acharius E. (1795) Nya och mindre kaenda Svenska Lafarter, beskrifne (V:te Fortsaettning). Kongl. Vetenskaps Academiens, Nya Handlingar 1795: 207-215.

Ahmadjian V. (1962) Investigations of lichen synthesis. American Journal of Botany 49: 277-283.

Ahmadjian V. (1966) Artificial reestablishment of the lichen *Cladonia cristatella*. Science 151: 199-201.

Ahmadjian V. (1982) The nature of lichens. Natural History 91: 31-36.

Ahmadjian V. & Jacobs J. B. (1981) Relationship between fungus and alga in the lichen *Cladonia cristatella* Tuck. Nature 289: 169-172.

Ahmadjian V., Jacobs J. B. & Russell L. A. (1978) Scanning electron microscope study of early lichen synthesis. Science 200: 1062-1064.

Ahti T., Stenroos S. & Moberg R. (2013) Nordic Lichen Flora, Vol. 5. Cladoniaceae. Museum of Evolution, Uppsala University, Uppsala, 117 pp.

Allen J. L. & Lendemer J. C. (2022) A call to reconceptualize lichen symbioses. Trends in Ecology and Evolution 37: 582-589.

Allen J. L. & Scheidegger C. (2022) Co-occurring *Lobaria pulmonaria* and *Ricasolia quercizans* share green algal photobionts: Consequences for conservation. Bryologist 125: 219-221.

Arnold A. E., Miadlikowska J., Higgins K. L., Sarvate S. D., Gugger P., Way A., Hofstetter V., Kauff F. & Lutzoni F. (2009) A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? Systematic Biology 58: 283-297.

Aschenbrenner I. A., Cardinale M., Berg G. & Grube M. (2014) Microbial cargo: do bacteria on symbiotic propagules reinforce the microbiome of lichens? Environmental Microbiology 16: 3743-3752.

Aschenbrenner I. A., Cardinale M., Berg G. & Grube M. (2016) Understanding microbial multi-species symbioses. Frontiers in Microbiology 7: 180.

Athukorala S. N. P., Huebner E. & Piercey-Normore M. D. (2014) Identification and comparison of the 3 early stages of resynthesis for the lichen *Cladonia rangiferina*. Canadian Journal of Microbiology 60: 41-52.

Athukorala S. N. P. & Piercey-Normore M. D. (2014) Effect of temperature and pH on the early stages of interaction of compatible partners of the lichen *Cladonia rangiferina* (Cladoniaceae). Symbiosis 64: 87-93.

Athukorala S. N. P. & Piercey-Normore M. D. (2015) Recognition- and defense-related gene expression at 3 resynthesis stages in lichen symbionts. Canadian Journal of Microbiology 61: 1-12.

Bačkor M. & Fahselt D. (2003) Cellulose-acetate disks as novel substrate for the resynthesis and culture of lichens. Bryologist 106: 439-442.

Beck A. (1999) Photobiont inventory of a lichen community growing on heavy-metal-rich rock. Lichenologist 31: 501-510.

Beck A., Friedl T. & Rambold G. (1998) Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. New Phytologist 139: 709-720.

Beck A., Kasalicky T. & Rambold G. (2002) Myco-photobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. New Phytologist 153: 317-326.

Beck A., Peršoh D. & Rambold G. (2014) First evidence for seasonal fluctuations in lichen-and bark-colonising fungal communities. Folia Microbiologica 59: 155-157.

Bubrick P., Galun M. & Frensdorff A. (1984) Observations on free-living *Trebouxia* de Puymaly and *Pseudotrebouxia* Archibald, and evidence that both symbionts from *Xanthoria parietina* (L.) Th. Fr. can be found free-living in nature. New Phytologist 97: 455-462.

Burgaz A. R. & Ahti T. (2009) Flora liquenológica Ibérica. Cladoniaceae. Sociedad Española de Liquenología, Madrid, 111 pp.

Cometto A., Leavitt S. D., Millanes A. M., Wedin M., Grube M. & Muggia L. (2022) The yeast lichenosphere: High diversity of basidiomycetes from the lichens *Tephromela atra* and *Rhizoplaca melanophthalma*. Fungal Biology 126: 587-608.

Crittenden P., David J. C., Hawksworth D. L. & Campbell F. S. (1995) Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. New Phytologist 130: 267-297.

Dal Grande F., Widmer I., Wagner H. H. & Scheidegger C. (2012) Vertical and horizontal photobiont transmission within populations of a lichen symbiosis. Molecular Ecology 21: 3159-3172.

Darienko T. & Pröschold T. (2017) Toward a monograph of non-marine Ulvophyceae using an integrative approach (Molecular phylogeny and systematics of terrestrial Ulvophyceae II.). Phytotaxa 324: 1-41.

Diederich P. (2011) Description of *Abrothallus parmotrematis* sp. nov. (lichenicolous Ascomycota). Bulletin de la Société des naturalistes luxembourgeois 112: 25-34.

Diederich P., Lawrey J. D. & Ertz D. (2018) The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. The Bryologist 121: 340-425.

Dillenius J. J. (1741) Historia Muscorum. Sheldonian Theatre, Oxford.

Dobson F. S. (2014) A field key to the coastal and seashore lichens. New Malden, UK, 100 pp.

Engelen A., Convey P. & Ott S. 2010. Life history strategy of *Lepraria borealis* at an Antarctic inland site, Coal Nunatak. Lichenologist 42: 339-346.

Fernández-Mendoza F., Domaschke S., García M. A., Jordan P., Martín M. P & Printzen C. (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. Molecular Ecology 20: 1208-1232.

Fernández-Mendoza F., Fleischhacker A., Kopun T., Grube M. & Muggia L. (2017) ITS1 metabarcoding highlights low specificity of lichen mycobiomes at a local scale. Molecular Ecology 26: 4811-4830.

Fleischhacker A., Grube M., Kopun T., Hafellner J. & Muggia L. (2015) Community analyses uncover high diversity of lichenicolous fungi in alpine habitats. Microbial Ecology 70: 348-360.

Fletcher A. (1975) Key for the identification of British marine and maritime lichens I. Siliceous rocky shore species. Lichenologist 7: 1-52.

Galun M. (1988) Lichenization. In: Galun M (Ed.) Handbook of lichenology, vol II. CRC Press, Boca Raton, pp. 153-169.

Galun M. & Garty J. (1988) Soredia formation of compatible and incompatible lichen symbionts. In: Scannerini S., Smith D., Bonfante-Fasolo P. & Gianinazzi-Pearson V. (Eds.) Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. NATO ASI Series (Series H: Cell Biology), vol 17. Springer, Berlin, pp. 207-218.

Garrido-Benavent I., Pérez-Ortega S. & de los Ríos A. (2017) From Alaska to Antarctica: species boundaries and genetic diversity of *Prasiola* (Trebouxiophyceae), a foliose chlorophyte associated with the bipolar lichen-forming fungus *Mastodia tessellata*. Molecular Phylogenetics and Evolution 107: 117-131.

Gasulla F., Guéra A., de los Ríos A. & Pérez-Ortega S. (2019) Differential responses to salt concentrations of lichen photobiont strains isolated from lichens occurring in different littoral zones. Plant and Fungal Systematics 64: 149-162.

Girlanda M., Isocrono D., Bianco C. & Luppi-Mosca A. M. (1997) Two foliose lichens as microfungal ecological niches. Mycologia 89: 531-536.

Gueidan C., Thüs H. & Pérez-Ortega S. (2011) Phylogenetic position of the brown algae-associated lichenized fungus *Verrucaria tavaresiae* (Verrucariaceae). Bryologist 114: 563-569.

Guzow-Krzemińska B. & Stocker-Wörgötter E. (2013) In vitro culturing and resynthesis of the mycobiont *Protoparmeliopsis muralis* with algal bionts. Lichenologist 45: 65-76.

Hawksworth D. L. (1978) The taxonomy of lichen-forming fungi: reflections on some fundamental problems. In: Street H. E. (Ed.) Essays in Plant Taxonomy. Academic Press, London, pp. 211-243.

Hawksworth D. L. (1983) A key to the lichen-forming, parasitic, parasymbiotic and saprophytic fungi occurring on lichens in the British Isles. Lichenologist 15: 1-44.

Hawksworth D. L. (1988) The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. Botanical journal of the Linnean Society 96: 3-20.

Hawksworth D. L. (2000) Freshwater and marine lichen-forming fungi. Fungal Diversity 5: 1-7.

Hawksworth D. L. & Grube (2020) Lichens redefined as complex ecosystems. New Phytologist 227: 1281-1283.

Hawksworth D. L., Coppins B. J. & James P. W. (1979) *Blarneya*, a lichenized hyphomycete from southern Ireland. Botanical Journal of the Linnean Society 79: 357-367.

Helms G. (2003) Taxonomy and symbiosis in associations of Physciaceae and *Trebouxia*. University of Göttingen (Doctoral thesis), 156 pp.

Helms G., Friedl T., Rambold G. & Mayrhofer H. (2001) Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. Lichenologist 33: 73-86.

Honegger R. (1992) Lichens: mycobiont–photobiont relationships. In: Reisser W. (Ed.) Algae and symbiosis. Biopress, Bristol, pp. 225-275.

Honegger R. (1993) Developmental biology of lichens. New Phytologist 125: 659-677.

Honegger R. (1996) Morphogenesis. In: Nash III. T. H. (Ed.) Lichen Biology. Cambridge University Press, Cambridge, UK, pp. 65-87.

Honegger R. (2012) The symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts. In: Hock B. (Ed.) The Mycota IX: Fungal Associations. Berlin and Heidelberg: Springer-Verlag, pp. 287-339.

Joneson S. & Lutzoni F. (2009) Compatibility and thigmotropism in the lichen symbiosis: a reappraisal. Symbiosis 47: 109-115.

Joneson S. & Lutzoni F. (2011) Fungal and algal gene expression in early developmental stages of lichen-symbiosis. Mycologia 103: 291-306.

Kaasalainen U., Tuovinen V., Mwachala G., Pellikka P. & Rikkinen J. (2021) Complex interaction networks among cyanolichens of a tropical biodiversity hotspot. Frontiers in Microbiology 12: 672333.

Kardish N., Silberstein L., Fleminger G. & Galun M. (1991) Lectin from the lichen *Nephroma laevigatum* Ach. Localization and function. Symbiosis 11: 47-62.

Kim J. I., Kim Y. J., Nam S. W., So J. E., Hong S. G., Choi H. G. & Shin W. (2020) Taxonomic study of three new Antarctic *Asterochloris* (Trebouxiophyceae) based on morphological and molecular data. Algae 35: 17-32.

Kim J. I., Shin W., Choi H. G. Hong S. G., So J. E. & Nam S. W. (2017) *Asterochloris sejongensis* sp. nov. (Trebouxiophyceae, Chlorophyta) from King George Island, Antarctica. Phytotaxa 295: 60-70.

Kohlmeyer J., Hawksworth D. L. & Volkmann-Kohlmeyer B. (2004) Observations on two marine and maritime 'borderline' lichens: *Mastodia tessellata* and *Collemopsidium pelvetiae*. Mycological Progress 3: 51-56.

Komárek S. (2017) Stručné dějiny biologie. Academia, Prague, 60 pp.

Kosecka M., Guzow-Krzemińska B., Černajová I., Škaloud P., Jabłońska A. & Kukwa M. (2021) New lineages of photobionts in Bolivian lichens expand our knowledge on habitat preferences and distribution of *Asterochloris* algae. Scientific Reports 11: 1-12.

Kotelko R. & Piercey-Normore M. D. (2010) *Cladonia pyxidata* and *C. pocillum*; genetic evidence to regard them as conspecific. Mycologia 102: 534-545.

Lawrey J. D. & Diederich P. (2003) Lichenicolous fungi: interactions, evolution, and biodiversity. Bryologist 106: 80-120.

Leliaert F., Rueness J., Boedeker C., Maggs C. A., Cocquyt E., Verbruggen H. & De Clerck O. (2009) Systematics of the marine microfilamentous green algae *Uronema curvatum* and *Urospora microscopica* (Chlorophyta). European Journal of Phycology 44: 487-496.

Lendemer J. C., Keepers K. G., Tripp E. A., Pogoda C. S., McCain C. M. & Kane N.C. (2019) A taxonomically broad metagenomic survey of 339 species spanning 57 families suggests cystobasidiomycete yeasts are not ubiquitous across all lichens. American Journal of Botany 106: 1090-1095.

Lewis J. R. (1961) The littoral zone on rocky shores – a biological or physical entity. Oikos 12: 280-301.

Li W.-C., Zhou J., Guo S.-Y. & Guo L.-D. (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Diversity 25: 69-80.

Lindgren H., Moncada B., Lücking R., Magain N., Simon A., Goffinet B., Sérusiaux E., Nelsen M. P., Mercado-Díaz J. A., Widhelm T. J. & Lumbsch H. T. (2020) Cophylogenetic patterns in algal symbionts correlate with repeated symbiont switches during diversification and geographic expansion of lichen-forming fungi in the genus Sticta (Ascomycota, Peltigeraceae). Molecular Phylogenetics and Evolution. 150: 106860.

Lindstrom S. C. & Hanic L. A. (2005) The phylogeny of North American *Urospora* (Ulotrichales, Chlorophyta) based on sequence analysis of nuclear ribosomal genes, introns and spacers. Phycologia 44: 194-201.

Lücking R., Hodkinson B. P. & Leavitt S. (2017) The 2016 classification of lichenized fungi in the Ascomycota and Basidiomycota – Approaching one thousand genera. Bryologist 119: 361-416.

Magain N., Miadlikowska J., Goffinet B., Sérusiaux E. & Lutzoni F. (2017) Macroevolution of specificity in cyanolichens of the genus *Peltigera* section *Polydactylon* (Lecanoromycetes, Ascomycota). Systematic Biology 66: 74-99.

Mark K., Laanisto L., Bueno C. G., Niinemets U., Keller C. & Scheidegger C. (2020) Contrasting co-occurrence patterns of photobiont and cystobasidiomycete yeast associated with common epiphytic lichen species. New Phytologist 227: 1362-1375.

McDonald T. R., Gaya E. & Lutzoni F. (2013) Twenty-five cultures of lichenizing fungi available for experimental studies on symbiotic systems. Symbiosis 59: 165-171.

Meeβen J., Eppenstein S. & Ott S. (2013) Recognition mechanisms during the pre-contact state of lichens: Influence of algal exudates and ribitol on the response of the mycobiont of *Fulgensia bracteata*. Symbiosis 59: 131-143.

Meeβen J. & Ott S. (2013) Recognition mechanisms during the pre-contact state of lichens: I. Mycobiont-photobiont interactions of the mycobiont of *Fulgensia bracteata*. Symbiosis 59: 121-130.

Miadlikowska J., Kauff F., Hofstetter V., Fraker E., Grube M., Hafellner J., Reeb V., Hodkinson B. P., Kukwa M., Lücking R., Hestmark G., Garcia Otalora M., Rauhut A., Büdel B., Scheidegger C., Timdal E., Stenroos S., Brodo I., Perlmutter G., Ertz D., Diederich P., Lendemer J. C., May P., Schoch C. L., Arnold A. E., Gueidan C., Tripp E., Yahr R., Robertson C. & Lutzoni F. (2006) New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA- and two protein-coding genes. Mycologia 98: 1088-1103.

Millanes A. M., Diederich P., Westberg M., Knutsson T. & Wedin M. (2014) *Tremella rhizocarpicola* sp. nov. and other interesting lichenicolous Tremellales and Filobasidiales in the Nordic countries. MycoKeys 8: 31-41.

Millanes A. M., Diederich P., Westberg M., Pippola E. & Wedin M. (2015) *Tremella cetrariellae* (Tremellales, Basidiomycota, Fungi), a new lichenicolous fungus on *Cetrariella delisei*. Lichenologist 47: 359-368.

Millanes A. M., Westberg M., Wedin M. & Diederich P. (2012) *Tremella diploschistina* (Tremellomycetes, Basidiomycota, Fungi), a new lichenicolous species growing on *Diploschistes*. Lichenologist 44: 321-332.

Moe R. L. (1997) *Verrucaria tavaresiae* sp. nov., a marine lichen with a brown algal photobiont. Bulletin of the California Lichen Society 4: 7-11.

Moya P., Molins A., Škaloud P., Divakar P. K., Chiva S., Dumitru C., Molina M. C., Crespo A. & Barreno E. (2021) Biodiversity patterns and ecological preferences of the photobionts associated with the lichen-forming genus *Parmelia*. Frontiers in Microbiology 12: 765310.

Moya P., Škaloud P., Chiva S., García-Breijo F. J., Reig-Armiñana J., Vančurová L. & Barreno E. (2015) Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems. International Journal of Systematic and Evolutionary Microbiology 65: 1838-1854.

Muggia L., Baloch E., Stabentheiner E., Grube M. & Wedin M. (2011) Photobiont association and genetic diversity of the optionally lichenized fungus *Schizoxylon albescens*. FEMS Microbiology Ecology 75: 255-272.

Muggia L., Fleischhacker A., Kopun T. & Grube M. (2016) Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Diversity 76: 119-142.

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

Muggia L., Pérez-Ortega S., Kopun T., Zellnig G. & Grube M. (2014) Photobiont selectivity leads to ecological tolerance and evolutionary divergence in a polymorphic complex of lichenized fungi. Annals of Botany 114: 463-475.

Nash III. T. H. (2008) Lichens as self-contained miniature ecosystems. In: Nash III. T. H. (Ed.) Lichen Biology. Cambridge University Press, Cambridge, UK, pp. 7-8.

Nelsen M. P. & Gargas A. (2006) Actin type I introns offer potential for increasing phylogenetic resolution in *Asterochloris* (Chlorophyta: Trebouxiophyceae). Lichenologist 38: 435-440.

Nelsen M. P. & Gargas A. (2009) Symbiont flexibility in *Thamnolia vermicularis* (Pertusariales: Icmadophilaceae). Bryologist 104: 404-417.

Nelsen M. P. (2021) Sharing and double-dating in the lichen world. Molecular Ecology 30: 1751-1754.

Nelsen M. P., Lücking R., Boyce C. K, Lumbsch H. T. & Ree R. H. (2020) The macroevolutionary dynamics of symbiotic and phenotypic diversification in lichens. Proceedings of the National Academy of Sciences 117: 21495-21503.

Nyati S., Beck A. & Honegger R. (2007) Fine structure and phylogeny of green algal photobionts in the microfilamentous genus *Psoroglaena* (Verrucariaceae, lichen-forming ascomycetes). Plant Biology 9: 390-399.

Oberwinkler F. (2017) Yeasts in Pucciniomycotina. Mycological Proggress 16: 831-856.

O'Brien H. E., Miadlikowska J. & Lutzoni F. (2013) Assessing population structure and host specialization in lichenized cyanobacteria. New Phytologist 198: 557-566.

Ott S. (1987) Reproductive strategies in lichens. Progress and Problems in Lichenology in the Eighties. Bibliotheca Lichenologica 25: 81-93.

Osyczka P. (2006) The lichen genus *Cladonia* (Cladoniaceae, lichenized Ascomycota) from Spitsbergen. Polish Polar Research 27: 207-242.

Osyczka P. (2011) The genus *Cladonia*, group *Cocciferae*, in Poland. Herzogia 24: 231-249.

Osyczka P., Lenart-Borón A., Borón P. & Rola K. (2021) Lichen-forming fungi in postindustrial habitats involve alternative photobionts. Mycologia 113: 43-55.

Palmqvist K., Franklin O. & Näsholm T. (2017) Symbiosis constraints: strong mycobiont control limits nutrient response in lichens. Ecology and Evolution 7: 7420-7433.

Paulsrud P. E. R., Rikkinen J. & Lindblad P. (2000) Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. New Phytologist 146: 291-299.

Peksa O. & Škaloud P. (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). Molecular Ecology 20: 3936-3948.

Peksa O., Gebouská T., Škvorová Z., Vančurová L. & Škaloud P. (2022) The guilds in green algal lichens – an insight into the life of terrestrial symbiotic communities. FEMS Microbiology Ecology 98: 1-17.

Pérez-Ortega S., de los Ríos A., Crespo A. & Sancho L. G. (2010) Symbiotic lifestyle and phylogenetic relationships of the bionts of *Mastodia tessellata* (Ascomycota, incertae sedis). American Journal of Botany 97: 738-752.

Pérez-Ortega S., Miller K. A. & de los Ríos A. (2018) Challenging the lichen concept: *Turgidusculum ulvae* (Verrucariaceae) represents an independent photobiont shift to a multicellular blade-like alga. Lichenologist 50: 341-356.

Peršoh D. & Rambold G. (2012) Lichen-associated fungi of the *Letharietum vulpinae*. Mycological Progress 11: 753-760.

Peters A. F. & Moe R. L. (2001) DNA sequences confirm that *Petroderma maculiforme* (Phaeophyceae) is the brown algal phycobiont of the marine lichen *Verrucaria tavaresiae* (Verrucariales, Ascomycota) from central California. Bulletin of the California Lichen Society 8: 41-43.

Petrini O., Hake U. & Dreyfuss M. M. (1990) An analysis of fungal communities isolated from fruticose lichens. Mycologia 82: 444-451.

Piercey-Normore M. D. & Athukorala S. N. P. (2017) Interface between fungi and green algae in lichen associations. Botany 95: 1005-1014.

Piercey-Normore M. D. & DePriest P. T. (2001) Algal switching among lichen symbioses. American Journal of Botany 88: 1490-1498.

Piercey-Normore M. D. (2006) The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. New Phytologist 169: 331-344.

Piercey-Normore M. D., Ahti T. & Goward T. (2010) Phylogenetic and haplotype analyses of four segregates within *Cladonia arbuscula* s.l. Botany 88: 397-408.

Pino-Bodas R. & Stenroos A. (2020) Global biodiversity patterns of the photobionts associated with the genus *Cladonia* (Lecanorales, Ascomycota). Microbial Ecology 82: 173-187.

Pino-Bodas R., Burgaz A. R. & Martín M. P. (2010) Elucidating the taxonomic rank of *Cladonia subulata* versus *C. rei* (Cladoniaceae). Mycotaxon 113: 311-326.

Pino-Bodas R., Martín M. P., Burgaz A. R. & Lumbsch T. (2013) Species delimitation in *Cladonia* (Ascomycota): a challenge to the DNA barcoding philosophy. Molecular Ecology Resources 13: 1058-1068.

Pino-Bodas R., Burgaz A. R., Martín M. P., Ahti T., Stenroos S., Wedin M. & Lumbsch H. T. (2015) The phenotypic features used for distinguishing species within the *Cladonia furcata* complex are highly homoplasious. Lichenologist 47: 287-303.

Pino-Bodas R., Sanderson N., Cannon P., Aptroot A., Coppins B., Orange A. & Simkin J. (2021) Lecanorales: Cladoniaceae, including the genera *Cladonia, Pilophorus* and *Pycnothelia*. Revisions of British and Irish Lichens 19: 1-45.

Pizarro D. (2019) Metagenomic sequencing with new bioinformatics approaches to understand the evolution of lichen forming fungi (Doctoral thesis). Complutense University of Madrid, Madrid.

Poelt J. (1977) Types of symbiosis with lichens. In: Bigelow H. E. & Simmons E.G. (Eds.) Abstracts, Second International Mycological Congress, Tampa, Florida, p. 526.

Pröschold.T & Darienko T. (2020) The green puzzle *Stichococcus* (Trebouxiophyceae, Chlorophyta): new generic and species concept among this widely distributed genus. Phytotaxa 441: 113-142.

Rambold G., Friedl T. & Beck A. (1998) Photobionts in lichens: possible indicators of phylogenetic relationships? Bryologist 101: 392-397.

Richardson D. H. S., Hill D. J. & Smith D. C. (1968) Lichen physiology. XI. The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts. New Phytologist 67: 469-486.

Rikkinen J. (1995) What's behind the pretty colours. A study on the photobiology of lichens. Bryobrothera 4: 1-239.

Rikkinen J., Oksanen I. & Lohtander K. (2002) Lichen guilds share related cyanobacterial symbionts. Science 297: 357.

Rikkinen J. (2003) Ecological and evolutionary role of photobiont-mediated guilds in lichens. Symbiosis 34: 99-110.

Rodriguez R. J., Henson J., Van Volkenburgh E., Hoy M., Wrigh, L., Beckwith F., Kim Y.-O. & Redman R. S. (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME journal 2: 404-416.

Rodriguez R. J., White Jr. J. F., Arnold A. E. & Redman A. R. A. (2009) Fungal endophytes: diversity and functional roles. New phytologist 182: 314-330.

Rodriguez-Flakus P. & Flakus A. (2021) Lichen symbioses in high Andean freshwater habitats: diversity, phylogenetic relationships and photobiont selectivity. International Association for Lichenology 9th Symposium (IAL 9), August 1 – 6, Brazil, online.

Rolshausen G., Dal Grande F., Sadowska-Deś A. D., Otte J. & Schmitt I. (2018) Quantifying the climatic niche of symbiont partners in a lichen symbiosis indicates mutualist-mediated niche expansions. Ecography 41: 1380-1392.

Rolshausen G., Hallman U., Dal Grande F., Otte J., Knudsen K. & Schmitt I. (2020) Expanding the mutualistic niche: parallel symbiont turnover along climatic gradients. Proceedings of the Royal Society B 287: 20192311.

Sanders W. B. (2014) Complete life cycle of the lichen fungus *Calopadia puiggarii* (Pilocarpaceae, Ascomycetes) documented in situ: propagule dispersal, establishment of symbiosis, thallus development, and formation of sexual and asexual reproductive structures. American Journal of Botany 101: 1836-1848.

Sanders W. B. & Lücking R. (2002) Reproductive strategies, relichenization and thallus development observed in situ in leaf-dwelling lichen communities. New Phytologist 155: 425-435.

Sanders W. B. & Masumoto H. (2021) Lichen algae: the photosynthetic partners in lichen symbioses. Lichenologist 53: 347-393.

Sanders W. B., Moe R. L. & Ascaso C. (2004) The intertidal marine lichen formed by the pyrenomycete fungus *Verrucaria tavaresiae* and the brown alga *Petroderma maculiforme* (Phaeophyceae): thallus organization and symbiont interaction. American Journal of Botany 91: 511-522.

Sanders W. B., Moe R. L. & Ascaso C. (2005) Ultrastructural study of the brown alga *Petroderma maculiforme* (Phaeophyceae) in the free-living state and in lichen symbiosis with the intertidal marine fungus *Verrucaria tavaresiae* (Ascomycotina). European Journal of Phycology 40: 353-361.

Schaper T. & Ott S. (2003) Photobiont selectivity and interspecific interactions in lichen communities. I. Culture experiments with the mycobiont *Fulgensia bracteata*. Plant Biology 5: 441-450.

Schuster G. (1985) Die Jugendentwicklung von Flechten. Ein Indikator fur Klimabedingungen und Umweltbelastung. Bibliotheca lichenologica 20: 1-206.

Schwendener S. (1867) Über die wahre Natur der Flechtengonidien. Verhandlungen der Schweizerischen Naturforschenden Gesellschaft 57: 9-11.

Seaward M. R. D. (2008) Environmental role of lichens. In: Nash III. T. H. (Ed.) Lichen Biology. Cambridge University Press, Cambridge, UK, pp. 274-298.

Selosse M.-A., Schneider-Maunoury L. & Martos F. (2018) Time to re-think fungal ecology? Fungal ecological niches are often prejudged. New Phytologist 217: 968-972.

Singh G., Dal Grande F., Divakar P. K., Otte J., Crespo A. & Schmitt I. (2017) Fungal–algal association patterns in lichen symbiosis linked to macroclimate. New Phytologist 214: 317-329.

Škaloud P. & Peksa O. (2010) Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta). Molecular Phylogenetics and Evolution 54: 36-46.

Škaloud P., Steinová J., Řidká T. & Vančurová L. (2015) Assembling the challenging puzzle of algal biodiversity: species delimitation within the genus *Asterochloris* (Trebouxiophyceae, Chlorophyta). Journal of Phycology 51: 507-527.

Škaloud P., Moya P., Molins A., Peksa O., Santos-Guerra A. & Barreno E. (2018) Untangling the hidden intrathalline microalgal diversity in *Parmotrema pseudotinctorum*: *Trebouxia crespoana* sp. nov. Lichenologist 50: 357-369.

Škvorová Z., Černajová I., Steinová J., Peksa O., Moya P. & Škaloud P. (2022) Promiscuity in lichens follows clear rules: partner switching in *Cladonia* is regulated by climatic factors and soil chemistry. Frontiers in Microbiology 12: 781585.

Smith H., Dal Grande F., Muggia L., Keuler R., Divakar P. K., Grewe F., Schmitt I., Lumbsch H. T. & Leavitt S. D. (2020) Metagenomic data reveal diverse fungal and algal communities associated with the lichen symbiosis. Symbiosis 82: 133-147.

Spribille T., Tuovinen V., Resl P., Vanderpool D., Wolinski H., Aime M. C., Schneider K., Stabentheiner E., Toome-Heller M., Thor G., Mayrhofer H., Johannesson H. & McCutcheon J. P. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353: 488-492.

Spribille T. (2018) Relative symbiont input and the lichen symbiotic outcome. Current Opinion in Plant Biology 44: 57-63.

Steinová J., Stenroos S., Grube M. & Škaloud P. (2013) Genetic diversity and species delimitation of the zeorin-containing red-fruited *Cladonia* species (lichenized Ascomycota) assessed with ITS rDNA and β-tubulin data. Lichenologist 45: 665-684.

Steinová J., Škaloud P., Yahr R., Bestová H. & Muggia L. (2019) Reproductive and dispersal strategies shape the diversity of mycobiont-photobiont association in *Cladonia* lichens. Molecular Phylogenetics and Evolution 134: 226-237.

Stenroos S. (1990) *Cladonia luteoalba* – an enigmatic *Cladonia*. Karstenia 30: 27-32.

Stenroos S., Pino-Bodas R., Weckman D. & Ahti T. (2015) Phylogeny of *Cladonia uncialis* (*Cladoniaceae*, *Lecanoromycetes*) and its allies. Lichenologist 47: 215-231.

Stenroos S., Pino-Bodas R., Hyvönen J., Lumbsch H. T. & Ahti T. (2019) Phylogeny of the family *Cladoniaceae* (*Lecanoromycetes*, *Ascomycota*) based on sequences of multiple loci. Cladistics 35: 351-384.

Stocker-Wörgötter E. (1991) Thallus formation of two cyanobacterial lichens: *Peltigera didactyla* and *Peltigera praetextata*, under laboratory conditions. Bulletin de la Société botanique de France 138, Lettres Botaniques 3: 179-187.

Stocker-Wörgötter E. (1995) Experimental cultivation of lichens and lichen symbionts. Canadian Journal of Botany 73: S579-S589.

Stocker-Wörgötter E. (2001) Experimental lichenology and microbiology of lichens: Culture experiments, secondary chemistry of cultured mycobionts, resynthesis, and thallus morphogenesis. Bryologist 104: 576-581.

Stocker-Wörgötter E. & Türk R. (1989) Artificial cultures of the cyanobacterial lichen *Peltigera didactyla* (Peltigeraceae) in the natural environment. Plant Systematics and Evolution 165: 39-48.

Suryanarayanan T. S., Govindarajulu M. B., Rajamani T., Tripathi M. & Joshi Y. (2017) Endolichenic fungi in lichens of Champawat district, Uttarakhand, northern India. Mycological progress, 16: 205-211.

Suryanarayanan T. S. & Thirunavukkarasu N. (2017) Endolichenic fungi: the lesser known fungal associates of lichens. Mycology 8: 189-196.

Suryanarayanan T. S., Thirunavukkarasu N., Hariharan G. N. & Balaji P. (2005) Occurrence of non-obligate microfungi inside lichen thalli. Sydowia 57: 120-130.

Tagirdzhanova G., Saary P., Tingley J. P., Díaz-Escandón D., Abbott D. W., Finn R. D. & Spribille T. (2021) Predicted input of uncultured fungal symbionts to a lichen symbiosis from metagenome-assembled genomes. Genome Biology and Evolution 13: evab047.

Thompson J. N. (1989) Concepts of coevolution. Trends in Ecology and Evolution 5: 179-183.

Thüs H., Muggia L., Pérez-Ortega S., Favero-Longo S. E., Joneson S., O'Brien H., Nelsen M. P., Duque-Thüs R., Grube M., Friedl T., Brodie J., Andrew C. J. Lücking R., Lutzoni F. & Gueidan C. (2011) Revisiting photobiont diversity in the lichen family Verrucariaceae (Ascomycota). European Journal of Phycology 46: 399-415.

Trembley M. L., Ringli C. & Honegger R. (2002) Morphologicaland molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. Mycological Research 106:7 68-776.

Tschermak-Woess E. (1989) The algal partner. In Galun M (Ed.), CRC Handbook of Lichenology. CRC Press, Boca Raton, Florida, pp. 39-92.

Tuovinen V., Ekman S., Thor G., Vanderpool D., Spribille T. & Johannesson H. (2019) Two basidiomycete fungi in the cortex of wolf lichens. Current Biology 29: 476-483.

Tuovinen V., Millanes A. M., Freire-Rallo S., Rosling A. & Wedin M. (2021) *Tremella macrobasidiata* and *Tremella variae* have abundant and widespread yeast stages in *Lecanora* lichens. Environmental Microbiology 23: 2484-2498.

U'Ren J. M., Lutzoni F., Miadlikowska J. & Arnold A. E. (2010) Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microbial Ecology 60: 340-353.

U'Ren J. M., Lutzoni F., Miadlikowska J., Laetsch A. D. & Arnold A. E. (2012) Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American Journal of Botany 99: 898-914.

U'Ren J. M., Riddle J. M., Monacell J. T., Carbone I., Miadlikowska J. & Arnold A. E. (2014) Tissue storage and primer selection influence pyrosequencing-based inferences of diversity and community composition of endolichenic and endophytic fungi. Molecular Ecology Resources 14: 1032-1048.

van Oppen M. J. H., Palstra F. P., Piquet A. M.-T. & Miller D. J. (2001) Patterns of coral–dinoflagellate associations in Acropora: significance of local availability and physiology of *Symbiodinium* strains and host–symbiont selectivity. Proceedings of the Royal Society of London B 268: 1759-1767.

Vančurová L., Muggia L., Peksa O., Řídká T. & Škaloud P. (2018) The complexity of symbiotic interactions influences the ecological amplitude of the host: a case study in *Stereocaulon* (lichenized Ascomycota). Molecular Ecology 27: 3016-3033.

Vančurová L., Kalníková V., Peksa O., Škvorová Z., Malíček J., Moya P., Chytrý K., Černajová I. & Škaloud P. (2020) Symbiosis between river and dry lands: phycobiont dynamics on river gravel bars. Algal Research 51: 102062.

Vančurová L., Malíček J., Steinová J. & Škaloud P. (2021) Choosing the right life partner: Ecological drivers of lichen symbiosis. Frontiers in Microbiology 12: 769304.

Voytsekhovich A. & Beck A. (2016) Lichen photobionts of the rocky outcrops of Karadag massif (Crimean Peninsula). Symbiosis 68: 9-24.

Watanabe S., Nakano T. & Deguchi H. (1997) Photobionts isolated from maritime lichens. Journal of Marine Biotechnology 5: 103-112.

Williams L., Colesie C., Ullmann A., Westberg M., Wedin M. & Büdel B. (2017) Lichen acclimation to changing environments: photobiont switching vs. climate-specific uniqueness in *Psora decipiens*. Ecology and Evolution 7: 2560-2574.

Wynne M. J. (1969) Life history and systematic studies of some Pacific North American Phaeophyceae (brown algae). University of California Publications in Botany 50: 1-88.

Yahr R., Vilgalys R. & DePriest P. T. (2004) Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. Molecular Ecology 13: 3367-3378.

Yahr R., Vilgalys R. & DePriest P. T. (2006) Geographic variation in algal partners of *Cladonia* tenuis (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. New Phytologist 171: 847-860.

Yamamoto Y., Mizuguchi R. & Yamada Y. (1985). Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agricultural and biological chemistry 49: 3347-3348.

Zakeri Z., Junne S., Jäger F., Dostert M., Otte V. & Neubauer P. (2022). Lichen cell factories: methods for the isolation of photobiont and mycobiont partners for defined pure and co-cultivation. Microbial Cell Factories 21: 1-11.

Zhang T., Wei X. L., Wei Y. Z., Liu H. Y. & Yu L. Y. (2016) Diversity and distribution of cultured endolichenic fungi in the Ny-Ålesund Region, Svalbard (High Arctic). Extremophiles 20: 461-470.

Zhang T., Wei X. L., Zhang Y. Q., Liu H. Y. & Yu L. Y. (2015) Diversity and distribution of lichen-associated fungi in the Ny-Ålesund Region (Svalbard, High Arctic) as revealed by 454 pyrosequencing. Scientific Reports 5: 1-10.

Zorer R., Türk R. & Stocker-Wörgötter E. (1997) Resynthesis of the lichen *Cladonia fimbriata* from the axenic cultures of the isolated symbionts. In: Kappen L (Ed.) New species and novel aspects in ecology and physiology of lichens. Bibliotheca Lichenologica 67: 85-89.

Zúñiga C., Leiva D., Carú M. & Orlando J. (2017) Substrates of *Peltigera* lichens as a potential source of cyanobionts. Microbial Ecology 74: 561-569.

6. Original papers

Paper 1

The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. nov.

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Abstract

The view of lichens as a symbiosis only between a mycobiont and a photobiont has been challenged by discoveries of diverse associated organisms. Specific basidiomycete yeasts in the cortex of a range of macrolichens were hypothesized to influence the lichens' phenotype. The present study explores the occurrence and diversity of cystobasidiomycete yeasts in the lichen genus *Cladonia*. We obtained seven cultures and 56 additional sequences using specific primers from 27 *Cladonia* species from all over Europe and performed phylogenetic analyses based on ITS, LSU and SSU rDNA loci. We revealed yeast diversity distinct from any previously reported. Representatives of Cyphobasidiales, Microsporomycetaceae and of an unknown group related to *Symmetrospora* have been found. We present evidence that the Microsporomycetaceae contains mainly lichen-associated yeasts. *Lichenozyma pisutiana* is circumscribed here as a new genus and species. We report the first known associations between cystobasidiomycete yeasts and *Cladonia* (both corticate and ecorticate), and find that the association is geographically widespread in various habitats. Our results also suggest that a great diversity of lichen associated yeasts remains to be discovered.

Keywords: endolichenic fungi, endothallic fungi, lichenicolous fungi, Microsporomycetaceae, third symbiont, yeast cultures

1. Introduction

All lichen thalli host a community of cryptic fungi (e.g., Arnold et al. 2009), which are commonly compared to plant endophytes and have been termed endothallic or endolichenic. These fungi are distinguished from lichenicolous fungi by the fact, that the later fruit or are otherwise symptomatic on thalli (U'Ren et al. 2010). However, many lichenicolous fungi are endothallic, i.e., form their mycelium inside the thallus, before the fruiting-body appears (e.g., *Abrothallus parmotrematis*, Diederich 2011). In addition, for fungi associated to the thallus surface, the distinction between endothallic and exothallic is problematic since lichens have no structure analogous to the plant cuticle to separate the interior of a thallus sharply from its outside. Thus, instead of endothallic,

lichenassociated fungi might be a more suitable term for the fungi living on or within the thallus without having any visible effect.

Most of the known lichen-associated fungi are filamentous ascomycetes, predominantly belonging to the subphylum Pezizomycotina (Ascomycota). Lichen-inhabiting yeasts and/or basidiomycetes have only rarely been isolated or were neglected or overlooked (e.g., Petrini et al. 1990, Girlanda et al. 1997, U'Ren et al. 2012, Muggia et al. 2016; but see Prillinger et al. 1997). However, Ekman (1999) stated that lichen-associated basidiomycetes are a common source of PCR errors in lichens. Zhang et al. (2015, 2016) identified up to 18 % of endolichenic taxa as representatives of Basidiomycota and Fernández-Mendoza et al. (2017) even showed that basidiomycetes are the dominant lichen associated fungi in some thalli. In addition, many teleomorphic filamentous basidiomycetes are parasites of lichens (Diederich 1996). Most of them belong to the Tremellomycetes (Millanes et al. 2011). Parallel classification of yeasts and filamentous forms of Tremellomycete fungi has caused a lot of confusion and the first integrated phylogeny was published only recently (Liu et al. 2016). It might be expected that many of these lichen-associated fungi have an endothallic yeast stage, as also demonstrated by Tuovinen et al. (2019).

Attention has been drawn to basidiomycete yeasts associated with lichens by Spribille et al. (2016). They detected yeasts of the class Cystobasiodiomycetes (Basidiomycota, Pucciniomycotina) in the cortex of a great taxonomic range of macrolichens. The authors suggested that these yeasts may play a role in the lichens' phenotype and hypothesized that the yeasts may represent yet another obligatory constituent of the lichen symbiosis (Spribille et al. 2016). Although not yet properly tested, the hypothesis has already been adopted by many authors (e.g., Palmqvist et al. 2017, Suryanarayanan and Thirunavukkarasu 2017, Zúñiga et al. 2017). On the contrary, it has also received a telling critique (Oberwinkler 2017). Later on, Spribille (2018) discussed superficial biofilms of fungi and bacteria that influence the lichen phenotype.

So far, few cystobasidiomycetes were reported as lichenassociated: cultures of *Cystobasidium laryngis* (Cystobasidiales) were obtained from *Usnea antarctica*, *U. aurantiaco-atra* and *Ramalina terebrata* collected from Antarctic islands (Santiago et al. 2015, Duarte et al. 2016), and from *Umbilicaria arctica* collected from Svalbard (Zhang et al. 2016). *Cystobasidium psychroaquaticum* was cultured from *Cladonia pocillum* also from Svalbard (Zhang et al. 2016). An undescribed *Rhodotorula* species was detected in *Usnea antarctica* from the South Shetland Islands, Antarctica (Duarte et al. 2016). Park et al. (2015) reported sequences corresponding to two unspecified Cystobasidiomycete taxa from environmental samples in *Cladonia borealis* and *C. gracilis* collected from King George Island, Antarctica but they were not deposited in GenBank, so this claim cannot be tested. Except for one lineage, all the yeasts found by Spribille et al. (2016) isolated from various macrolichens from all over the world grouped into the newly described order Cyphobasidiales (Spribille et al. 2016). However, no living material was acquired. The genus *Cyphobasidium*, which gives the order its name, is parasitic and produces galls on *Hypogymnia* and *Usnea*.

In our study of lichen symbiosis, we sampled *Cladonia* species from all over Europe. We screened the lichens for Cystobasidiomycetes using specific primers and also succeeded in culturing a few strains of these yeasts. The aim of the present paper was to give the first-ever report on occurrence, diversity and morphology of Cystobasidiomycete yeasts in the lichen genus *Cladonia*, leading to description of a new genus of these fungi. We focused on both corticate and ecorticate *Cladonia* species to verify whether the yeasts are strictly cortex-inhabiting.

2. Materials and methods

2.1. Sampling

Terricolous *Cladonia* species were collected all over Europe from diverse vegetation types, different bedrocks and soil types and various climatic conditions from April to November 2017. Lichen species, their taxonomic position within the genus *Cladonia* and locality details for specimens from which Cystobasidiomycete sequences or cultures were obtained are given in Table 1. The lichen specimens are deposited in the Herbarium PRC (Department of Botany, Charles University, Prague, Czech Republic).

2.2. Isolation, culturing and characterization of the yeasts

The lichen thalli were air-dried and processed within three weeks of collection. Two isolation methods were used: 1) the thalli were washed with Tween and rinsed with water thoroughly several times in a magnetic stirrer, then using a mortar and pestle they were ground into small pieces (isolates Pol13-14 CKV, Pol14-2 CKV, Pol 14-13 CKV). 2) The upper part of a thallus was removed with a sterile razorblade and minute pieces of alga-free tissue were extracted with a sterile preparation needle (isolates CSA5A CKV1, LNV4A CKV1, SNI4A CKV1, SSB6A CKV). While in method 2) the isolates were derived from the medulla, in method 1) their origin cannot be given with certainty. However, the rinsing should eliminate the epithallic biota and thus, all the isolates are considered endothallic. In both cases the thallus fragments were placed onto cultivation media. The media used were malt-yeast agar (MYA), Sabouraud 2 % agar (SAB) or Bold's Basal Medium (BBM) enriched with 1 % glucose (Stocker-Wörgötter and Hager 2008). Yeasts grown from the lichen tissue were isolated into axenic cultures and kept at 16.5 _C in dark. Morphological characterization was noted from colonies grown on YM agar (yeast extract-malt extract-peptone-glucose agar) following Kurtzman et al. (2011). Tests for ballistoconidia, hyphae or pseudohyphae formation were performed on YM, MYA, potato-dextrose agar (PDA) and corn meal agar (CMA) according to Kurtzman et al. (2011) at 4 °C, 12 °C, 17 °C and 24 °C.

2.3. DNA isolation, amplification and sequencing

DNA from the lichens was isolated following the modified CTAB protocol (Cubero et al. 1999) with minor adjustments. DNA from the cultures was isolated using Chelex following Ferencova et al. (2017). The yeast ITS rDNA from the lichen DNA was amplified using the Cystobasidiomycete-specific primers ITS_symrho_2F and LR0_symrho_R, designed by Spribille et al. (2016). PCR amplification began with denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 1 min and elongation at 72 °C for 1 min, and finished with extension at 72 °C for 2 min. ITS of the yeast cultures was amplified using the primers ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993) with 30 cycles of 94 °C denaturation for 45 s, 54 °C annealing for 1 min and 72 °C elongation for 1 min. The ITS rDNA of the host *Cladonia* species was amplified using the same primers and the same PCR conditions, except the elongation was for 2 min and the final extension for 10 min. LSU rDNA of the cultures was amplified with the LR0R and LR6 primers (Vilgalys and Hester 1990) with 35 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 1 min. SSU rDNA from both cultures and overall lichen DNA was amplified using the Cystobasidiomycete-specific primers SSU_symrho_2F and NS6 (Spribille et al. 2016) with 30 cycles of 95 °C denaturation for 30 s, 56 °C annealing for 30 s and 72 °C elongation for 45 s. The PCR products were sequenced by Macrogen Europe, Amsterdam, the Netherlands. The obtained sequences are

Table 1 List of specimens from which Cystobasidiomycetes yeasts were obtained. *Cladonia* host species identification, GenBank accession numbers, and group to which the host species belong (clades sensu Stenroos et al. 2018), PRC accession number and locality details are given. Sequence origin states the source (total lichen DNA or from cultures).

Yeast strain	Sequence origin	Yeast identity	**Host Cladonia sp.**	Host GenBank accession	Clade	PRC	Locality	Locality type	GPS coordinates	Altitude (m)	Collection date
CSA5A_CKV1	Culture	*Lichenozyma pisutiana*	***C. rei***	MK508912	*cladonia*	4314	Hungary, Csákberény	Abandoned limestone quarry	N47.329388 E18.313593	169.5	4 Jun 2017
EBP4BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. furcata***	MK508913	*cladonia*	--	Spain, Barranc de la Pegunta	Calcareous soil	N40.246528 W0.351806	1300	18 Aug 2017
EBP6BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. rangiformis***	MK508914	*cladonia*	--	Spain, Barranc de la Pegunta	Calcareous soil	N40.246528 W0.351806	1300	18 Aug 2017
ECS3DY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. rangiformis***	MK508915	*cladonia*	--	Spain, Camarena de la Sierra	Triassic gypsum	N40.132404 W1.043856	1300	10 Aug 2017
EJA2BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. chlorophaea gr.***	MK508916	*cladonia*	--	Spain, near Javalambre peak	Calcareous soil	N40.161043 W1.007792	1500	10 Aug 2017
EXV1EY	Total DNA	Uncultured *Lichenozyma pisutiana*	***Cladonia sp.***	MK508917	*cladonia*	--	Spain, between Xodos and Vistabella	Calcareous soil	N40.250517 W0.317483	1300	18 Aug 2017
KAL3CY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. polycarpoides***	MK508918	*cladonia*	4264	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	17 May 2017
KAL7AY	Total DNA	Uncultured Microsporomycetaceae	***C. humilis***	MK508919	*cladonia*	4254	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	17 May 2017
LNV4A_CKV1	Culture	*Lichenozyma pisutiana*	***C. phyllophora***	MK508920	*cladonia*	4257	Slovakia, Lakšárska Nová Ves	Sand dune	N48.582857 E17.176843	225	5 Jun 2017
NAG1CY	Total DNA	Uncultured Microsporomycetaceae	***C. subulata***	MK508921	*cladonia*	4320	Hungary, Nagytevel	Grassland on sand	N47.269014 E17.600788	238	4 Jun 2017
NAG5EY	Total DNA	Uncultured Microsporomycetaceae	***C. rangiformis***	MK508922	*cladonia*	4305	Hungary, Nagytevel	Grassland on sand	N47.269014 E17.600788	238	4 Jun 2017
NEU1Y	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. verticillata***	MK508923	*cladonia*	4150	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. deformis***	MK508924	*erythrocarpae*	4182	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU5CY	Total DNA	Uncultured *Lichenize pisutiana*	***C. diversa***	MK508925	*erythrocarpae*	4184	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU6AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. merochlorophaea***	MK508926	*cladonia*	4255	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU7BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. cf. subulata***	MK508927	*cladonia*	4151	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU8CY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. floerkeana***	MK508928	*erythrocarpae*	4185	Germany, Neuhausen	Early succesional sands	N51.671095 E14.387173	72	11 Apr 2017

NFJ10AY	Total DNA	Uncultured Cystobasidiomycetes	***C. cf. rangiferina***	MK508929	*implexae*	4265	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ14AY	Total DNA	Uncultured Cystobasidiomycetes	***C. bellidiflora***	MK508930	*erythrocarpae*	4142	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ16AY	Total DNA	Uncultured Cystobasidiomycetes	***C. cornuta***	MK508931	*cladonia*	4158	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ17AY	Total DNA	Uncultured Cystobasidiomycetes	***C. sulphurina***	MK508932	*erythrocarpae*	4325	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ3AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. gracilis***	MK508933	*cladonia*	4242	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NKA2AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. pyxidata***	MK508934	*cladonia*	4266	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. pocillum***	MK508935	*cladonia*	4261	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA4AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. arbuscula***	MK508936	*arbuscula*	4141	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA5AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. furcata***	MK508937	*cladonia*	4227	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA6AY	Total DNA	Uncultured Microsporomycetaceae	***C.* cf. *macroceras***	MK508938	*cladonia*	4152	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NTN1BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. pocillum***	MK508939	*cladonia*	4262	Norway, Trøsen	Limestone outcrops	N68.569387 E16.649329	4	8 Jul 2017
Pol12-14_CKV	Culture	Microsporomycetaceae isolate	***C. foliacea***	MK508940	*cladonia*	4186	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	27 Jul 2017
Pol14-13_CKV	Culture	Microsporomycetaceae isolate	***C. subulata***	MK508941	*cladonia*	4321	Slovakia, Sitno	Andesite rock outcrops in a forest	N48.404301 E18.874294	929	29 Jul 2017
Pol14-3_CKV	Culture	*Lichenozyma pisutiana*	***C. subulata***	MK508942	*cladonia*	4321	Slovakia, Sitno	Andesite rock outcrops in a forest	N48.404301 E18.874294	929	29 Jul 2017
SAL5DY	Total DNA	Uncultured Microsporomycetaceae	***C. furcata***	MK508943	*cladonia*	4217	Hungary, Salfőld	Early succession stages of sand quarry	N46.834682 E17.562669	149	3 Jun 2017
SCK3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. rangiferina***	MK508944	*crustaceae*	4278	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SCK4BY	Total DNA	Uncultured Cystobasidiomycetes	***C. deformis***	MK508945	*erythrocarpae*	4183	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017

SCK7BY	Total DNA	Uncultured Cystobasidiomycetes	***C. gracilis***	MK508946	*cladonia*	4246	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SCK8BY	Total DNA	Uncultured Cystobasidiomycetes	***C. deformis***	MK508947	*erythrocarpae*	4258	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SDA1BY	Total DNA	Uncultured Cystobasidiomycetes	***C. cariosa***	MK508948	*cladonia*	4147	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDA3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. pocillum***	MK508949	*cladonia*	4263	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDA8AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. furcata***	MK508950	*perviae*	4172	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDJ13AY	Total DNA	Uncultured Cystobasidiomycetes	***C. rangiferina***	MK508951	*crustaceae*	4272	Sweden, Djurmo Klack	Granite boulder scree	N60.556239 E15.181526	351	31 Aug 2017
SEP12AY	Total DNA	Uncultured Cystobasidiomycetes	***C. rangiferina***	MK508952	*crustaceae*	4276	Sweden, Paktajåkaluobbalah	Tundra on rock outcrops	N68.439601 E18.631060	352	7 Jul 2017
SEP8AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. coccifera/borealis***	MK508953	*erythrocarpae*	4155	Sweden, Paktajåkaluobbalah	Tundra on rock outcrops	N68.439601 E18.631060	352	7 Jul 2017
SGA2AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. subulata***	MK508954	*cladonia*	4324	Sweden, Garpenbergs gård	Metavolcanic boulders	N60.285947 E16.203372	136	24 Aug 2017
SLI2AY	Total DNA	Uncultured Microsporomycetaceae	***C. pocillum***	MK508955	*cladonia*	4153	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI3AY	Total DNA	Uncultured Cystobasidiomycetes	***C. cariosa***	MK508956	*cladonia*	4148	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI5AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***Cladonia sp.***	MK508957	*perviae*	4175	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI6BY	Total DNA	Uncultured Microsporomycetaceae	***C. furcata***	MK508958	*cladonia*	4234	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SNI4A_CKV1	Culture	*Lichenozyma pisutiana*	***C. cornuta***	MK508959	*cladonia*	4160	Sweden, Nittsjö	Clear-cut in pine forests	N60,926643 E15,064329	223.5	28 Aug 2017
SNI4BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. cornuta***	MK508960	*cladonia*	4160	Sweden, Nittsjö	Clear-cut in pine forests	N60.926643 E15.064329	223.5	28 Aug 2017
SSB4BY	Total DNA	Uncultured Cyphobasidiales	***C. rangiferina***	MK508961	*crustaceae*	4270	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017
SSB6A_CKV	Culture	*Lichenozyma pisutiana*	***C. cariosa***	MK508962	*cladonia*	4149	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017
SSB6AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. cariosa***	MK508963	*cladonia*	4149	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017
SSO5AY	Total DNA	Uncultured Microsporomycetaceae	***C. cornuta***	MK508964	*cladonia*	4164	Sweden, Sollerön	A former Viking burial heap	N60.977837 E14.613716	185	26 Aug 2017

SUS5BY	Total DNA	Uncultured Microsporomycetaceae	***C. rei***	MK508965	*cladonia*	4319	Czech Republic, Sušice	Former limestone quarry	N49.2547483 E13.5522144	467	19 May 2017
SYT3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. furcata***	MK508966	*cladonia*	4191	Czech Republic, Sytno	Mine spoil heap	N49.738951 E13.027498	450	3 Nov 2017
TIH1AY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. rangiformis***	MK508967	*cladonia*	4312	Hungary, Tihany	Basalt outcrops with dry grassland	46.918950N E17870927	128.5	2 Jun 2017
TIH1BY	Total DNA	Uncultured Microsporomycetaceae	***C. rangiformis***	MK508968	*cladonia*	4311	Hungary, Tihany	Basalt outcrops with dry grassland	46.918950N E17870927	128.5	2 Jun 2017
WLT2EY	Total DNA	Uncultured Microsporomycetaceae	***C. rangiformis***	MK508969	*cladonia*	4291	Wales, Little Tor	Limestone grassland with rock outcrops	N51.57034244 W4.1293128 6	101	14 Oct 2017
WLT4CY	Total DNA	Uncultured Microsporomycetaceae	***C. pocillum***	MK508970	*cladonia*	4260	Wales, Little Tor	Limestone grassland with rock outcrops	N51.57034244 W4.1293128 6	101	14 Oct 2017
WST4HY	Total DNA	Uncultured Cyphobasidiales	***C. rangiformis***	MK508971	*cladonia*	4295	Wales, Stackpole	Limestone sand dune	N51.6105296 0 W4.9199659 9	74	16 Oct 2017
ZAV2CY	Total DNA	Uncultured Microsporomycetaceae	***C. cf. pocillum***	MK508972	*cladonia*	4259	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017
ZAV3BY	Total DNA	Uncultured Microsporomycetaceae	***C. rangiformis***	MK508973	*cladonia*	4282	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017
ZAV5BY	Total DNA	Uncultured *Lichenozyma pisutiana*	***C. cf. pocillum***	MK508974	*cladonia*	4267	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017

deposited in GenBank under the accession numbers MK491194 – MK491271 (yeasts, Table 3) and MK508912 – MK508974 (host species, Table 1).

2.4. Sequence analyses

To infer the phylogenetic position of our sequences within the class Cystobasidiomycetes, we first performed the multigene phylogenetic analysis using the dataset of Wang et al. (2015a), which is the basis of the currently accepted classification (Wang et al. 2015b, Oberwinkler 2017). Additionally, we included i) sequences from the type material of both currently recognized *Cyphobasidium* species (Millanes et al. 2016), ii) two representatives of each lineage of the order Cyphobasidiales, and iii) two representatives of clade I sensu Spribille et al. (2016). These sequences retrieved from GenBank (Table 2) together with the newly obtained sequences (Table 3) were aligned using MAFFT v.7 (Katoh et al. 2017) using the Q-INS-I method, aligning each locus separately. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and eliminated. The final concatenated alignment comprised of 88 unique sequences and 866 SSU rDNA, 313 ITS rDNA, 419 LSU rDNA, 658 RPB1, 1033 RPB2, 925 TEF1 and 392 CYTB alignment positions. Substitution models were estimated with Bayesian Information Criterion using JModelTest v. 2.1.4 (Darriba et al. 2012) as follows: TrN + I + G for SSU rDNA (gamma shape 0.0.787), TPM1 + G for ITS1 rDNA (gamma shape 1.241), K80 + G for 5.8S rDNA (gamma shape 0.178), SYM + G for ITS2 rDNA (gamma shape 0.467), TrN + G for LSU rDNA (gamma shape 0.296); SYM + I + G (gamma shape 0.661), TrN + I + G (gamma shape 0.565) and HKY + I + G (gamma shape 0.652) for the first, second and third codon positions of RPB1, respectively; HKY + I + G (gamma shape 0.343), GTR + I + G (gamma shape 1.299) and SYM + I + G (gamma shape 0.514)) for the first, second and third codon positions of RPB2, respectively; GTR + G (gamma shape 0.463), K80 + I + G (gamma shape 0.487) and GTR + G (gamma shape 0.338) for the first, second and third codon positions of TEF1 respectively; and finally GTR + I + G (gamma shape 0.690), TPM1uf + I + G (gamma shape 0.655) and GTR + I + G (gamma shape 0.544) for the first, second and third codon positions of CYTB, respectively.

All our cultures grouped within the family Microsporomycetaceae. Accordingly, in the second analysis we reconstructed its phylogeny based on three rDNA loci. In addition to sequences of the type material of the five currently accepted species (Nakase et al. 2003, Pohl et al. 2011, Wang et al. 2015b, Bai et al. 2016), we also included sequences of the eight uncultured Cyphobasidiomycete clones that form clade I in Spribille et al. (2016) and one sequence of a *Rhodotorula* sp. isolated by Duarte et al. (2016) from *U. antarctica*, all of which are apparently closely related to *Microsporomyces* (Tables 2 and 3). *Erythrobasidium elongatum* was selected as the outgroup. The sequences were processed as described above. The final concatenated alignment was composed of 51 sequences and 396 ITS rDNA, 468 LSU rDNA and 635 SSU rDNA positions. Estimated substitution models were: K80 + I for SSU rDNA, HKY + G (gamma shape 0.643) for ITS1 rDNA, K80 for 5.8S rDNA, SYM + G (gamma shape 0.528) for ITS2 rDNA and K80 + G (gamma shape 0.122) for LSU rDNA.

The phylogenetic trees were inferred by Bayesian Inference (BI) using MrBayes v. 3.2.6 (Ronquist et al. 2012), using the 17 and 5 partitions for Cystobasidiomycetes and Microsporomycetaceae, respectively. Two parallel MCMC runs, with one cold and three heated chains, were carried out for 50 and 10 million generations for Cystobasidiomycetes and Microsporomycetaceae, respectively. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The

Table 2 List of sequences downloaded from GenBank used for phylogenetic analyses. The last column (Analysis) indicates if they were used in phylogeny of the order Cystobasidiomycetes (C) or the family Microsporomycetaceae (M).

Taxon	Strain /voucher	ITS	LSU	SSU	RPB1	RPB2	TEF1	CYTB	An.
Bannoa bischofiae	JCM 10338	AB035721	AB082572	AB035721	KJ708018	KJ708292	KJ707777	KJ707684	C
Bannoa hahjimensis	JCM 10336	AB035897	AB082571	AB035897	KJ708014	KJ708146	KJ707750	KJ707682	C
Bannoa ogasawarensis	JCM 10326	AB035713	AB082570	AB035713	KJ708017	KJ708323	KJ707781	KJ707681	C
Bannoa syzygii	JCM 10337	AB035720	AB082573	AB035720	KJ708011	KJ708338	KJ707778	KJ707683	C
Buckleyzyma armeniaca	JCM 8977	AF444523	AF189920	AB126644	KP216521	KJ708211	KJ707762	AB040615	C
Buckleyzyma aurantiaca	JCM 3771	AF444538	AF189921	KJ708436	KJ707970	KJ708212	KJ707757	AB040616	C
Buckleyzyma kluyveri-nielii	JCM 6356	AF444544	AF189988	AB021674	KJ707977	KJ708310	KJ707760	–	C
Buckleyzyma phyllomatis	JCM 7549	AF444515	AF189991	AB021685	KJ707976	KJ708328	KJ707761	KJ707728	C
Buckleyzyma salicina	JCM 2959	AF444511	AF189995	AB021687	–	–	KJ707758	KJ707703	C
Cyphobasidium hypogymniicola	S-F264671	KU587700	KU587694	KU587705	–	–	–	–	C
Cyphobasidium usneicola	S-F264675	KU587704	KU587699	KU587706	–	–	–	–	C
Cyrenella elegans	CBS 274.82	KJ778626	KJ708454	KJ708360	KJ708080	KJ708168	KJ707830	KJ707620	C
Cystobasidium benthicum	JCM 10901	AB026001	AB026001	AB126647	KJ708081	KJ708214	KJ707842	KJ707691	C
Cystobasidium calyptogenae	JCM 10899	AB025996	AB025996	AB126648	KJ708075	KJ708218	KJ707840	KJ707690	C
Cystobasidium fimentarium	DB1489	–	AY512843	AY124479	–	–	LM644071	–	C
Cystobasidium laryngis	JCM 10953	AB078500	AB078500	AB126649	KJ708055	KJ708240	KJ707824	KJ707619	C
Cystobasidium lysinophilum	JCM 5951	AB078501	AB078501	AB126650	KJ708074	KJ708243	KJ707845	KJ707721	C
Cystobasidium minutum	AS 2.1516	AF190011	AF189945	D45367	KJ708059	KJ708246	KJ707825	KJ707562	C
Cystobasidium oligophagum	KM1106	AB702968	AB702967	–	–	–	–	–	C
Cystobasidium pallidum	JCM 3780	AB078492	AF189962	AB126651	KJ708056	KJ708253	KJ707826	KJ707621	C
Cystobasidium pinicola	AS 2.2193	AF444292	AF444293	AB126652	KJ708057	KJ708257	KJ707827	KJ707579	C
Cystobasidium portillonense	071209-Pi2-frotapiedra-7-lev	JQ769323	JQ769312	–	–	–	–	–	C
Cystobasidium psychroaquaticum	CBS:11769	KY103148	KY107444	LM644062	–	–	LM644068	–	C
Cystobasidium ritchiei	CBS:12324	KY103149	KY107445	LM644063	–	–	LM644069	–	C
Cystobasidium slooffiae	JCM 10954	AF444627	AF444722	AB126653	KJ708058	KJ708266	KJ707828	KJ707629	C
Erythrobasidium elongatum	AS 2.1949	AF444561	AF189983	AB021669	KJ708012	KJ708300	KJ707782	KJ707570	C, M
Erythrobasidium hasegawianum	AS 2.1923	AF444522	AF189899	D12803	KF706506	KF706534	KJ707776	KJ707563	C
Erythrobasidium yunnanensis	AS 2.2090	AB030353	AY335162	AF229176	KJ708015	KJ708344	KJ707779	KJ707576	C
Hasegawazyma lactosa	CBS 5826	NR_073295	NG_057668	D45366	KJ708016	KJ708239	AB127098	AB040633	C
Microsporomyces bloemfonteinensis	CBS 8598	EU075189	EU075187	KJ708359	KJ708082	KJ708215	–	KJ707657	C
Microsporomyces hainanensis	CICC 33066	KU296948	KU296947	–	–	–	–	–	C
Microsporomyces magnisporus	JCM 11898	AB112078	AB111954	KJ708428	KJ708013	KJ708317	KJ707780	KJ707695	C
Microsporomyces orientis	CBS 8594	HM559719	HM559718	KJ708358	KJ708078	KJ708249	KJ707843	KJ707656	C
Microsporomyces pini	CBS 107345	EU075190	EU075188	KJ708357	KJ708084	KJ708258	KJ707832	KJ707601	C
Naohidea sebacea	CBS 8477	DQ911616	DQ831020	KP216515	KF706508	KF706535	KF706487	KJ707654	C
Occultifur brasiliensis	UFMG-CM-Y376	KM248526	KM248525	–	–	–	–	–	C
Occultifur externus	JCM 10725	AF444567	AF189910	AB055193	KJ708060	KJ708199	KJ707829	KJ707689	C
Occultifur kilbournensis	NRRL Y-63695	NR_155564	KP413160	–	–	–	–	–	C
Occultifur tropicalis	DMKU SE59	NR_148062	–	–	–	–	–	–	C

Rhodotorula sp.	10.10.L31	KU057818	KT970781	–	–	–	–	–	M
Sakaguchia cladiensis	CBS 10878	FJ008055	FJ008049	KJ708354	–	KJ708219	KJ707847	KJ707603	C
Sakaguchia dacryoidea	JCM 3795	AF444597	AF189972	D13459	KJ708102	KJ708348	KP216514	KJ707709	C
Sakaguchia lamellibrachii	CBS 9598	AB025999	AB025999	AB126646	KJ708098	KJ708314	KJ707876	KJ707667	C
Sakaguchia meli	CBS 10797	FJ807683	KJ708452	KJ708355	KJ708085	KJ708245	KJ707855	KJ707602	C
Sakaguchia oryzae	AS2.2363	AY335160	AY335161	KJ708352	KJ708100	KJ708250	KJ707853	KJ707587	C
Symmetrospora coprosmae	JCM 8772	AF444577	AF189980	D66880	KJ707966	KJ708296	KJ707798	KJ707742	C
Symmetrospora foliicola	AS 2.2527	AF444521	AF189984	AB021671	KJ707969	KJ708302	KJ707797	KJ707589	C
Symmetrospora gracilis	JCM 2963	AF444578	AF189985	KJ708433	KJ707968	KJ708304	KJ707799	KJ707705	C
Symmetrospora marina	JCM 3776	AF444504	AF189944	AB126645	KJ707973	KJ708244	KJ707795	AB040635	C
Symmetrospora symmetrica	AS 2.2299	AY364836	AY364836	KJ708350	KJ707975	KJ708337	KJ707800	KJ707582	C
Symmetrospora vermiculatus	JCM 10224	AB030335	AF460176	AB030322	KJ707967	KJ708342	KJ707801	KJ707675	C
Symmetrospora oryzicola	JCM 5299	AF444546	AF189990	AB021677	KJ707974	KJ708324	KJ707955	KJ707712	C
Uncultured *Cyphobasidiales*	T1433	KU948752	KU948880	KU948829	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1385	KU948738	KU948871	KU948820	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1390	KU948743	–	KU948825	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1587	KU948731	KU948890	KU948834	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1645	KU948778	KU948917	KU948855	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1397	KU948744	KU948912	–	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1630	KU948770	KU948924	KU948845	–	–	–	–	C
Uncultured *Cystobasidiomycetes*	T1613	KU948765	–	KU948843	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1402	KU948747	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1400	KU948746	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T770	KU948735	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1388	KU948741	–	KU948823	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1667	KU948788	–	KU948865	–	–	–	–	C, M
Uncultured *Cystobasidiomycetes*	T1646	KU948779	–	KU948856	–	–	–	–	C, M
Uncultured *Cystobasidiomycetes*	T1615	KU948766	–	–	–	–	–	–	M

Table 3 List of newly obtained sequences and their GenBank accession numbers. The last column shows if they were used in phylogeny of the order Cystobasidiomycetes (C) or the family Microsporomycetaceae (M). Cultures are in bold.

Strain /voucher	Taxon	ITS	LSU	SSU	Analysis
CSA5A_CKV1	*Lichenozyma pisutiana*	MK491194	MK491265	MK491257	C, M
EBP4BY	Uncultured *Lichenozyma pisutiana*	MK491201	–	–	C, M
EBP6BY	Uncultured *Lichenozyma pisutiana*	MK491202	–	–	C, M
ECS3DY	Uncultured *Lichenozyma pisutiana*	MK491203	–	–	C, M
EJA2BY	Uncultured *Lichenozyma pisutiana*	MK491204	–	–	C, M
EXV1EY	Uncultured *Lichenozyma pisutiana*	MK491205	–	–	C, M
KAL3CY	Uncultured *Lichenozyma pisutiana*	MK491206	–	–	C, M
KAL7AY	Uncultured *Microsporomycetaceae*	MK491207	–	–	C, M
LNV4A_CKV1	*Lichenozyma pisutiana*	MK491196	MK491266	–	C, M
NAG1CY	Uncultured *Microsporomycetaceae*	MK491208	–	–	C, M
NAG5EY	Uncultured *Microsporomycetaceae*	MK491209	–	–	C, M
NEU1Y	Uncultured *Lichenozyma pisutiana*	MK491210	–	–	C, M
NEU3BY	Uncultured *Lichenozyma pisutiana*	MK491211	–	MK491258	C, M
NEU5CY	Uncultured *Lichenozyma pisutiana*	MK491212	–	–	C, M
NEU6AY	Uncultured *Lichenozyma pisutiana*	MK491213	–	–	C, M
NEU7BY	Uncultured *Lichenozyma pisutiana*	MK491214	–	MK491259	C, M
NEU8CY	Uncultured *Lichenozyma pisutiana*	MK491215	–	–	C, M
NFJ10AY	Uncultured *Cystobasidiomycetes*	MK491216	–	–	C
NFJ14AY	Uncultured *Cystobasidiomycetes*	MK491217	–	–	C
NFJ16AY	Uncultured *Cystobasidiomycetes*	MK491218	–	–	C
NFJ17AY	Uncultured *Cystobasidiomycetes*	MK491219	–	–	C
NFJ3A	Uncultured *Lichenozyma pisutiana*	MK491220	–	–	C, M
NKA2AY	Uncultured *Lichenozyma pisutiana*	MK491221	–	–	C, M
NKA3BY	Uncultured *Lichenozyma pisutiana*	MK491222	–	–	C, M
NKA4AY	Uncultured *Lichenozyma pisutiana*	MK491223	–	–	C, M
NKA5AY	Uncultured *Lichenozyma pisutiana*	MK491224	–	–	C, M
NKA6AY	Uncultured *Microsporomycetaceae*	MK491225	–	–	C, M
NTN1BY	Uncultured *Lichenozyma pisutiana*	MK491226	–	–	C, M
Pol12-14_CKV	*Microsporomycetaceae* isolate	MK491199	MK491267	–	C, M
Pol14-13_CKV	*Microsporomycetaceae* isolate	MK491200	MK491268	MK491260	C, M
Pol14-3_CKV	*Lichenozyma pisutiana*	MK491198	MK491269	–	C, M
SAL5DY	Uncultured *Microsporomycetaceae*	MK491227	–	–	C, M
SCK3BY	Uncultured *Lichenozyma pisutiana*	MK491228	–	–	C, M
SCK4BY	Uncultured *Cystobasidiomycetes*	MK491229	–	–	C
SCK7BY	Uncultured *Cystobasidiomycetes*	MK491230	–	–	C
SCK8BY	Uncultured *Cystobasidiomycetes*	MK491231	–	–	C
SDA1BY	Uncultured *Cystobasidiomycetes*	MK491232	–	–	C
SDA3BY	Uncultured *Lichenozyma pisutiana*	MK491233	–	–	C, M
SDA8AY	*Lichenozyma pisutiana*	MK491234	–	–	C, M
SDJ13AY	Uncultured *Cystobasidiomycetes*	MK491235	–	–	C
SEP12AY	Uncultured *Cystobasidiomycetes*	MK491236	–	MK491261	C
SEP8AY	Uncultured *Lichenozyma pisutiana*	MK491237	–	–	C, M

SGA2AY	Uncultured *Lichenozyma pisutiana*	MK491238	–	–	C, M
SLI2AY	Uncultured *Microsporomycetaceae*	MK491239	–	–	C, M
SLI3AY	Uncultured *Cystobasidiomycetes*	MK491240	–	–	C
SLI5AY	Uncultured *Lichenozyma pisutiana*	MK491241	–	–	C, M
SLI6BY	Uncultured *Microsporomycetaceae*	MK491242	–	–	C, M
SNI4A_CKV1	*Lichenozyma pisutiana*	MK491197	MK491270	MK491262	C, M
SNI4BY	Uncultured *Lichenozyma pisutiana*	MK491243	–	–	C, M
SSB4BY	Uncultured *Cyphobasidiales*	MK491244	–	–	C
SSB6A_CKV	*Lichenozyma pisutiana*	MK491195	MK491271	MK491263	C, M
SSB6AY	Uncultured *Lichenozyma pisutiana*	MK491245	–	–	C, M
SSO5AY	Uncultured *Microsporomycetaceae*	MK491246	–	MK491264	C, M
SUS5BY	Uncultured *Microsporomycetaceae*	MK491247	–	–	C, M
SYT3BY	Uncultured *Lichenozyma pisutiana*	MK491248	–	–	C, M
TIH1AY	Uncultured *Lichenozyma pisutiana*	MK491249	–	–	C, M
TIH1BY	Uncultured *Microsporomycetaceae*	MK491250	–	–	C, M
WLT2EY	Uncultured *Microsporomycetaceae*	MK491251	–	–	C, M
WLT4CY	Uncultured *Microsporomycetaceae*	MK491252	–	–	C, M
WST4HY	Uncultured *Cyphobasidiales*	MK491253	–	–	C
ZAV2CY	Uncultured *Microsporomycetaceae*	MK491254	–	–	C, M
ZAV3BY	Uncultured *Microsporomycetaceae*	MK491255	–	–	C, M
ZAV5BY	Uncultured *Lichenozyma pisutiana*	MK491256	–	–	C, M

SDSF value between simultaneous runs was 0.002 and 0.001 for Cystobasidiomycetes and Microsporomycetaceae, respectively. Finally, the burn-in values were determined using the 'sump' command. Bootstrap analyses were also performed by maximum likelihood (ML) using GARLI v. 2.0 (Zwickl 2006) for Cystobasidiomycetes and RAxML v. 8.0.0 (Stamatakis 2014) for Microsporomycetaceae on partitioned datasets. ML analysis consisted of 1000 rapid bootstrap inferences with automatic termination. RAxML analysis was run on the CIPRES Science Gateway v.3.3 web portal (Miller et al. 2010). The resulting trees were visualized using FigTree v. 1.4.3 (Rambaut 2016). The final visualizationwas done in the free software R v. 3.4.3 (R Core Team 2017) using the packages ape (Paradis et al. 2004) and phytools (Revell 2012).

3. Results

Using the specific primers, we successfully obtained 56 ITS sequences matching Cystobasidiomycetes from 104 *Cladonia* samples. They were apparent in other 43 specimens but we could not obtain legible sequences due to technical reasons (data not shown). In addition to the ITS rDNA region, SSU rDNA was amplified only in three cases (Table 3). We further successfully cultured seven strains of Cystobasidiomycete yeasts from six lichen specimens. The cultures were identified by sequencing the ITS, LSU and SSU rDNA (Table 3). Despite the effort (combinations of various cultivation media and temperatures), only the yeast stage was observed; no conidia, pseudohyphae or hyphae were formed. BI and ML analyses of Cystobasidiomycetes gave identical topologies. Our phylogeny (Fig. 1) supports most of the major groups described by Wang et al. (2015a, 2015b). However, their

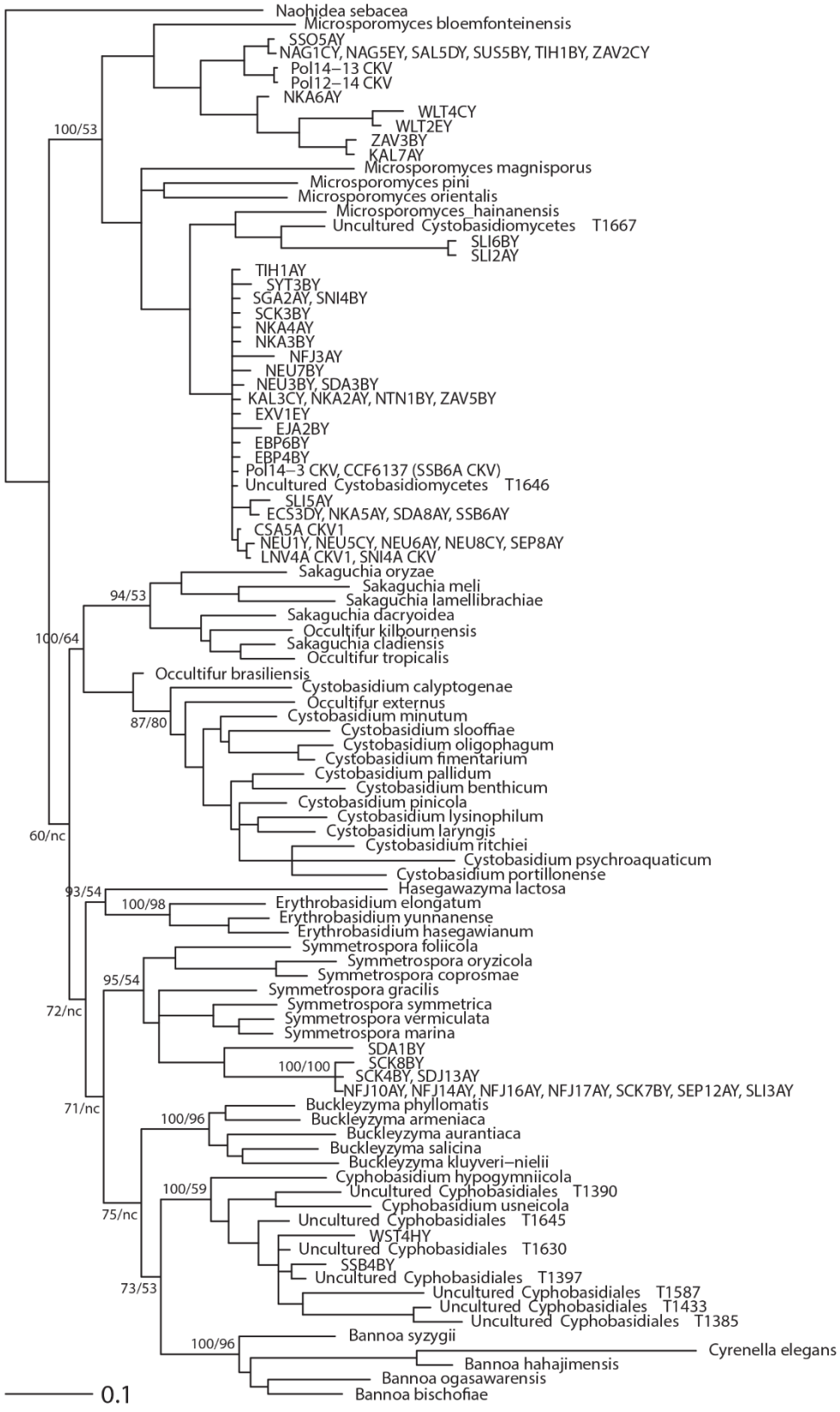

Figure 2 Phylogeny of the Cystobasidiomycetes obtained by Bayesian inference of concatenated seven-locus dataset. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Values at lower taxonomic rank not shown. Newly obtained sequences are marked in bold. Scale bar represents the expected number of substitutions per site. nc = not calculated.

relationships differ and the analysis found no support for the clustering of genera *Bannoa* and *Erythrobasidium*, nor did it resolve the genus *Occultifur* as monophyletic.

The newly obtained sequences grouped into three distinct lineages within the Cystobasidiomycetes (Fig. 1). First, two sequences (SSB4BY and WST4HY) grouped within the lichen-associated order Cyphobasidiales. Second, a group of eleven sequences grouped into a lineage that appears to be related to *Symmetrospora*. Finally, all the remaining sequences, including those obtained from the cultures, grouped into a monophyletic lineage including the genus *Microsporomyces*. These were further treated in the second analysis.

The phylogeny of Microsporomycetaceae (Fig. 2) suggests that the genus *Microsporomyces*, as defined by Wang et al. (2015b) and Bai et al. (2016), is polyphyletic. At least four monophyletic groups were defined within the family: i) *Microsporomyces magnisporus* together with *M. orientalis* and *Microsporomyces pini*, ii) *Microsporomyces bloemfonteinensis* and *Microsporomyces hainanensis* together with a *Rhodotorula* sp. (Duarte et al. 2016), uncultured Cystobasidiomycetes T1402 (Spribille et al. 2016), two of our cultures (Pol12-14_CKV and Pol14-13_CKV) and twelve of the sequences obtained from lichen DNA, iii) five uncultured Cystobasidiomycetes clones (Spribille et al. 2016) along with two of ours, SLI2AY and SLI6BY, iv) five of our cultures (Fig. 3), 29 clones, and two uncultured clones sequences by Spribille et al. (2016). We propose a new genus, *Lichenozyma*, with a single species *L. pisutiana* for this lineage. The descriptions follow.

4. Taxonomy

***Lichenozyma* gen. nov.**
MycoBank No.: MB 829658

Etym.: referring to its yeast form and its first known occurrence in association with lichens.

The genus is described based on rDNA-derived phylogenic results, which show *Lichenozyma* as a close relative of the genus *Microsporomyces* Q.M. Wang, F.Y. Bai, M. Groenew. and Boekhout.

Colonies ochraceous to pale salmon coloured; sexual reproduction unknown; budding cells present; formation of ballistoconidia, pseudohyphae or hyphae not observed.

The genus is associated to lichens. It has been reported from various *Cladonia* species and *Cetraria ericetorum* collected from diverse habitats and widely separated geographic regions.

Type species: L. pisutiana.

***Lichenozyma pisutiana* sp. nov.**
MycoBank No.: MB 829659

Fig. 3.

Etym.: In memory of Slovak lichenologist Ivan Pišút (1935 – 2017).

Type: Sweden, Dalarna Province, Rättvik Municipality, Solberga, N60.983492, E15.212700, abandoned limestone quarry, 211 m a.s.l., 27 August 2017, J. Steinová and I. Černajová SSB6A;

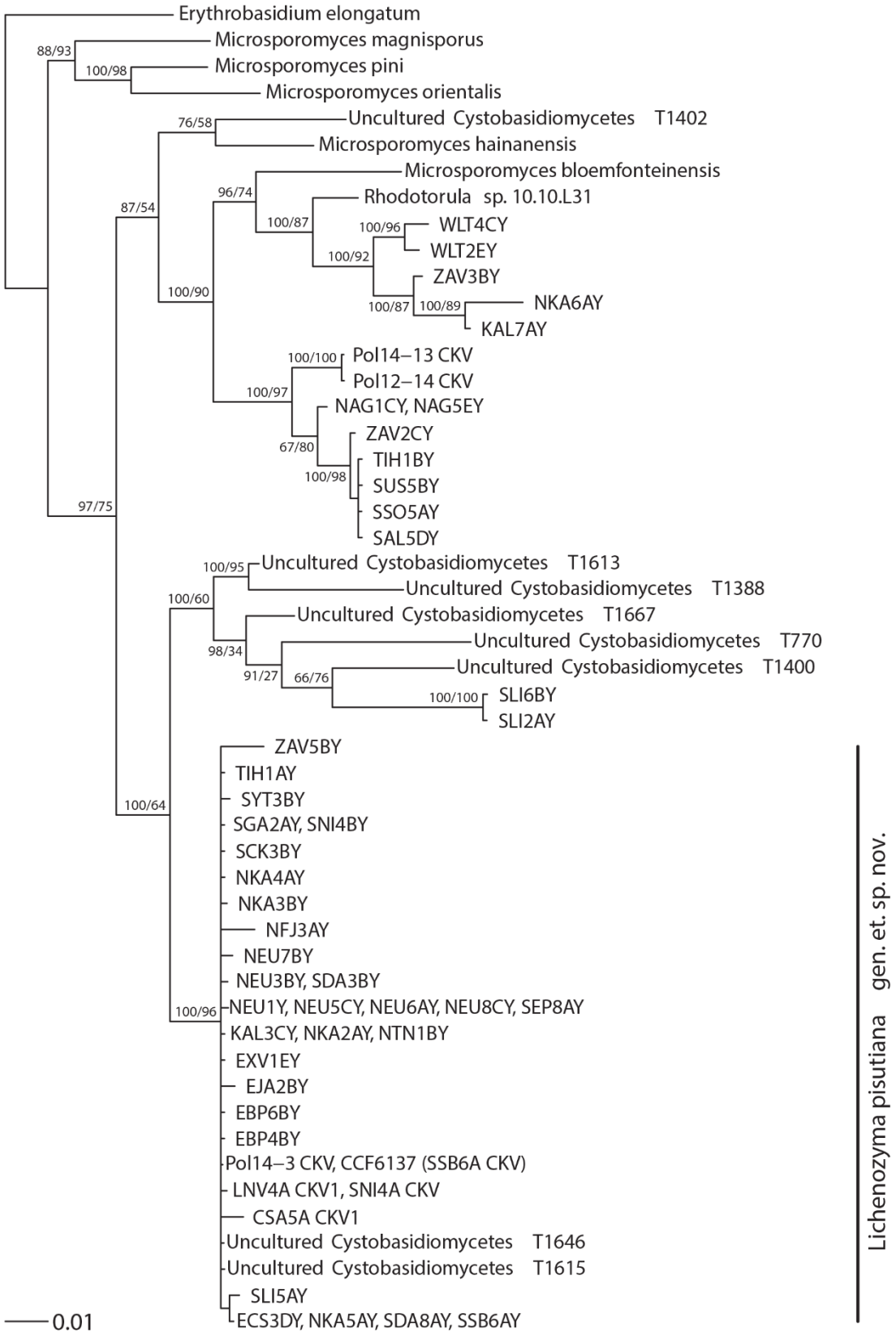

Figure 3 Phylogeny of the Microsporomycetaceae obtained by Bayesian inference of concatenated SSU, ITS and LSU rDNA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Newly obtained sequences are named by codes only. Cultures are in bold. Scale bar represents the expected number of substitutions per site. nc = not calculated.

isolated as strain SSB6A_CKV, 5 September 2017 by I. Černajová from *Cladonia cariosa* PRC4149 (holotype PRC4294 dried culture, isotype CCF6137 stored in liquid nitrogen).

Molecular characteristics: SSU rDNA, ITS rDNA (including 5.8S exon) and LSU rDNA sequences of the type are deposited in NCBI/EMBL (GenBank) under the accession numbers MK491270, MK491195 and MK491263, respectively.

Morphological description: Colonies small, up to 4 – 7 mm in diameter after ten days, even older cultures < 1 cm in diameter; ochraceous to pale salmon coloured, smooth, margin entire (Fig. 3a, b); on YM after 10 d at room temperature cells ellipsoidal 5.3 – 6.6 × 3.2 – 3.7 mm (Fig. 3c, d), budding polar (Fig. 3c); production of ballistoconidia, pseudohyphae or hyphae not observed (neither on MYA, YM, CMA nor PDA, at 4 °C, 12 °C, 17 °C nor 24 °C).

Ecology: Associated with lichens, not producing visible symptoms on the thalli.

Host range: Various *Cladonia* species listed in Table 1, also reported from *C. multiformis* and *Cetraria ericetorum* (in Spribille et al. 2016).

Distribution: Here reported from Norway, Sweden, Germany, Czech Republic, Slovakia, Hungary, Wales (UK), Spain (Table 1). Also reported from Montana, USA (Spribille et al. 2016).

Additional material examined: Strain CSA5A_CKV1 isolated from *Cladonia rei* (PRC4314), SNI4A_CKV1 from *C. cornuta* (PRC4160), LNV4A_CKV1 from *C. phyllophora* (PRC4257) and Pol14-3_CKV from *C. subulata* (PRC4321), see Tables 1 and 3.

5. Discussion

In this study we focused on Cystobasidiomycete yeasts in lichens. Our results show that these yeasts are common associates/inhabitants of the lichen genus *Cladonia*, as we detected them in 95 % of the studied specimens collected in various climatic conditions and habitats (Table 1). Additionally, seven strains were obtained in culture, so the fungi are culturable and can be studied further to detect any function they may have in the lichen symbiosis.

Cystobasidiomycete yeasts had been detected in the upper cortex of macrolichens (Spribille et al., 2016) and were hypothesized to play a role in the lichens' phenotype. However, we found them in corticate (e.g., *Cladonia furcata*), partly corticate (e.g., *C. pocillum*), and ecorticate (e.g., *C. rangiferina*) species. It is thus likely that in *Cladonia* these fungi are rather either constituents of a superficial biofilm (as suggested by Spribille 2018) or live within the thallus without association with the cortex.

Studies of endolichenic fungi have been intensive in the last decade (e.g., Arnold et al. 2009, U'Ren et al. 2010, 2012, 2014, Peršoh and Rambold 2012, Muggia et al. 2016, Fernández-Mendoza et al. 2017, Banchi et al. 2018). Despite this, there are only few reports of cystobasidiomycete fungi (see introduction). Culture-dependent studies mostly concentrated on filamentous ascomycetes, thus neglecting basidiomycetes or yeasts in general. Metabarcoding using ITS1 and ITS2 is biased against the detection of basidiomycetes (Banchi et al. 2018). Nevertheless, studies of endolichenic fungi based on metabarcoding (Fernández-Mendoza et al. 2017, Banchi et al. 2018) did reveal basidiomycetes (but not Cystobasidiomycetes). Interestingly, Banchi et al. (2018) and Fernández-Mendoza et al. (2017) did not surface-sterilize the lichens prior to DNA sampling for legitimate

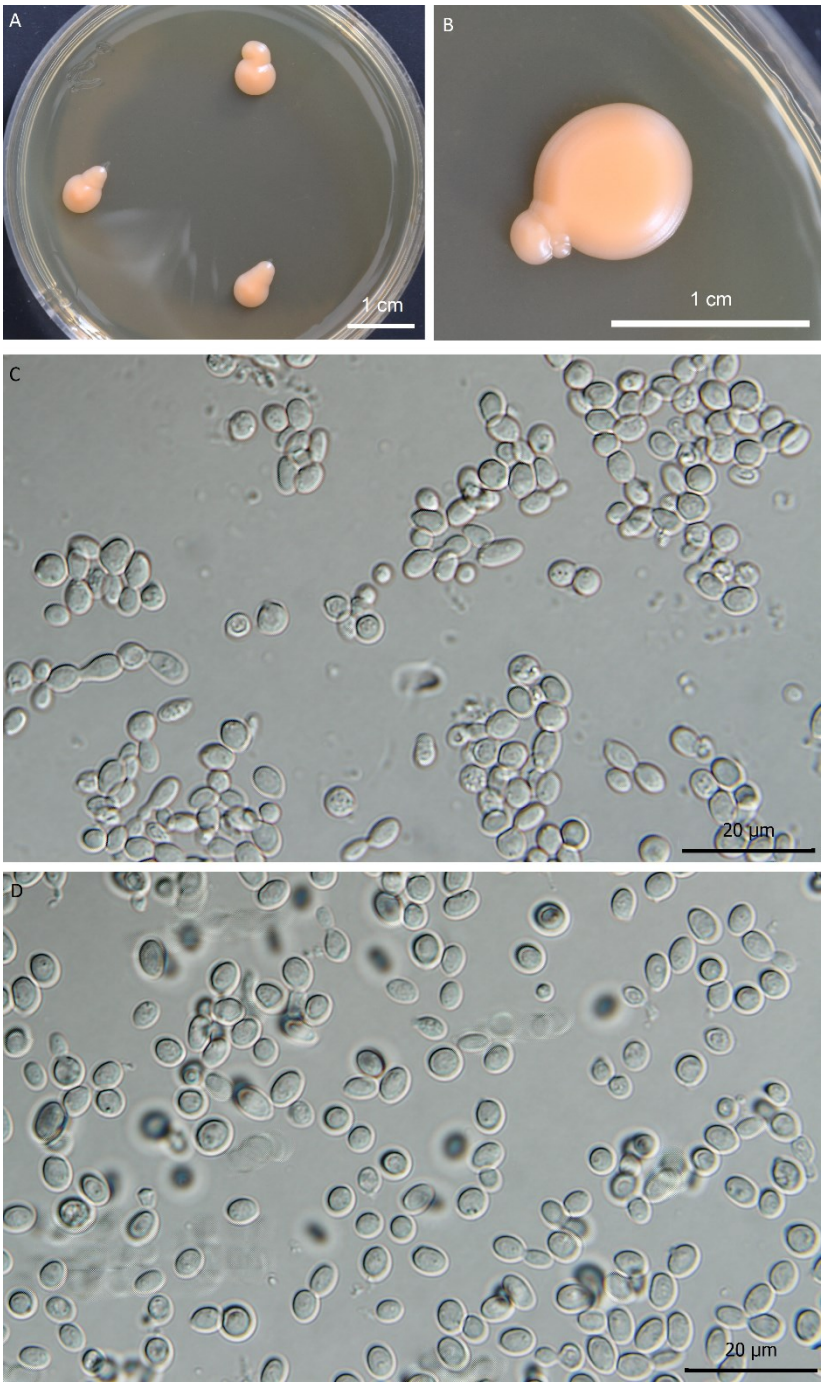

Figure 4 *Lichenozyma pisutiana* sp. nov. (A) colonies of CCF6137 on SAB after two weeks, (B) colony of strain Pol14-3 CKV on SAB after two weeks, (C) budding cells of strain Pol14-3

reasons explained in Fernández-Mendoza et al. (2017). Thus, the other tenable explanation of the rarity of basidiomycetes detection is that these fungi are killed by surface sterilization showing that lichen-associated fungi should not be approached with the same methods as plant endophytes. This supports the hypothesis that cystobasidiomycete yeasts are associated with the surfaces of lichens. The hypothesis is further supported in the case of *Bryoria capillaris*. While in some lichens (e.g,. *Bryoria fremontii, Usnea hirta, Hypogymnia tubulosa*) the cystobasidiomycete yeasts embedded in

the cortex are scattered, in *B. capillaris* they are actually what we are looking at when looking at the lichen. They have been shown to form an entire layer above the layers of the mycobiont hyphae (Spribille et al. 2016). The fact that we isolated cultures from the medulla is contradictory. We suggest that at least some of the lichen-associated yeasts are not exclusively limited to the surface.

Generally, our knowledge of basidiomycete yeasts is still poor, although taxa with a yeast stage occur in all three subphyla of Basidiomycota (Boekhout et al. 2011). The class Cystobasidiomycetes includes asexual yeast species and dimorphic species. Their life strategies are diverse, including mycoparasites, endophytes, saprophytes, lichen-associates and fungi adapted to aquatic environment, both marine and freshwater. It may be assumed that a large portion of species diversity in this class remains to be discovered.

We recovered a distinct diversity of yeasts compared to that reported by Spribille et al. (2016) who sampled mainly parmelioid lichens. First, Cyphobasidiales were found in two thalli only. Second, we report a previously unknown phylogenetic clade within the class, that appears to be related to *Symmetrospora* (Fig. 1). Its representatives were found in various *Cladonia* species, all collected in Scandinavia, suggesting that these fungi might prefer cold environments. Finally, most of our sequences, however, belong to Microsporomycetaceae. These have a broader distribution range, including Scandinavia, central Europe and Spain. Also, two out of three *Cladonia* specimens studied by Spribille et al. (2016) contained yeasts belonging to the same family (as clade I therein). These data suggest a certain degree of specificity of the cystobasidiomycete yeast lineages to their hosts at higher taxonomic ranks. Although no species specificity was found, the lichen-inhabiting Microsporomycetaceae might be specific to the genus *Cladonia*. Likely, Cyphobasidiales might be specific to Parmeliaceae.

The family Microsporomycetaceae was described based on molecular data by Wang et al. (2015b) as monogeneric, containing five species. However, according to our analysis, novel and undescribed taxa should be included in the family. The clade that includes the type species of Microsporomyces, *M. magnisporus*, also includes only *M. orientalis* and *M. pini*. The other two species - *M. bloemfonteinensis*, a saprophyte of pine needles from South Africa (Pohl et al. 2011) and *M. hainanensis*, isolated from rice seeds from China (Bai et al. 2016) - form a distinct, unrelated lineage. It also includes a *Rhodotorula* strain isolated from *U. antarctica* by Duarte et al. (2016) and uncultured Cystobasidiomycete clone T1402 detected in *Thamnolia vermicularis* by Spribille et al. (2016) and sequences obtained from *Cladonia* samples in this study. Another lineage within the family is composed of the reduced clade I in Spribille et al. (2016) and two sequences obtained in this study. According to our data, it is a sister lineage to the genus *Lichenozyma*. Given that most of the representatives of the clade we found to correspond with the Microsporomycetaceae are lichen-associated, we can infer that is the common ecological setting of the family, though most of the previously known species are not found in lichens.

Here we propose a new monotypic genus *Lichenozyma*, with the newly described species *L. pisutiana*. Phylogenetic analyses showed that their closest known relatives are the species of *Microsporomyces* and supported the recognition of this new lineage as a novel genus. It is thus defined phylogenetically and ecologically as associated with lichens, mainly of the genus *Cladonia*. It was cultured from five *Cladonia* species, and using specific primers it was further detected in 27 specimens belonging to 17 other *Cladonia* species in this study (Table 1). Uncultured Cystobasidiomycete clones T1615 and T1646 from *C. multiformis* and *Cetraria ericetorum*,

respectively (Spribille et al., 2016) also belong to the species. The yeasts could be suspected to be an anamorphic form of a known lichenicolous fungus which might be a common case as suggested by Fernández-Mendoza et al. (2017) and recently shown in the case of *Tremella* by Tuovinen et al. (2019). However, our sequence data show that this is not possible, as the only known teleomorphic lichenicolous fungi in Cystobasidiomycetes are *Cyphobasidium hypogymniicola* and *Cyphobasidium usneicola*. The only yeasts previously circumscribed from lichens are the species of *Fellomyces* (Prillinger et al. 1997) of the Tremellales. Thus, it is not likely that *L. pisutiana* is conspecific with any taxon described in the past and its circumscription as a new taxon is justified. The teleomorph might be discovered in the future.

In conclusion, the present study shows that Cystobasidiomycete yeasts are commonly associated with the lichen genus *Cladonia*. Notably, they occur in both corticate and ecorticate species. Any biological relationship to the host still remains unknown and their diversity can be expected to be remarkable.

Acknowledgements

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References

Arnold A. E., Miadlikowska J., Higgins K. L., Sarvate S. D., Gugger P., Way A., Hofstetter V., Kauff F. & Lutzoni F. (2009) A phylogenetic estimation of trophic transitions networks for Ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? Systematic Biology 58: 293-297.

Bai F., Liu Y., Yao S., Li N., WangW. & Cheng C. (2016) *Microsporomyces hainanensis* sp. nov., isolated from hybrid rice (*Oryza sativa* L.) seeds. Current Microbiology 73: 569-573.

Banchi E., Stankovic D., Fernández-Mendoza F., Gionechetti F., Pallavicini A. & Muggia L. (2018) ITS2 metabarcoding analysis complements lichen mycobiont diversity data. Mycological Progress 17: 1049-1066.

Boekhout T., Fonseca A., Sampaio J. P., Bandoni R. J., Fell J. W. & Kwon-Chung K. J. (2011) Discussion of teleomorphic and anamorphic basidiomycetous yeasts. In: Kurtzman C. P., Fell J. W. & Boekhout T. (Eds.) The Yeasts. A Taxonomic Study, 5th edition, Elsevier Science, pp. 1339-1372.

Castresana J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540-552.

Cubero O. F., Crespo A., Fatehi J. & Bridge P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243-249.

Darriba T., Taboada G. L., Doallo R. & Posada D. (2012) jModelTest2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Diederich P. (1996) The lichenicolous heterobasidiomycetes. Bibliotheca Lichenologica 61: 1-198.

Diederich P. (2011) Description of *Abrothallus parmotrematis* sp. nov. (lichenicolous Ascomycota). Bulletin de la Société des Naturalistes Luxembourgeois 112: 25-34.

Duarte A. W. F., Passari M. R. Z., Delforno T. P., Pellizzari F. M., Cipro C. V. Z., Montone R. C., Petry M. V., Putzke J., Rosa L. H. & Sette L. D. (2016) Yeasts from macroalgae and lichens that inhabit the South Shetland Islands, Antarctica. Environmental Microbiology Reports 8: 874-885.

Ekman S. (1999) PCR optimalization and troubleshooting with special reference to the amplification of ribosomal DNA in lichenized fungi. Lichenologist 31: 517-531.

Ferencova Z., Hawksworth D. L. & Rico V. J. (2017) Extraction of DNA from lichen-forming and lichenicolous fungi: a low-cost fast protocol using Chelex. Lichenologist 49: 521-525.

Fernández-Mendoza F., Fleischhacker A., Kopun T., Grube M. & Muggia L. (2017) ITS1 metabarcoding highlights low specificity of lichen mycobiomes at a local scale. Molecular Ecology 26: 4811-4830.

Gardes M. & Bruns T. (1993) ITS primers with enhanced specificity for Basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Girlanda M., Isocrono D., Bianco C. & Luppi-Mosca A. M. (1997) Two foliose lichens as microfungal ecological niches. Mycologia 89: 531-536.

Katoh K., Rozewicki J. & Yamada K. D. (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 18: 1-7.

Kurtzman C. P., Fell J. W., Boekhout T. & Robert V. (2011) Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman C. P., Fell J. W. & Boekhout T. (Eds.) The Yeasts. A Taxonomic Study, 5th edition. Elsevier Science, pp. 87-110.

Liu X.-Z., Wang Q.-M., Göker M., Groenwald M., Kachalkin A. V., Lumbsch H. T., Millanes A. M., Wedin M., Yurkov A. M., Boekhout T. & Bai F.-Y. (2016) Towards an integrated phylogenetic classification of the Tremellomycetes. Studies in Mycology 81: 85-147.

Millanes A., Diederich P., Ekman S. & Wedin M. (2011) Phylogeny and character evolution in the jelly fungi (Tremellomycetes, Basidiomycota, Fungi). Molecular Phylogenetics and Evolution 61: 12-28.

Millanes A. M., Diederich P. & Wedin M. (2016) *Cyphobasidium* gen. nov., a new lichen-inhabiting lineage in the Cystobasidiomycetes (Pucciniomycotina, Basidiomycota, Fungi). Fungal Biology 120: 1468-1477.

Miller M. A., Pfeiffer W. & Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, USA, pp. 1-8.

Muggia L., Fleischhacker A., Kopun T. & Grube M. (2016) Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Diversity 76: 119-142.

Nakase T., Tsuzuki S., Lee F.-L., Sugita T., Jindamorakot S., Jan-ngam H., Potacharoen W., Tanticharoen M., Kudo T. & Takashima M. (2003) *Sporobolomyces magnisporus* sp. nov., a new yeast species in the *Erythrobasidium* cluster isolated from plants in Taiwan. Journal of General and Applied Microbiology 49: 337-344.

Oberwinkler F. (2017) Yeasts in Pucciniomycotina. Mycological Progress 16: 831-856.

Palmqvist K., Franklin O. & Näsholm, T. (2017) Symbiosis constraints: strong mycobiont control limits nutrient response in lichens. Ecology and Evolution 7: 7420-7433.

Paradis E., Claude J. & Strimmer K. (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20: 289-290.

Park C. H., Kim K. M., Elvebakk A., Kim O.-S., Jeong G., Hong S. G. (2015) Algal and fungal diversity in Antarctic lichens. Journal of Eukaryotic Microbiology 62: 196-205.

Peršoh D. & Rambold G. (2012) Lichen-associated fungi of the *Letharietum vulpinae*. Mycological Progress 11: 753-760.

Petrini O., Hake U. & Dreyfuss M. M. (1990) An analysis of fungal communities isolated from fruticose lichens. Mycologia 82: 444-451.

Pohl C. H., Smit M. S. & Albertyn J. (2011) *Rhodotorula bloemfonteinensis* sp. nov., *Rhodotorula eucalyptica* sp. nov., *Rhodotorula orientis* sp. nov. and *Rhodotorula pini* sp. nov., yeasts isolated from monoterpene-rich environments. International Journal of Systematic and Evolutionary Microbiology 61: 2320-2327.

Prillinger H., Kraepelin G., Lopandic K., Schweigkofler W., Molnar O., Weigang F. & Dreyfuss, M. M. (1997) New species of *Fellomyces* isolated from epiphytic lichen species. Systematic and Applied Microbiology 20: 572-584.

R Core Team (2017) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rambaut A. (2016) FigTree. Tree Figure Drawing Tool. University of Edinburgh. http://tree.bio.ed.ac.uk/.

Revell L. J. (2012) phytools: an R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3: 217-223.

Ronquist F., Teslenko M., van der Mark P., Ayres D., Darling A., Höhna S., Larget B., Liu L., Suchard M. A. & Huelsenbeck J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539-542.

Santiago I. F., Soares M. A., Rosa C. A. & Rosa L. H. (2015) Lichenospere: a protected natural microhabitat of the non-lichenised fungal communities living in extreme environments of Antarctica. Extremophiles 19: 1087-1097.

Spribille T., Tuovinen V., Resl P., Vanderpool D., Wolinski H., Aime M.C., Schneider K., Stabentheiner E., Toome-Heller M., Thor G., Mayrhofer H., Johannesson H. & McCutcheon J. P. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353: 488-492.

Spribille T. (2018) Relative symbiont input and the lichen symbiotic outcome. Current Opinion in Plant Biology 44: 57-63.

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

Stenroos S., Pino-Bodas R., Hyvönen J., Lumbsch H. T. & Ahti T. (2019) Phylogeny of the family *Cladoniaceae* (*Lecanoromycetes*, *Ascomycota*) based on sequences of multiple loci. Cladistics 35: 351-384.

Stocker-Wörgötter E. & Hager A. (2008) Appendix: culture methods for lichens and lichen symbionts. In: Nash III T.H. (Ed.) Lichen Biology, 2nd edition, Cambridge University Press, Cambridge, pp. 353-363.

Suryanarayanan T. S. & Thirunavukkarasu N. (2017) Endolichenic fungi: the lesser-known fungal associates of lichens. Mycology 8: 189-196.

Tuovinen V., Ekman S., Göran T., Vanderpool D., Spribille T. & Johannesson H. (2019) Two Basidiomycete fungi in the cortex of wolf lichens. Current Biology 29: 1-8.

U'Ren J. M., Lutzoni F., Miadlikowska J. & Arnold A. E. (2010) Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microbial Ecology 60: 340-353.

U'Ren J. M., Lutzoni F., Miadlikowska J., Laetsch A. D. & Arnold A. E. (2012) Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American Journal of Botany 99: 898-914.

U'Ren J. M., Riddle J. M., Monacell J. T., Carbone I., Miadlikowska J. & Arnold A. E. (2014) Tissue storage and primer selection influence pyrosequencing-based inferences of diversity and community composition of endolichenic and endophytic fungi. Molecular Ecology Resources 14: 1032-1048.

Vilgalys R. & Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238-4246.

Wang Q.-M., Groenewald M., Takashima M., Theelen B., Han P.-J., Liu X.-Z., Boekhout T. & Bai F.-Y. (2015a) Phylogeny of yeasts and related filamentous fungi within Pucciniomycotina determined from multigene sequence analyses. Studies in Mycology 81: 27-53.

Wang Q.-M., Yurkov A. M., Göker M., Lumbsch H. T., Leavitt S. D., Groenewald M., Theelen B., Liu X.-Z., Boekhout, T. & Bai F.-Y. (2015b) Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. Studies in Mycology 81: 149-189.

White T. J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (Eds.) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp. 315-322.

Zhang T., Wei X.-L., Zhang Y.-Q., Liu H.-Y. & Yu L.-Y. (2015) Diversity and distribution of lichen-associated fungi in the Ny-Ålesund region (Svalbard, High Arctic) as revealed by 454 pyrosequencing. Scientific Reports 5: 14850.

Zhang T., Wei X.-L., Wei Y.-Z., Liu H.-Y. & Yu L.-Y. (2016) Diversity and distribution of cultured endolichenic fungi in the Ny-Ålesund region, Svalbard (High Arctic). Extremophiles 20: 461-470.

Zúñiga C., Leiva D., Carú M. & Orlando J. (2017) Substrates of *Peltigera* lichens as a potential source of cyanobionts. Microbial Ecology 74: 561-569.

Zwickl D. J. (2006) Genetic Algorithm Approaches for the Phylogenetic Analysis of Large Biological Sequence Datasets under the Maximum Likelihood Criterion. The University of Texas, Austin.

Paper 2

Lessons from culturing lichen soredia

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Abstract

Vegetative propagules play various important roles in lichen biology. We cultured soredia of *Cladonia* lichens in vitro and obtained three noteworthy results. Firstly, soredia are a beneficial source for the isolation of lichen symbionts. The mycobiont was obtained from 66% and the photobiont from 67% of the cultured soredia that were not contaminated. Secondly, the development of soredia followed a previously recognized pattern, arachnoid stage – soredium field – primordium, but a thalline structure was not achieved. We suggest that thallus formation in vitro is a question of favourable environmental factors, not partners compatibility. Finally, we discovered that fungi, other than the mycobiont, as well as airborne contaminants are dispersed together with lichen soredia. This is the first-ever report of such a phenomenon. The possible ecological consequences are discussed. Cystobasidiomycete yeasts were found among these fungi. We isolated representatives of three different lineages from a single thallus suggesting a low specificity for this association.

1. Introduction

Vegetative dispersal propagules are an exclusive expression of the lichen symbiotic phenotype (Ahmadjian 1993b). Soredia are small (20–50 μm) spherical clumps of a few algal cells and short hyphae, and among the most common means of reproduction in many lichens (Büdel and Scheidegger 2008). Their role in lichen biology is quite well-understood. Soredia provide a lichen the clear advantage of co-dispersal of both symbiotic partners, eliminating the need for recruitment of compatible algae, which are considered to be rare in the environment (Vančurová et al. 2020). As a result, however, sorediate lichen-forming *Cladonia* species have been shown to be more specific towards their photobionts, i.e., their potential range of compatible partners is lower (Steinová et al. 2019), which may limit their ecological niches and distribution ranges (Rolshausen et al. 2018, Vančurová et al. 2018). In addition to dispersal, soredia also serve as photobiont source for other lichens (Ahmadjian 1993a). This fact plays an important role in establishment of whole lichen communities. According to the core-fringe species hypothesis (Rikkinen et al. 2002), sexual lichen species (fringe) depend on the dispersal of suitable photobionts by asexual species (core). This hypothesis has been supported by recent studies (Belinchón et al. 2015, Cardós et al. 2019). Soredia are dispersed continuously in large amounts, often landing near the parent lichen thallus (Armstrong 1987). However, they are also carried by the wind up to distances of tens of meters (Armstrong 1987,

Werth et al. 2006), and exceptionally hundreds or thousands of kilometers (Harmata and Olech 1991). Soredia are also effectively dispersed by invertebrates, such as mites, ants or snails (Stubbs 1995, Lorentsson and Mattsson 1999, Boch et al. 2011). Success of reestablishment of lichen thalli from soredia has been demonstrated in various transplantation experiments (Armstrong 1990, Scheidegger 1995, Kon and Ohmura 2010). Soredia have been studied experimentally mainly with the purpose of lichen synthesis in vitro (e.g., Stocker-Wörgötter 1995, Valarmathi and Hariharan 2007) or viability testing (e.g., Hauck and Zöller 2003, Buldakov 2010). Successful syntheses of lichens in vitro are infrequent and factors that determine the underlying processes are still only partially understood (as reviewed, e.g., by Stocker-Wörgötter 2001, Joneson and Lutzoni 2009). Thus, procedures for such experiments are not standardized and need to be established for each experimental series anew. Additionally, although the contamination rate associated with culturing lichen material is generally high (Crittenden et al. 1995), studies about culturing soredia did not provide information on contaminating fungi (see, e.g., Stocker-Wörgötter and Türk 1988, Stocker-Wörgötter 1995, Zorer et al. 1997, Trembley et al. 2002 and references above). It might, however, be expected that besides airborne and laboratory contaminants, fungi associated with lichen thalli (Hawksworth and Grube 2020) are co-dispersed with soredia, as has already been shown for bacteria (Aschenbrenner et al. 2014). We cultured soredia to observe their development with the objective of setting a reference frame for future in vitro compatibility testing. Specifically, our aims were to 1) evaluate suitability of soredia for the isolation of symbiont cultures, 2) inspect their development in vitro, and 3) have a first-ever look into diversity of fungi spread with soredia.

2. Materials and Methods

2.1 Materials

Cladonia rei was collected from soil on silicate rock outcrops in Svatá, Czech Republic, N49.9399972 E13.9607781, 480 m a. s. l. on 5 March 2019 and *C. fimbriata* from soil on the lower edge of an oak-pine forest in Černošice, Czech Republic, N49.9470725 E14.3388042, 200 m a. s. l. on 11 September 2019. The specimens were deposited in PRC (PRC 4638 and PRC 4639, respectively). *C. rei* was processed the day after collection, *C. fimbriata* after two days and then again three weeks after collection. Both were used for evaluation of isolation success of the mycobiont and photobiont. Only soredia from *C. fimbriata* were used to study development and to collect information on the associated fungi.

2.2 Isolation

The thalli were used unwashed. In addition to soredia development, we were also interested in fungi co-dispersed with soredia, both within the soredia and on their surface (see Discussion). We are aware of disadvantages of not washing, but we believe molecular methods give us a powerful tool for distinguishing airborne fungi and laboratory contaminants. Also, thorough washing could result in detachment of the most mature soredia that might be expected to germinate most readily. Under a binocular microscope, the soredia were separated directly from the thalli using a sterile needle and placed onto cultivation media. Sterile 12 well cell culture plates (Cellstar, USA) were used, each well 22 mm in diameter. Care was taken to separate as little lichen material as possible, resulting in removal of individual soredia or clusters of a few. Media recipes are to be found in Stocker-Wörgötter and Hager (2008). The media used were Bold's Basal medium (BBM) and Malt-yeast extract medium (MYA) with no sugars added, or BBM, Trebouxia organic medium (TOM) and

Sabouraud agar (SAB) with the addition 1% or 2% of ribitol, mannitol or glucose. Ribitol and mannitol were used as alternatives to glucose because they have been reported to stimulate the growth of lichen-forming fungi (Guzow-Krzemińska and Stocker-Wörgötter 2013, Meeβen et al. 2013). The inoculated plates were kept in an incubator (Electrolux, ERC2543, 250CI, with thermostat TS-3, FK technics and fluorescent bulb controlled by a digital time switch TR610, Theben) at 16.5 °C and 12 h light regime (18 µmol photons m^2s^1). Cultures of lichen mycobiont (*Cladonia* spp.) and photobionts (*Asterochloris* spp.) were identified morphologically. Ten representative isolates of the mycobiont were chosen for molecular study and confirmation. We expressed isolation success in two ways; as isolation rate and viability rate. The isolation rate was calculated as the percentage of inoculates from which the mycobiont/photobiont grew. The viability rate was calculated as the percentage of the number of obtained isolates from the number of inoculated wells minus the number of contaminated wells. A well was considered contaminated if it was overgrown by common airborne fungi, such as Cladosporium, Penicillium etc. We believe that the number of contaminated plates provides information about the state of laboratory equipment and skilfulness of the isolator rather than the quality of the studied material. Thus, the isolation rate provides information about how fruitful the effort was, and the viability rate allows us to compare our results with other methods of isolation. Contamination rates are high in all of them. Fungal isolates that were neither airborne and ubiquitous fungi, nor lichen mycobionts, were considered as soredia-associated fungi.

2.3 Soredia cultivation on natural substrates

Soil or pieces of sandstone, both collected from natural *Cladonia* habitats were autoclaved. They were placed in glass petri dishes (4 cm in diameter) and autoclaved again after one week. The material was fixed in the petri dish with water agar. After three months of culturing, most soredia had developed into primordia (see Results and Discussion). Six of them were picked, divided into 22 smaller pieces and transferred onto sterile soil or sandstone. At first, they underwent four drying and re-wetting cycles. Drying was done in the following way: the agar surrounding the natural substrata was cut out from the petri dishes, open dishes were then placed in a running laminar flow box for four hours. During this time the air flow dried both the developing soredia and the substrata completely. The petri dishes were closed and sealed with parafilm. After four days, they were re-wetted as follows: hot water agar was carefully poured inside to surround the soil/sandstone. After the agar stiffened, the dishes were sealed with parafilm. This way 100% moisture was kept inside until the dishes were open again four days later. The developing soredia thus absorbed the humidity from the air surrounding them. They were inspected microscopically before each drying and photos were taken under a stereomicroscope. After the first month (four drying and re-wetting cycles), they were rewetted monthly as described above. After each wetting the petri dishes were covered with their lids but were not sealed with parafilm, so that slow continual drying was allowed. They were completely dry after about three weeks. The final evaluation was made after six months of culturing on the natural media (December 2019 – June 2020). The petri dishes were kept at circa 22 °C on a window sill to simulate natural light conditions.

2.4 Molecular methods

DNA from both cultures and the original thalli was isolated using the CTAB protocol (Cubero et al 1999) with minor modifications. ITS rDNA was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR began with denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C denaturation for 45 s, 54 °C annealing for 1 min and 72 °C elongation for 2 min and finished with extension at 72 °C for 10 min. For the cultures of yeast belonging to

Cystobasidiomycetes SSU and LSU rDNA was amplified too. For SSU the primers SSU_symrho_2F and NS6 (Spribille et al. 2016) were used and the PCR consisted of 35 cycles of 95 °C denaturation for 30 s, 56 °C annealing for 30 s and 72 °C elongation for 45 s. The primers LR0R and LR6 (Vilgalys and Hester 1990) were used for LSU and the PCR consisted of 35 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 1 min. The PCR products were sequenced by Macrogen Europe, Amsterdam, the Netherlands. GenBank accession numbers of the newly obtained sequences are given in Table 1.

2.5 Identification of associated fungi

Because all the isolates were sterile, we used ITS rDNA to designate their taxonomic position. The obtained chromatographs were examined and sequences were edited if needed. They were compared to GenBank using BLASTn. For each isolate the closest match was recorded. If the closest match was an unidentified fungus and/or from environmental sample, the closest reliably identified match, e. g. a sequence from type specimen or from a curated culture collection, was recorded as well (Table 1). Provisional names were given to the isolates based on sequence similarity; at the similarity of at least 98% the isolate was given a species names, at similarities between 90 and 97% the isolate was classified into an order and at lower similarities it was classified either to a subclass or class (Table 1).

2.6 Phylogeny of Cystobasidiomycetes

Cystobasidiomycetes yeasts were previously hypothesized to form specific symbiosis with lichens (Spribille et al. 2016). So, in order to position isolates SOR11c5, SOR11d6, SOR12b5 and SOR12d2 within the class a phylogeny based on the three ribosomal DNA markers was performed. Representatives of all main lineages of the class (Wang et al. 2015, Spribille et al. 2016, Černajová and Škaloud 2019) were included in the dataset taking into account the closet BLAST matches of our sequences (Table 2). *Sporidiobolus salmonicolor* (Microbotryomycetes) was used as the outgroup. Each marker was processed separately. Sequences downloaded from the GenBank were aligned with the newly obtained sequences using MAFFT v.7 (Katoh et al. 2017) using the Q-INS-I method. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and removed. Final datasets consisted of 866 SSU, 314 ITS and 536 LSU positions. Substitution models were estimated with Bayesian Information Criterion using JModelTest v. 2.1.4 (Darriba et al. 2012) as follows: TrN + I + G for SSU (p-inv 0.588, gamma shape 0.71), JC + G for ITS1 (gamma shape 1.645), K80 for 5.8S, TVMef + G for ITS2 (gamma shape 0.648) and TIM2ef + I + G for LSU (p-inv 0.414, gamma shape 0.55). The phylogenetic trees were inferred by Bayesian Inference in MrBayes v. 3.2.6 (Ronquist et al. 2012), initially for each locus separately. All three loci gave congruent topologies. So, the final analysis was performed on a concatenated dataset using the five partitions. Two parallel MCMC runs, with one cold and three heated chains, were run. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF), which was 0.001 after final 11 million generations. The first 25% of the trees were discarded as burn-in in each run. 50% majority-rule consensus trees were obtained using the sumt option. The analyses were run on the CIPRES Science Gateway v. 3.3 web portal (Miller et al. 2010).

Table 1. Identification based od ITS rDNA and best GenBank matches of the isolates obtained from soredia of *Cladonia fimbriata*.

Isolate	GenBank Accession [1]	Class	Identification	Best GenBank matches				
				similarity	e-value	Accession	Strain/Clone	Habitat
Lichen mycobiont								
SOR6a2[2]	MT981770-MT981779	Lecanoromycetes	*Cladonia fimbriata*	544/546(99%)	0.0	MK811629	*Cladonia fimbriata* O-L-200909	terricolous[3]
Ascomycota								
SOR6c3	MT981780	Dothideomycetes	Dothideomycetes sp.	543/546(99%)	0.0	MT236889	Uncultured fungus 4248_906	irrigation water from the pond[4]
				394/460(86%)	7E-101	NR_155853	*Saccharata eucalyptorum* CPC 29222	holotype culture, from *Eucalyptus bigalerita*[5]
SOR8a3	MT981784	Dothideomycetes	Dothideomycetes sp.	414/423(98%)	0.0	GU993541	Uncultured Capnodiales A11	energy transmission tower (corrosion dust)[6]
				443/496(89%)	2E-170	GU570527	*Devriesia pseudoamericana* CPC:16174	fruit surface[7]
SOR11b6	MT981786	Dothideomycetes	Pleosporomycetidae sp.	473/495(96%)	0.0	KC222749	Uncultured fungus Toohyp3	soil in eucalyptus forest[8]
				435/502(87%)	1E-147	NR_154080	*Hermatomyces thailandicus* MFLUCC 14-1143	holotpye culture, from *Tectonia grandis*[9]
SOR12b1	MT981790	Dothideomycetes	Pleosporales sp	421/435(97%)	0.0	JX457096	Uncultured fungus HIC6	forest soil[10]
				411/435(94%)	0.0	MN421854	*Lophiostoma chamaecyparidis* isolate 4	culture from *Nectandra lineatifolia*[11]
SOR12c3	MT981792	Dothideomycetes	Venturiales sp.	238/238(100%)	4E-119	KX194025	Uncultured fungus 1604	soil[12]
				312/341(91%)	4E-94	NR_168748	*Parafusicladium amoenum* CBS 254.95	holotype, from leaf litter of *Eucalyptus grandis*[13]
SOR13b1	MT981795	Dothideomycetes	*Pseudocamaropycnis pini*	502/507(99%)	0.0	NR_153459	*Pseudocamaropycnis pini* CBS:115589	holotype culture, from a *Pinus elliotii* leaf[14]
SOR6d2	MT981781	Leotiomycetes	Helotiales sp.	464/479(97%)	0.0	KX908215	Leotiomycetes sp. 780 JMUR-2016	endophyte culture, from a leaf of *Pinus strobus*[15]
				454/501(91%)	0.0	MH221525	*Ciliolarina ligniseda* SBRH847	dead pinus log on the ground[16]
SOR8a2	MT981783	Leotiomycetes	Helotiales sp.	447/455(98%)	0.0	EF619699	Uncultured Helotiales 3S2.16.F04	forest soil[17]
				457/483(95%)	0.0	NR_156207	*Hyalodendriella betulae* CBS 261.82	*Alnus glutinosa*[13]

SOR12d3	MT981794	Leotiomycetes	*Lachnellula pulverulenta*	469/476(99%)	0.0	AB481260	*Lachnellula pulverulenta* FC-2025	*L. pulvurentula* fruit body[18]
SOR8d2	MT981785	Eurotiomycetes	Chaetothyriomycetidae sp.	509/509(100%)	0.0	KX147893	Uncultured fungus PO.1.69	pine sapwood [19]
				466/552(84%)	3E-139	NR_153652	*Bacillicladium lobatum* CCF 5200	type culture (walls of metro station)[20]
Basidiomycota								
SOR7c1	MT981782	Spiculogloeomycetes	Pucciniomycotina sp.	459/525(87%)	1E-162	MT236898	Uncultured fungus clone 4248_1241	irrigation water from the pond[4]
				299/370(81%)	3E-69	NR_121215	*Phyllozyma producta* MAFF 654001	holotype culture, from a leaf of *Citrus unshiu*[21]
SOR11c4	MT981787	Exoboasidiomycetes	*Microstroma bacarum*	632/633(99%)	0.0	NR_153481	*Microstroma bacarum* CBS 6526	type, from fruit[22]
	MT990521/MT981788/MT974387	Cystobasidiomycetes	Cystobasidiomycetes sp.	504/531(95%)	0.0	KT581825	Uncultured Rhodotorula clone MDW-OTU-12	*Quercus deserticola* litter[23]
SOR11c5 = SOR12b5	MT990522/MT981791/MT974388			393/439(90%)	7E-151	KY104259	*Microsporomyces orientalis* culture CBS:8594	holotype, from soil [22]
SOR11d6	MT990523/MT981789/MT974389	Cystobasidiomycetes	Cystobasidiomycetes sp.	470/529(89%)	2E-176	AB263120	*Sakaguchia lamellibrachiae* strain: SY-298	on *Calyptogena* in deep sea[24]
SOR12d2	MT990524/MT981793/MT974390	Cystobasidiomycetes	*Cystobasidium pinicola*	519/520(99%)	0.0	MH380197	*Cystobasidium pinicola* strain ICMP 2924	*Prunus persica* leaf[25]

[1]Accession numbers of newly obtained ITS rDNA sequences are provided except for Cystobasidiomycetes where SSU/ITS/LSU rDNA are given.

[2]also SOR6c2, SOR6d3, SOR7a2, SOR8c1, SOR10-1, SOR10-3, SOR11d4, SOR12c4, SOR12c6

[3-25]References:[3]Marthinsen et al. 2019, [4]Marčiulynas et al. 2020, [5]Crous et al. 2016, [6]Sette et al. 2010, [7]Frank et al. 2010, [8]Greenlaw 2012 unpubl., [9]Doilom et al. 2016, [10]Liu and Qiu 2012, unpubl., [11]Nelson et al. 2019 unpubl., [12]Beck et al. 2016 unpubl., [13]Crous et al. 2007, [14]Crous and Groenewald 2016 , [15]U'Ren and Arnold 2016, [16]Helleman 2018, unpubl., [17]Parrent and Vilgalys 2007, [18]Hosoya et al. 2010, [19]van Nieuwenhuijzen et al. 2017, [20]Reblová et al. 2016, [21]Furuya et al. 2012, [22]Vu et al. 2016, [23]Rosales-Castillo et al. 2018, [24]Sampaio et al. 2006 unpubl., [25]Weir and Park 2018 unpubl.

Table 2. List of GenBank sequences used for the phylogeny of Cystobasidiomycetes.

taxon	strain/clone	ITS	LSU	SSU
Bannoa hahjimensis	JCM 10336	AB035897	–	AB035897
Bannoa ogasawarensis	JCM 10326	AB035713	AB082570	AB035713
Bannoa syzygii	JCM 10337	AB035720	AB082573	AB035720
Buckleyzyma armeniaca	JCM 8977	AF444523	AF189920	AB126644
Buckleyzyma aurantiaca	JCM 3771	AF444538	AF189921	KJ708436
Buckleyzyma salicina	JCM 2959	AF444511	AF189995	AB021687
Cyphobasidium hypogymniicola	S-F264671	KU587700	KU587694	KU587705
Cyphobasidium usneicola	S-F264675	KU587704	KU587699	KU587706
Cystobasidium laryngis	JCM 10953	AB078500	AB078500	AB126649
Cystobasidium pinicola	AS 2.2193	AF444292	AF444293	AB126652
Cystobasidium ritchiei	CBS 12314	NR_154854	KY107445	NG_063085
Erythrobasidium elongatum	AS 2.1949	AF444561	AF189983	AB021669
Erythrobasidium hasegawianum	AS 2.1923	AF444522	AF189899	D12803
Lichenozyma pisutiana	CCF 6137	MK491195	MK491271	MK491263
Microsporomyces magnisporus	JCM 11898	AB112078	AB111954	KJ708428
Microsporomyces pini	CBS 107345	EU075190	EU075188	KJ708357
Occultifur externus	JCM 10725	AF444567	AF189910	AB055193
Occultifur tropicalis	DMKU SE59	NR_148062	–	–
Sakaguchia dacryoidea	JCM 3795	AF444597	AF189972	D13459
Sakaguchia lamellibrachii	CBS 9598	AB025999	AB025999	AB126646
Sakaguchia oryzae	AS2.2363	AY335160	AY335161	KJ708352
Sporidiobolus salmonicolor	CBS 490	NR_149325	NG_056268	NG_063452
Symmetrospora coprosmae	JCM 8772	AF444577	AF189980	D66880
Symmetrospora foliicola	AS 2.2527	AF444521	AF189984	AB021671
Symmetrospora gracilis	JCM 2963	AF444578	AF189985	KJ708433
Uncultured Cyphobasidiales	T1385	KU948738	KU948871	KU948820
Uncultured Cyphobasidiales	T1645	KU948778	KU948917	KU948855

3 Results

3.1 Mycobiont (*Cladonia* spp.) and photobiont (*Asterochloris* spp.) isolation success

Either the lichen symbionts or other fungi grew from all the soredia. Bacteria grew on only a few plates; the colonies were very small and did not perturb the symbionts. The isolation and viability rates are given in Table 3. Overall, isolation/viability rate was 46/66% for the mycobiont and 50/67% for the photobiont. Mycobiont viability rate was 75% from the soredia of *C. rei* and 62% from the soredia of *C. fimbriata*. Photobiont (*Asterochloris* sp.) viability rate was 83% and 59% from the soredia of *C. rei* and *C. fimbriata*, respectively. Viability of the soredia of *C. fimbriata* did not decrease after three weeks. The viability rate of the mycobiont was 62% in both cases. The viability rate of the photobiont was 57% two days and 62% three weeks after collection. Interestingly, for both species the isolation and viability rates were the highest on BBM, i.e., mineral medium with no source of carbohydrates. The mycobiont viability rate was 83% for *C. rei* and 80% for *C. fimbriata*. On the other

Table 3. Isolation success; numbers of obtained isolates, contamination, isolation and viability rates are given.

	inoculates	obtained isolates				contaminated	**contamination rate**	**mycobiont isolation rate[3]**	**mycobiont viability rate[4]**	**photobiont isolation rate[3]**	**photobiont viability rate[4]**
		mycobiont only	photobiont only	mycobiont + photobiont	other fungi						
Cladonia rei											
BBM	12	0	1	10	0	1	**8%**	**83%**	**91%**	**92%**	**100%**
+ glucose [1]	10	0	2	5	2	1	**10%**	**50%**	**56%**	**70%**	**78%**
+ mannitol [1]	8	0	0	5	3	0	**0%**	**63%**	**63%**	**63%**	**63%**
+ ribitol [1]	9	0	0	7	1	1	**11%**	**78%**	**88%**	**78%**	**88%**
total	39	0	3	27	6	3	**8%**	**69%**	**75%**	**77%**	**83%**
Cladonia fimbriata											
BBM	10	0	2	8	0	0	**0%**	**80%**	**80%**	**100%**	**100%**
MYA	26	3	1	3	4	15	**58%**	**23%**	**55%**	**15%**	**36%**
+ glucose [1]	52	0	3	21	14	15	**29%**	**40%**	**57%**	**46%**	**65%**
+ mannitol [1]	17	5	1	2	4	6	**35%**	**41%**	**64%**	**18%**	**27%**
+ ribitol [1]	10	1	0	6	3	0	**0%**	**70%**	**70%**	**60%**	**60%**
sum 2 days [2]	45	17	6	4	12	8	**18%**	**51%**	**62%**	**47%**	**57%**
sum 3 weeks [2]	70	23	3	3	13	28	**40%**	**37%**	**62%**	**37%**	**62%**
total	115	40	9	7	25	36	**31%**	**43%**	**62%**	**41%**	**59%**
C. rei + C. fimbriata											
total	154	40	12	34	31	39	**25%**	**49%**	**66%**	**50%**	**67%**

[1] combined numbers of isolates for BBM/SAB/TOM with glucose/mannitol/ribitol

[2] sum of isolates on all media inoculated two days or three weeks after collection

[3] isolation rate was calculated as number of obtained isolates / number of inoculates

[4] viability rate was calculated as number of obtained isolates / (number of inoculates - number of contaminated inoculates)

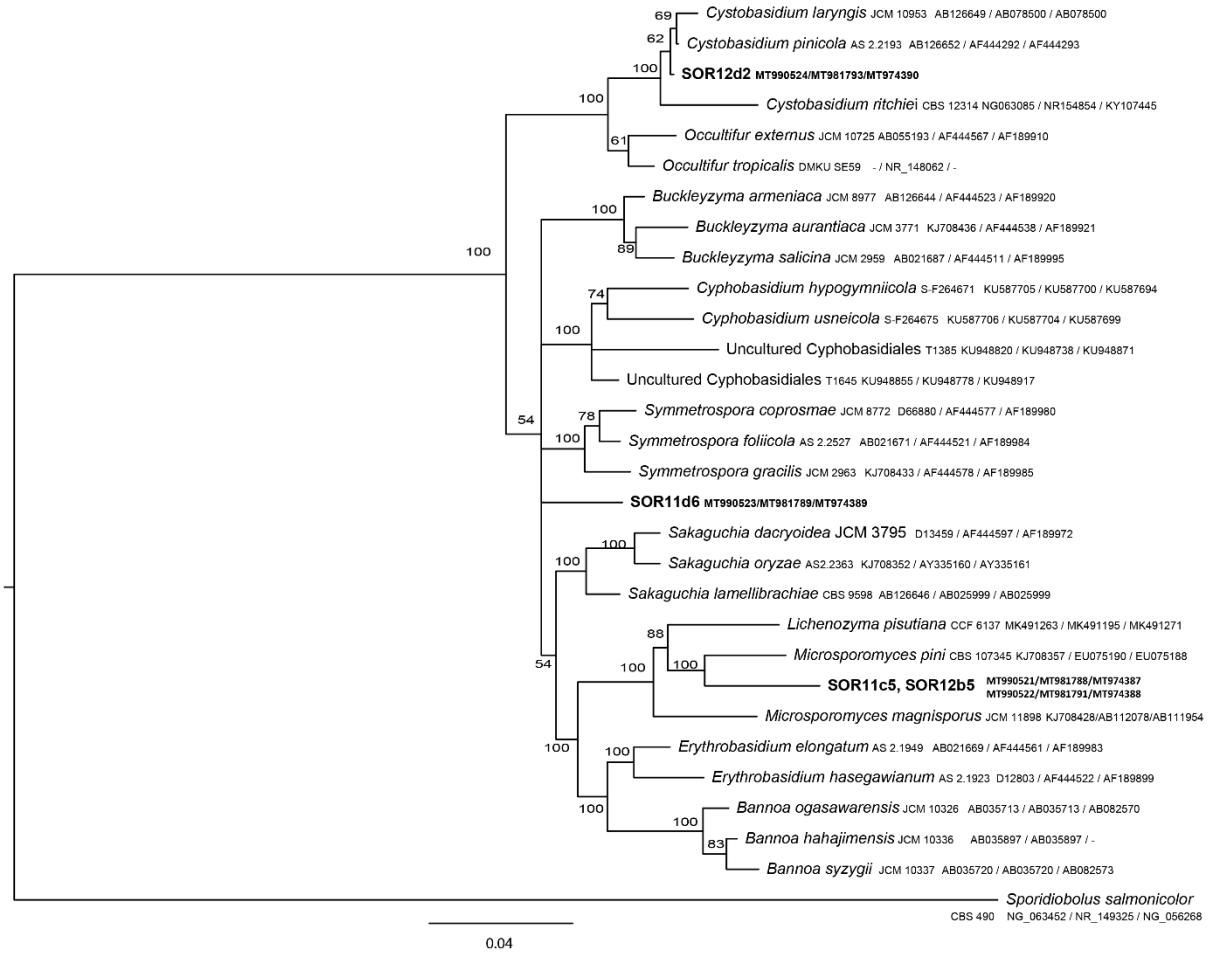

Figure 1 Phylogeny of the Cystobasidiomycetes obtained by Bayesian inference of concatenated SSU, ITS and LSU rDNA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability. Newly obtained sequences are marked in bold. Scale bar represents the expected number of substitutions per site

hand, it was 68% for *C. rei* and 60% for *C. fimbriata* on media with sugars added. The photobiont viability rate of both species was 100% on BBM and 76% for *C. rei* and 57% for *C. fimbriata* on media with sugars added (Table 3).

3.2 Diversity of associated fungi

Altogether 73 fungal isolates were obtained from the soredia of *C. fimbriata*. 47 of them were isolates of the lichen mycobiont, i.e., *C. fimbriata* and 26 were different from the lichen mycobiont. Ten of the former and 16 of the latter were successfully sequenced. The closest BLAST matches together with the closest reliably identified matches are shown in Table 1. Ten Ascomycota and six Basidiomycota isolates were obtained. Among them, Dothideomycetes (six isolates) and Cystobasidiomycetes (four isolates) prevailed respectively (Table 1). Majority of the isolates could not be assigned to a species or genus based on ITS rDNA. Sequences of only four isolates gave matches of 99% similarity: SOR11c4 matched *Microstoma baccarum* (632/633 bp), SOR12d2

Cystobasidium pinicola (519/520 bp), SOR12d3 *Lachnellula pulverulenta* (469/476 bp) and SOR13b1 *Pseudocamaropycnis pini* (502/507). Sequences from other cultures gave high matches (96–100%) with sequences of unidentified mostly uncultured fungi (Table 1) found in soil (SOR8a2, SOR11b6, SOR12b1, SOR12c3), pine needles and wood (SOR6d2 and SOR8d2) or even in corrosion dust (SOR8a3) and water (SOR6c3). We obtained four isolates belonging to three distinct genotypes of Cystobasidiomycetes. Their position within the class as inferred by Bayesian Inference is shown in Fig. 1. SOR11c5 and SOR12b5 represent an unrecognized species related to *Microsporomyces pini* with full bootstrap support. SOR11d6 formed a unique lineage of uncertain position within the class. And SOR12d2 belongs to the genus *Cystobasidium* with full bootstrap support. It is conspecific with *Cystobasidium pinicola* based on the similarity of ITS sequence, but it appears as a separate species in the phylogram probably because of the fact that the similarity in both SSU and LSU is very high among various species within the genus.

3.3 Soredia development

The structure of soredia disintegrated after germination on all media. The symbionts grew in close association with one another but separately, each in its own way; the photobiont grew in an elevated globular form, typical of *Asterochloris* spp. and the mycobiont formed a loose arachnoid radial mycelium tightly fixed to the substrate (Fig. 2a). After five weeks, the diameter of these flat mycelia was about 2 mm, ranging between 1.4 mm and 2.4 mm, regardless of presence or absence of sugars in the medium (Wilcoxon sum rank test, W = 37, p = 0.54, n = 20, not shown). The exceptions were two particularly large mycelia (4.7 and 5.6 mm in diameter) that developed on media with glucose. After three months, the development differed depending on the medium. On BBM, there was no progress from the small arachnoid radial mycelia. On TOM and SAB both with glucose (n = 21), the symbionts came together and developed further in association (see below). It is uncertain whether this development was a result of the presence of glucose or organic nitrogen compounds (peptone) in the medium, because on BBM with glucose and on SAB with ribitol/mannitol growth of both symbionts unfortunately was not achieved, with only one exception. On BBM with ribitol (n = 6) or mannitol (n = 2) and on SAB with mannitol (n = 1) both symbionts grew, the mycobiont formed a dense and compact mycelium and the photobiont a compact colony that did not seem to interact in five of the soredia (Fig. 2b). In two others a soredium field (see below) was observed, and from one of these a primordium (see below) developed. However, it is impossible to draw conclusions about the effect of carbohydrate type in the medium as the contamination rate was very uneven (Table 3) and there were only a few soredia developing to these stages. The mycobiont cultures formed numerous lateral branches (Fig. 2c) and encircled algal cells (Fig. 2d). A well-developed so-called soredium field (Schuster 1985, Stocker-Wörgötter and Türk 1988), was observed that consisted of a layer of undifferentiated algal-fungal tissue after two–three months of inoculation. At the beginning it consisted of a mass of predominantly algal cells with a few interwoven fungal hyphae (Fig. 2e). If there were more soredia sown on a plate, they usually fused into one mass/tissue at this stage. Gradually, the network of hyphae became denser and the mycobiont started to dominate either in parts of the soredium field (Fig. 2f) or over the whole tissue at once (Fig. 2g). In the next stage, a so-called primordium appeared; the mycobiont enclosed the algal cells inside the tissue, thus forming the basis of thallus stratification (Fig. 2h). Rather than distinct phases, the development was a continuum. The various stages could be observed simultaneously even within one well (Fig. 2i). Some of the soredia reached the primordium phase very quickly, the soredium field phase being very short. Others remained in the soredium field phase for a very long time and did not enter the primordium

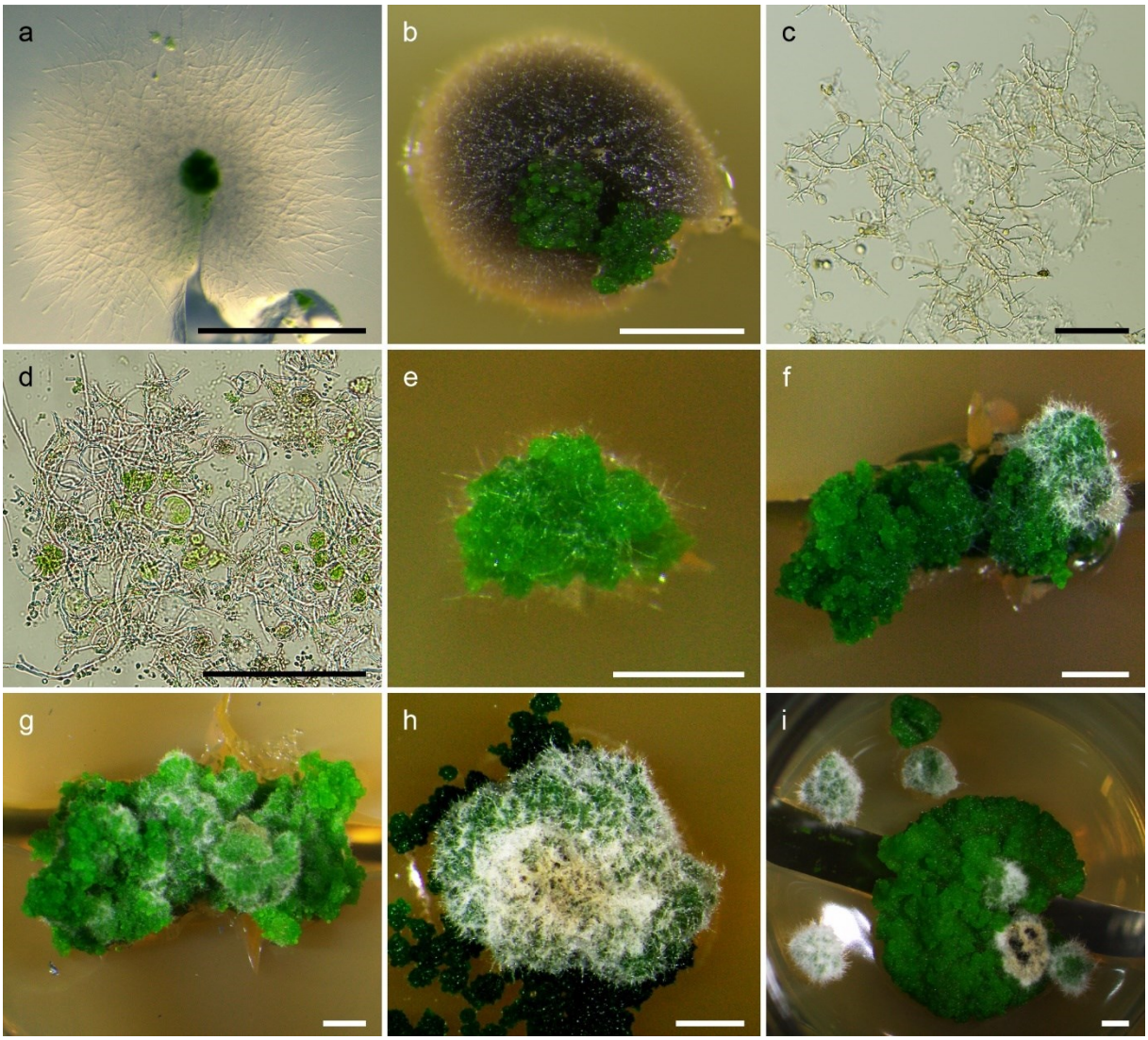

Figure 2 Development of soredia in vitro: **a** Arachnoid stage, after one month of culturing; **b** No interaction between the mycobiont and photobiont; **c** Frequent lateral branching with short internodes of the mycobiont hyphae; **d** Mycobiont hyphae encircling photobiont cells; **e** Soredium field, a mass of predominantly algal cells with a few interwoven fungal hyphae; **f** Soredium field, mycobiont dominates in a part; **g** Soredium field, mycobiont takes over; **h** Primordium, mycobiont forms a superficial layer enclosing the photobiont inside; **i** Different stages of soredium field and a primordium developing on a single plate. Scale bars represent 1 mm (**a, b, e-i**) or 100 µm (**c, d**)

phase during the course of our experiment. This variation was observed even though all cultures were kept in the same conditions. No further development was observed on agar media even months later. After reaching the stage of primordium, no further development occurred. Although a layer of mycobiont tissue was formed on the surface it still consisted of loose hyphae with aerial hyphal strands sticking out (Fig. 3a) and thus the cultures did not form a cortical layer and no podetia or squamules were observed. After the soredium field phase was transferred onto soil or sandstone, the mycobiont started to take over. It quickly increased the network of hyphae within the tissue (Fig. 3b) and fixed it to the substrate with hyphal strands resembling rhizines (Fig. 3c). A layer of only

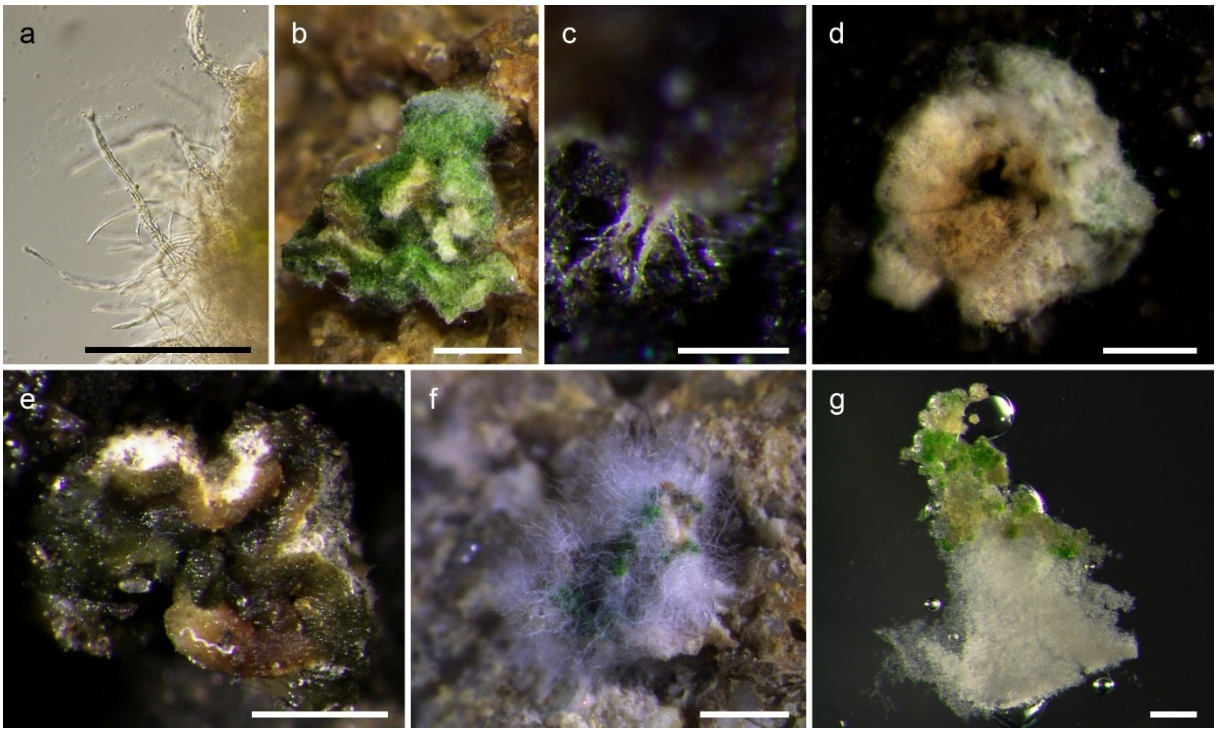

Figure 3 Development of soredia in vitro: **a** Superficial mycobiont layer, strands of aerial hyphae stick out; **b-g** Development on soil: **b** Mycobiont quickly multiplied the network of hyphae within the tissue; **c** Rhizine-like structures fasten the developing soredium to its substrate; **d** Mycobiont layer completely enclosed the photobiont quickly, the figure shows primordium in Fig. 2h two weeks after transfer to soil; **e** Horizontal cut of primordium shown in Fig. 3d, photobiont layer enclosed by a mycobiont layer; **f** Strands of hyphae protruding in all directions after re-wetting; **g** Loose white medullary tissue formed below the mycobiont-photobiont interaction layer. Scale bars represent 1 mm (**b, d-f**) or 200 µm (**a, c, g**)

mycobiont was formed on the substratum, enclosing the inner photobiont layer (Fig. 3d, e). In contrast to agar media, the primordium phase was reached on all plates with natural substrata within the first month after the transfer. However, as on agar media, the cultures still lacked cortical structure in the strict sense. After each rewetting the mycobiont formed strands of hyphae protruding in all directions (Fig. 3f), thus colonizing the surrounding substratum. In some of them, formation of a white loose medullary tissue could also be observed (Fig. 3g). However, we did not observe any podetia or squamules.

4 Discussion

4.1 Isolation success

Lichen vegetative propagules, such as soredia and isidia, have been shown to be highly viable in laboratory testing (Buldakov 2010), resynthesis experiments (StockerWörgötter and Türk 1988) and transplantation experiments (Ott 1987). Here we show that soredia are also a good source of mycobiont cultures. Mycobionts were obtained from 88% of the soredia that were not contaminated or overgrown by other fungi. From the remaining 12%, only photobionts grew. In contrast, isolation

from spores and thallus fragments is, to a great extent, hindered by problems with obtaining spores discharge, inducing germination and the failure of thallus fragments to grow (Crittenden et al. 1995). In our experiments, both symbionts often grew together but their separation by subculturing was not difficult. Contamination remains the biggest problem but we found for soredia that it is comparable to other isolation methods (Crittenden et al. 1995). Soredia culturing thus offers a straightforward and effective approach to the isolation of lichen mycobionts. Armaleo and May (2009) used soredia of *C. grayi* to obtain cultures that were the basis for genomes sizing. We believe that soredia culturing would facilitate studies, such as the recognition of signalling, secondary metabolites production, or whole genome sequencing.

4.2 Soredia development

Development of soredia in culture was comparable to the development of soredia in the natural environment (Schuster et al. 1985, Stocker-Wörgötter and Türk 1988, 1989). The developmental series appears to be universal, including (1) arachnoidal stage, (2) soredium field, (3) primordium and (4) thalline stage (Schuster 1985, Stocker-Wörgötter and Türk 1989, Stocker-Wörgötter 1991, see below). The soredium germinates into a loose arachnoid mycelium, the symbiotic partners thus come apart first (Fig. 2a, StockerWörgötter and Türk 1988). Interestingly, isidia, which have stratified thalline structure, also disintegrate at the beginning of their development (Schuster 1985). This fact implies that after a vegetative propagule germinates, the partners need to recognize each other anew before further development. Thus, the initial processes are analogous to reestablishment of the symbiosis de novo from mycobiont spores and photobiont cells (Athukorala et al. 2014). Although the molecular mechanisms of recognition are still largely unknown, it is clear that a complex pre-contact signalling is involved (Meeβen and Ott 2013). Initial steps of this signalling lead to release of specific polyols by the alga, i.e. ribitol in the case of *Asterochloris* (Richardson et al. 1968). Ribitol is not only the source of carbohydrates for the mycobiont but is probably the transformation signal that triggers lichenization (Ahmadjian 1993b, Meeβen et al. 2013). At the end of the pre-contact signalling, morphological changes in the mycobiont are induced; the hyphae grow forming numerous lateral branches with short internodes and encircle the photobiont (Fig. 2c and d; Athukorala et al. 2014, Joneson and Lutzoni 2009). However, this response has also been observed in co-cultures of certain incompatible partners (Ahmadjian and Jacobs 1981, Meeβen and Ott 2013), indicating low specificity of the pre-contact signalling. The next stage of the development is an undifferentiated mass of mingling symbionts that was termed a soredium field by Schuster (1985) (Fig. 2e-g). Soredium-like stages have also been reported from de-novo resyntheses (Galun and Garty 1988; 1995), even in incompatible partners (Ahmadjian et al. 1980, Galun and Garty 1988, Guzow-Krzemińska and Stocker-Wörgötter 2013). However, only under compatible combinations is the relative growth of the symbionts gradually balanced during this stage (Fig. 2f-i; Galun 1988, StockerWörgötter and Türk 1989). This leads to turning the soredium field into a primordium. A primordium (Fig. 2h and 3d) exhibits stratification; most importantly a layer of dense fungal network is formed on its surface enclosing the photobiont inside (Fig. 3e). In some cases, photobiont cells are continuously organized into a layer (Fig. 3e) and a loose white medullary tissue is formed below (Fig. 3g) from hyphae already in the primordium. In our experiments, the superficial mycobiont-only layer has a cottony appearance with long strands of aerial hyphae (Fig. 3a, also in Stocker-Wörgötter and Türk 1988). Although this layer is, for sure, the basis for the cortex, we think it cannot be termed as such (cf. Stocker-Wörgötter and Türk 1988.) until it develops the typical cortical tissue structure composed of tightly adhering hyphae (Büdel and Scheidegger 2008). Thus, the primordia in our

experiments did not develop the cortex *sensu stricto*. On contrary, after each rewetting the superficial aerial hyphae expanded, enlarging the primordium and colonizing more substratum (Fig. 3f). In contrast to previous studies (Ahmadjian 1966, StockerWörgötter and Türk 1988, Stocker-Wörgötter 1995) no further structures, anatomical (cortex) or morphological (squamules or podetia), developed in our experiments, so the thalline stage was not reached. Obviously, the development of these structures, as well as reproductive structures, is not a question of compatibility but of environmental factors. The use of soil substrata and alternation of wetting and drying cycles are considered crucial in this aspect (Ahmadjian 1966, Jahns 1993, Stocker-Wörgötter 1995, Zorer et al. 1997). Both were tried in our experiments but still the development did not proceed. Thus, the conditions for further development remain poorly understood and might involve environmental stresses other than drying, for example night temperature drops, or air movement as a mechanical stimulus, could be important. In conclusion, we have shown that the development of soredia of *C. fimbriata* follows the same pattern as described previously (Stocker-Wörgötter 1995), also for other species, e. g. *Peltigera didactyla, Hypogymnia physodes* and *Physcia tenella* (Schuster et al. 1985, Stocker-Wörgötter and Türk 1988, 1989). The same developmental stages as described here for soredia were observed in de-novo lichen resynthesis from spores (see Zorer et al. 1997 for C. fimbriata in vitro and Galun and Garty 1988 for *Xanthoria parietina* in situ). Thus, our observations can serve as a reference-frame for studies of compatibility of the mycobiont with diverse photobionts. Compatibility of the partners is not disproved by the lack of formation of advanced morphological structures, as long as the primordium stage is formed.

4.3 Soredia-associated fungi

Considering the limited number of isolates obtained we do not mean to give an exhaustive list of associated fungi but rather to look at the ecological groups they represent and indicate ecological consequences of such associations. There are two possible causes of the association of other fungi with lichen soredia. First, they might be spores, conidia or other diaspores originating from fungi present in the surroundings of the lichen. This is probably the case of SOR12d3 matching *Lachnellula pulverulenta*, which forms fruit bodies on pine needles. Second, they might be derived from the interior of the lichen thallus. The hyphae that form soredia are of medullary origin (Darbishire 1927, Lallemant 1972). During the morphogenesis some of the numerous symptomless fungi present within the lichen thallus (e.g., Petrini et al. 1990, U'Ren et al. 2010, Honegger 2012) might be accidentally incorporated. The functional relationship of most of the endothallic fungi to their host is not known (U'Ren et al. 2010, Chagnon et al. 2016). Some are symptomless stages of strictly lichen-associated (lichenicolous) fungi (Oberwinkler 2017, Tuovinen et al. 2019) or other fungi with multiple ecological niches (Honegger 2012, Selosse et al. 2018). Others may be just inactive diaspores accidentally trapped within the thallus (Hawksworth and Grube 2020). For the latter two groups, vegetative propagules may be the only means of leaving the thallus and proceeding with their life cycle. Majority of the fungi we found in association with soredia can be divided into three categories; firstly, fungi previously isolated from pine trees (SOR8d2, *Cystobasidium pinicola* SOR12d2 and *Pseudocamaropycnis pini* SOR13b1 known from pine sapwood, xylem and needles, respectively), secondly, fungi previously found in the soil (SOR8a2, SOR11b6, SOR12b1, SOR12c3) and thirdly, fungi that did not match any sequences deposited in GenBank (SOR7c1, SOR11c5, SOR11d6). Considering the current stage of knowledge, it is impossible to say for the former two groups whether they come from the surroundings or the interior of the thalli. The last group may be strictly lichen-associated, but we should avoid drawing conclusions before more is known about them. Spribille et al. (2016)

introduced cystobasidiomycetous yeasts as close and specific associates of lichens, even claiming them obligatory constituents of the lichen cortex. While mainly yeasts of Cyphobasidiales were found in Parmeliaceae lichens (Spribille et al. 2016), Microsporomycetaceae and another, yet undescribed, family-level lineage of Cystobasidiomycetes were found in a wide range of *Cladonia* species (Černajová and Škaloud 2019). This specificity was opposed by Mark et al. (2020) who found certain genotypes of Cystobasidiomycetes in several lichens and also single lichen species in association with various lineages of the yeast. They also anticipated yeast multiplicity in a thallus due to frequent mixed signal in Sanger sequencing chromatograms. In the present study we confirm this hypothesis; isolates of Cystobasidiomycetes yeast belonging to three distantly related lineages (Fig. 1) were obtained from a single thallus. This multiplicity indeed implies very limited specificity of the lichen-yeast association. It also suggests that lichens are commonly inhabited by diverse cystobasidiomycetous yeasts, contrarily to Lendemer et al. (2019) who found no evidence for such ubiquity. However, it is worth remembering that these yeast-like fungi occur on other hosts where they thrive on released carbohydrates and nutrients from tree leaves (Richardson et al. 1985, Richardson and Dowding 1988), and in the lichen context could be using carbohydrates released by dried lichens upon rewetting. Regardless of their link to the host, whether they come from the surroundings and are attached at the surface, or come from inside of the lichen thallus and are incorporated within the soredium, it is apparent that other fungi are capable of co-dispersal with lichen soredia. Vertical transmission of endophytic fungi is also known in plants, for example grasses and forbs (White et al. 1993, Hodgson et al. 2014). It has also been shown that whole communities of bacteria are dispersed with lichen vegetative propagules (Aschenbrenner et al. 2014). At present, we can only speculate about consequences of such co-dispersal. Aschenbrenner et al. (2014) showed that bacterial communities on isidioid soredia of *Lobaria pulmonaria* are similar in composition to those on the original thalli and suggested that the newly developing thallus thus does not depend on de novo recruitment of bacteria, which may provide many essential functions to the lichen holobiont (Grube et al. 2015, Cernava et al. 2017). On the other hand, it has been suggested that lichen thalli host plant pathogens, including viruses and bacteria (Petrzik et al. 2014, Vilhelmsson et al. 2016). The vegetative propagules of lichens could be the way of their transmission. Here, we present evidence that other fungi are co-dispersed with lichen diaspores. The extent to which this co-dispersal influences life histories of both the lichen and the fungus remains to be explored.

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References

Ahmadjian V. (1966) Artificial reestablishment of the lichen *Cladonia cristatella*. Science 151: 199-201.

Ahmadjian V. (1993)a The lichen photobiont: What Can It Tell Us about Lichen Systematics? Bryologist 96: 310-313.

Ahmadjian V. (1993)b The Lichen Symbiosis. John Wiley & Sons, New York, 266 pp.

Ahmadjian V., Jacobs J. B. (1981) Relationship between fungus and alga in the lichen *Cladonia cristatella* Tuck. Nature 289: 169-172.

Ahmadjian V., Russel L. A. & Hildreth K. C. (1980) Artificial re-establishment of lichens. I. Morphological interactions between the phycobiont of different lichens and the mycobionts of *Cladonia cristatella* and *Lecanora chrysoleuca*. Mycologia 72: 73-89.

Armaleo D. & May S. (2009) Sizing the fungal and algal genomes of the lichen *Cladonia grayi* through quantitative PCR. Symbiosis 49: 43-51.

Armstrong R. A. (1987) Dispersal in a population of the lichen *Hypogymnia physodes*. Environmental and Experimental Botany 27: 357-363.

Armstrong R. A. (1990) Dispersal, establishment and survival of soredia and thallus fragments of the lichen, *Hypogymnia physodes* (L.) Nyl. New Phytologist 114: 239-245.

Aschenbrenner I. A., Cardinale M., Berg G. & Grube M. (2014) Microbial cargo: do bacteria on symbiotic propagules reinforce the microbiome of lichens? Environmental Microbiology 16: 3743-3752.

Athukorala S. N. P., Huebner E. & Piercey-Normore M. D. (2014) Identification and comparison of the 3 early stages of resynthesis for the lichen *Cladonia rangiferina*. Canadian Journal of Microbiology 60: 41-52.

Belinchón R., Yahr R. & Ellis C. J. (2015) Interactions among species with contrasting dispersal modes explain distributions for epiphytic lichens. Ecography 37: 762-768.

Boch S., Prati D., Werth S., Rüetschi J. & Fischer M. (2011) Lichen endozoochory by snails. PlosOne 6: e18770.

Büdel B. & Scheidegger C. (2008) Thallus morphology and anatomy. In: Nash III. TH (Ed.) Lichen Biology, 2nd edition, Cambridge University Press, New York, pp. 40-68.

Buldakov M. S. (2010) Intraspecific variation in the viability of soredia in *Hypogymnia physodes* (L.) Nyl. (Ascomycota: Lecanorales). Russian Journal of Ecology 41: 211-217.

Cardós J. L. H, Prieto M., Jylhä M., Aragón G., Molina M. C., Martínez I. & Rikkinen J. (2019) A case study on the re-establishment of the cyanolichen symbiosis: where do the compatible photobionts come from? Annals of Botany 124: 379-388.

Castresana J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540-552.

Černajová I. & Škaloud P. (2019) The first survey of cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. nov. Fungal Biology 123: 625-637.

Cernava T., Erlacher A., Aschenbrenner I. A., Krug L., Lassek C., Riedel K., Grube M. & Berg G. (2017) Deciphering functional diversification within the lichen microbiota by meta-omics. Microbiome 5: 82.

Chagnon P.-L., U'Ren J. M., Miadlikowska J., Lutzoni F. & Arnold A. E. (2016) Interaction type influences ecological network structure more than local abiotic conditions: evidence from endophytic

and endolichenic fungi at a continental scale. Oecologia 180: 181–191.

Crittenden P. D., Hawksworth D. L. & Campbell F. S. (1995) Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. New Phytologist 130: 267-297.

Crous P. W., Braun U., Schubert K. & Groenewald J. Z. (2007) Delimiting *Cladosporium* from morphologically similar genera. Studies in Mycology 58: 33-56.

Crous P. W. & Groenewald J. Z. (2016) They seldom occur alone. Fungal Biology 120: 1392-1415.

Crous P. W., Schubert K., Braun U., de Hoog G. S., Hocking A. D., Shin H. D. & Groenewald J. Z. (2007) Opportunistic, human-pathogenic species in the Herpotrichiellaceae are phenotypically similar to saprobic or phytopathogenic species in the Venturiaceae. Studies in Mycology 58: 185-217.

Crous P. W., Wingfield M. J., Burgess T. I., Hardy G. S. J., Crane C., Barrett S. et al. & Groenewald J. Z. (2016) Fungal Planet description sheets: 469–557. Persoonia 37: 218-403.

Cubero O. F., Crespo A., Fatehi J. & Bridge P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243-249.

Darbishire O. V. (1927) The soredia of *Peltigera erumpens* Wain. and *P. scutata* Kbr. Transactions of the British Mycological Society 12: 52-70.

Darriba T., Taboada G. L., Doallo R. & Posada D. (2012) jModelTest2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Doilom M., Dissanayake A. J., Wanasinghe D. N., Boonmee S., Liu J.-K., Bhat D. J., Taylor J. E., Bahkali A. H., McKenzie E. H. C. & Hyde K. D. (2017) Microfungi on Tectona grandis (teak) in Northern Thailand. Fungal Diversity 82: 107-182.

Frank J., Crous P. W., Groenewald J. Z., Oertel B., Hyde K. D., Phengsintham P. & Schroers H. J. (2010) *Microcyclospora* and *Microcyclosporella*: novel genera accommodating epiphytic fungi causing sooty blotch on apple. Persoonia 24: 93-105.

Furuya N., Takashima M. & Shiotani H. (2012) Reclassification of citrus pseudo greasy spot causal yeasts, and a proposal of two new species, *Sporobolomyces productus* sp. nov. and *S. corallinus* sp. nov. Mycoscience 53: 261-269.

Galun M. (1988) Lichenization. In: Galun M. (Ed.) CRC Handbook of Lichenology, Vol. 2. CRC Press, Boca Raton, pp. 153-169.

Galun M. & Garty J. (1988) Soredia formation of compatible and incompatible lichen symbionts. In: Scannerini S., Smith D., Bonfante-Fasolo P. & Gianinazzi-Pearson V. (Eds.) Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. NATO ASI Series (Series H: Cell Biology), vol 17. Springer, Berlin, pp. 207-218.

Gardes M. & Bruns T. (1993) ITS primers with enhanced specificity for Basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Grube M., Cernava T., Soh J., Fuchs S., Aschenbrenner I. A., Lassek C., Wegner U., Becher D., Riedel K., Sensen C. W. & Berg G. (2015) Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. ISME Journal 9: 412-424.

Guzow-Krzemińska B. & Stocker-Wörgötter E. (2013) In vitro culturing and resynthesis of the mycobiont *Protoparmeliopsis muralis* with algal bionts. Lichenologist 45: 65-76.

Harmata K. & Olech M. (1991) Transect for aerobiological studies from Antarctica to Poland. Grana 30: 458-463.

Hauck M. & Zöller T. (2003) Copper sensitivity of soredia of the epiphytic lichen *Hypogymnia physodes.* Lichenologist 35: 271-274.

Hawksworth D. L. & Grube M. (2020) Lichens redefined as complex ecosystems. New Phytologist 227: 1281-1283.

Hodgson S., de Cates C., Hodgson J., Morley N. J., Sutton B. C. & Gange A. C. (2014) Vertical transmission of fungal endophytes is widespread in forbs. Ecology and Evolution 4: 1199-1208.

Honegger R. (2012) The symbiotic phenotype of lichen-forming Ascomycetes and their endo- and epibionts. In: Hock B. (Ed.) The Mycota. IX. Fungal Associations, 2nd edition. Springer, Berlin, pp. 287-340.

Hosoya T., Sasagawa R., Hosaka K., Gi-Ho S., Hirayama Y., Yamaguchi K., Toyama K. & Kakishima M. (2010) Molecular phylogenetic studies of Lachnum and its allies based on the Japanese material. Mycoscience 51: 170-181.

Jahns H. M. (1993) Culture experiments with lichens. Plant Systematics and Evolution 187: 145-174.

Joneson S. & Lutzoni F. (2009) Compatibility and thigmotropism in the lichen symbiosis: A reappraisal. Symbiosis 47: 109-115.

Katoh K., Rozewicki J. & Yamad K. D. (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 18: 1-7.

Kon Y. & Ohmura Y. (2010) Regeneration of juvenile thalli from transplanted soredia of *Parmotrema clavuliferum* and *Ramalina yasudae.* Bulletin of the National Museum of Nature and Science, Series B 36**:** 65-70.

Lallemant R. (1972) Etude de la formation des sorédies chez le Discolichen *Buellia canescens* (Dicks.) D. Notrs. Bulletin de la Société Botanique de France 119: 463-475.

Lendemer J. C., Keepers K. G., Tripp E. A., Pogoda C. S., McCain C. M. & Kane N. C. (2019) A taxonomically broad metagenomic survey of 339 species spanning 57 families suggests cystobasidiomycete yeasts are not ubiquitous across all lichens. American Journal of Botany 106: 1090-1095.

Lorentsson S. & Mattsson J.-E. (1999) New reports of soredia dispersed by ants, *Formica cunicularia*. Lichenologist 31: 204-207.

Marčiulynas A., Marčiulynienė D., Lynikienė J., Gedminas A., Vaičiukynė M. & Menkis A. (2020) Fungi and Oomycetes in the Irrigation Water of Forest Nurseries. Forests 11: 459.

Mark K., Laanisto L., Bueno C. G., Niinemets U., Keller C. & Scheidegger C. (2020) Contrasting co-occurrence patterns of photobiont and cystobasidiomycete yeast associated with common epiphytic lichen species. New Phytologist 227: 1362-1375.

Marthinsen G., Rui S. & Timdal E. (2019) OLICH: A reference library of DNA barcodes for Nordic lichens. Biodiversity Data Journal 7: 1-146.

Meeβen J., Eppenstein S. & Ott S. (2013) Recognition mechanisms during the pre-contact state of lichens: Influence of algal exudates and ribitol on the response of the mycobiont of *Fulgensia bracteata*. Symbiosis 59: 131-143.

Meeβen J. & Ott S. (2013) Recognition mechanisms during the pre-contact state of lichens: I. Mycobiont-photobiont interactions of the mycobiont of *Fulgensia bracteata*. Symbiosis 59: 121-130.

Miller M. A., Pfeiffer W. & Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop, 14 Nov 2010: 1-8.

Oberwinkler F. (2017) Yeasts in Pucciniomycotina. Mycological Progress 16: 831-856.

Ott S. (1987) Differences in the developmental rates of lichens. Annales Botanici Fennici 24: 385-393.

Parrent J. L. & Vilgalys R. (2007) Biomass and compositional responses of ectomycorrhizal fungal hyphae to elevated CO2 and nitrogen fertilization. New Phytologist 176: 164-174.

Petrini O., Hake U. & Dreyfuss M. M. (1990) An analysis of fungal communities isolated from fruticose lichens. Mycologia 82: 444-451.

Petrzik K., Vondrák J., Barták M., Peksa O. & Kubešová O. (2014) Lichens - a new source or yet unknown host of herbaceous plant viruses? European Journal of Plant Pathology 138: 549-559.

Réblová M., Hubka V., Thureborn O., Lundberg J., Sallstedt T., Wedin M. & Ivarsson M. (2016) From the tunnels into the treetops: new lineages of black yeasts from biofilm in the Stockholm metro system and their relatives among ant-associated fungi in the Chaetothyriales. PLoS One 11: e0163396.

Richardson D. H. S, Hill D. J. & Smith D. C. (1968) Lichen physiology. XI. The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts. New Phytologist 67: 469-486.

Richardson D. H. S. & Dowding P. (1988) The processes involved in the leaching of leaves under non-polluted conditions and the influence of leachates on leaf-surface micro-organisms. In: Cape N. & Mathy P. (Eds.) Scientific Basis of Forest Decline Symptomology. Air pollution Research Report 15: 133-148.

Richardson D. H. S., Dowding P. & Ni Lamhna E. (1985) Monitoring air quality with leaf yeasts. Journal of Biological Education 19: 299-303.

Rikkinen J., Oksanen I. & Lohtander K. (2002) Lichen guilds share related cyanobacterial symbionts. Science 297: 357.

Rolshausen G., Dal Grande F., Sadowska-Deś A. D., Otte J. & Schmitt I. (2018) Quantifying the climatic niche of symbiont partners in a lichen symbiosis indicates mutualist-mediated niche expansions. Ecography 41: 1380-1392.

Ronquist F., Teslenko M., van der Mark P., Ayres D., Darling A., Hohna S., Larget B., Liu L., Suchard M. A. & Huelsenbeck J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539-542.

Rosales-Castillo J. A., Oyama K., Vázquez-Garcidueñas M. S., Aguilar-Romero R., García-Oliva F. & Vázquez-Marrufo G. (2018) Fungal community and ligninolytic enzyme activities in *Quercus deserticola* Trel. litter from forest fragments with increasing levels of disturbance. Forests 9: 11.

Scheidegger C. (1995) Early development of transplanted isidioid soredia of *Lobaria pulmonaria* in an endangered populaton. Lichenologist 27: 361-374.

Schuster G. (1985) Die Jugendentwicklung von Flechten. Ein Indikator fur Klimabedingungen und Umweltbelastung. Bibliotheca lichenologica 20: 1-206.

Schuster G., Ott S. & Jahns H. M. (1985) Artificial cultures of lichens in the natural environment. Lichenologist 17: 247-253.

Selosse M.-A., Schneider-Maunoury L. & Martos F. (2018) Time to re-think fungal ecology? Fungal ecological niches are often prejudged. New Phytologist 217: 968-972.

Sette L. D., Passarini M. R. Z., Rodrigues A., Leal R. R., Simioni K. C. M., Nobre F. S., Brito B. R., Rocha A. J. & Pagnocca F. C. (2010) Fungal diversity associated with Brazilian energy transmission towers. Fungal Diversity 44: 53-63.

Spribille T., Tuovinen V., Resl P., Vanderpool D., Wolinski H., Aime M. C., Schneider K., Stabentheiner E., Toome-Heller M., Thor G., Mayrhofer H., Johannesson H. & McCutcheon J. P. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353: 488-492.

Steinová J., Škaloud P., Yahr R., Bestová H. & Muggia L. (2019) Reproductive and dispersal strategies shape the diversity of mycobiontphotobiont association in *Cladonia* lichens. Molecular Phylogenetics and Evolution 134: 226-237.

Stocker-Wörgötter E. (1991) Thallus formation of two cyanobacterial lichens: *Peltigera didactyla* and *Peltigera praetextata*, under laboratory conditions. Bulletin de la Société botanique de France 138, Lettres Botaniques 3: 179-187.

Stocker-Wörgötter E. (1995) Experimental cultivation of lichens and lichen symbionts. Canadian Journal of Botany 73: S579-S589.

Stocker-Wörgötter E. (2001) Experimental lichenology and microbiology of lichens: Culture experiments, secondary chemistry of cultured mycobionts, resynthesis, and thallus morphogenesis. Bryologist 104: 576-581.

Stocker-Wörgötter E. & Hager A. (2008) Appendix: culture methods for lichens and lichen symbionts. In: Nash III. TH (Ed.) Lichen Biology, 2nd edition. Cambridge University Press, New York, pp. 353-363.

Stocker-Wörgötter E. & Türk R. (1988) Culture of the cyanobacterial lichen *Peltigera didactyla* from soredia under laboratory conditions. Lichenologist 20: 369-375.

Stocker-Wörgötter E. & Türk R. (1989) Artificial cultures of the cyanobacterial lichen *Peltigera didactyla* (Peltigeraceae) in the natural environment. Plant Systematics and Evolution 165: 39-48.

Stubbs C. S. (1995) Dispersal of soredia by the oribatid mite, *Humerobater arborea*. Mycologia 87: 454-458.

Trembley M. L., Ringli C. & Honegger R. (2002) Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. Mycological Research 106: 768-776.

Tuovinen V., Ekman S., Thor G., Vanderpool D., Spribille T. & Johannesson H. (2019) Two basidiomycete fungi in the cortex of wolf lichens. Current Biology 29: 476-483.

U'Ren J. M. & Arnold A. E. (2016) Diversity, taxonomic composition, and functional aspects of fungal communities in living, senesced, and fallen leaves at five sites across North America. PeerJ 4: e2768.

U'Ren J. M., Lutzoni F., Miadlikowska J. & Arnold A. E. (2010) Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microbial Ecology 60: 340-353.

van Nieuwenhuijzen E. J., Houbraken J. A. M. P., Punt P. J., Roeselers G., Adan O. C. G. & Samson R. A. (2017) The fungal composition of natural biofinishes on oil-treated wood. Fungal Biology and Biotechnology 4: 2.

Vančurová L., Muggia L., Peksa O., Řídká T. & Škaloud P. (2018) The complexity of symbiotic interactions influences the ecological amplitude of the host: a case study in *Stereocaulon* (lichenized Ascomycota). Molecular Ecology 27: 3016-3033.

Vančurová L., Kalníková V., Peksa O., Škvorová Z., Malíček J., Moya P., Chytrý K., Černajová I. & Škaloud P. (2020) Symbiosis between river and dry lands: phycobiont dynamics on river gravel bars. Algal Research 51: 102062.

Valarmathi R. & Hariharan G. N. (2007) Soredial culture of *Dirinaria applanata* (Fee) Awasthi: Observations on developmental stages and compound production. Symbiosis 43: 137-142.

Vilgalys R. & Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238-4246.

Vilhelmsson O., Sigurbjörnsdóttir A., Grube M. & Höfte M. (2016) Are lichens potential natural reservoirs for plant pathogens? Molecular Plant Pathology 17: 143-145.

Vu D., Groenewald M., Szöke S., Cardinali G., Eberhardt U., Stielow B., de Vries M., Verkleij G. J. M., Crous P. W., Boekhout T. & Robert V. (2016) DNA barcoding analysis of more than 9000 yeast isolates contribute to quantitative thresholds for yeast species and genera delimitation. Studies in Mycology 85: 95-105.

Wang Q.-M., Yurkov A. M., Göker M., Lumbsch H. T., Leavitt S. D., Groenewald M., Theelen B., Liu X.-Z., Boekhout T. & Bai F.-Y. (2015) Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. Studies in Mycology 81: 149-189.

Werth S., Wagner H. H., Gugerli F., Holderegger R., Csencsics D., Kalwij J. M. & Scheidegger C. (2006) Quantifying dispersal and establishment limitation in a population of an epiphytic lichen. Ecology 87: 2037-2046.

White T. J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (Eds.) PCR Protocols: a guide to methods and applications. Academic Press, San Diego, pp. 315-322.

White Jr. J. F., Morgan-Jones G. & Morrow A. C. (1993) Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. Agriculture, Ecosystems & Environment 44: 13-37.

Yurkov A. M., Kachalkin A. V., Daniel H. M., Groenewald M., Libkind D., de Garcia V., Zalar P., Gouliamova D. E., Boekhout T. & Begerow D. (2015) Two yeast species *Cystobasidium psychroaquaticum f.a.* sp. nov. and *Cystobasidium rietchieii f.a*. sp. nov. isolated from natural environments, and the transfer of *Rhodotorula minuta* clade members to the genus *Cystobasidium*. Antonie van Leeuwenhoek 107: 173185.

Zorer R., Türk R. & Stocker-Wörgötter E. (1997) Resynthesis of the lichen *Cladonia fimbriata* from the axenic cultures of the isolated symbionts. In: Kappen L. (Ed.) New species and novel aspects in ecology and physiology of lichens. Bibliotheca Lichenologica 67: 85-89.

Paper 3

Lichens from the littoral zone host diverse Ulvophycean photobionts

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Abstract

Crustose Verrucariaceae lichens form a distinctive black belt on seashores all over the world. This lifestyle is apparently enabled by a specific set of photobionts. However, their diversity is understudied. We sampled these lichens from the northern Patagonian Pacific coast of Chile. Using molecular markers, we identified both mycobionts and photobionts. The lichens, belonging to the genus *Hydropunctaria* and to the *Wahlenbergiella* group, hosted solely Ulvophycean photobionts. *Pseudendoclonium submarinum* (Kornmanniaceae, Ulvales) was the most common, but representatives of other closely related, yet undescribed, lineages were also found. *Undulifilum symbioticum* gen. et sp. nov. is described within Kornmanniaceae based on culture morphology and DNA sequence data. Furthermore, the free-living macroscopic genus *Urospora* (Acrosiphoniaceae, Ulotrichales) is reported as a lichen photobiont for the first time and is the first of its kind in the order. These results indicate that undescribed algal diversity is waiting to be uncovered in seashore lichens.

Key index words: Chile, *Hydropunctaria*, intertidal rocks, *Pseudendoclonium*, symbiosis, *Undulifilum symbioticum* gen. et sp. nov., *Urospora*, *Verrucaria*

Abbreviations: ASW, artificial sea water medium; BI, Bayesian inference; BBM, Bold's Basal Medium; CAUP, Culture Collection of Algae of Charles University in Prague; CTAB, cetyltrimethylammonium bromide; MCMC, Markov Chain Monte Carlo; ML, maximum likelihood; Pi, parsimony informative; PP, posterior probability; PRC, Herbarium collection of the Charles University in Prague; SAG, Culture Collection of Algae at Goettingen University; SDSF, standard deviation of split frequencies; SPRI, solid phase reversible immobilization; V, variable positions

1. Introduction

The remarkable ability of lichens to tolerate abiotic stresses allows them to dominate various hostile habitats, most notably not only bare rocks and soil in arctic and alpine regions (Beckett et al. 2008) but also rocky seashores all over the world (Fletcher 1973, Brodo and Slone 2004). Some seashore lichen species can be found as low as the littoral zone, where they undergo periodic submersion or

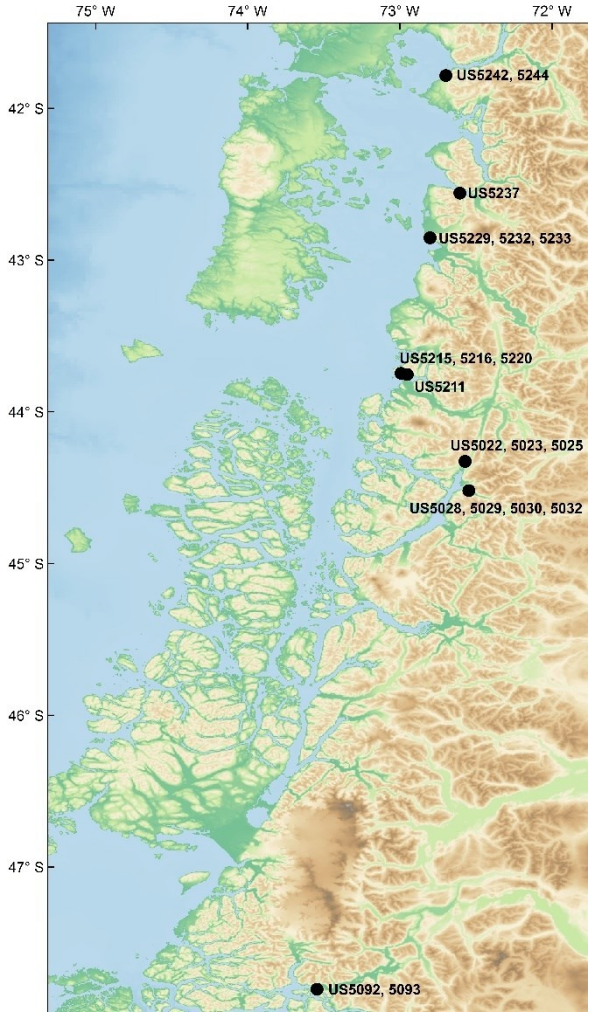

Figure 1 Map of sampling sites on the Northern Patagonian Pacific Coast in Chile. For details, see Table S1.

continual splashing by waves, acclimatizing to both inundation and exposure, associated with high solar radiation, desiccation, osmotic stress, and also wave action. They often form distinctly colored vertical zones. The so-called black belt on the littoral fringe, formed almost exclusively by black crustose Verrucariaceae lichen species, is so striking that it is often confused with oil contamination (Dobson 2014). The ecology of lichens is significantly influenced by their photobionts (Helms 2003, Peksa and Škaloud 2011). Expansion of the ecological niche of a lichen is often facilitated by its ability to switch to a photobiont adapted to specific environmental conditions (Ertz et al. 2018, Rolshausen et al. 2018, Vančurová et al. 2018). It has also been shown that the pool of adapted photobionts is shared by lichens with a similar ecology, regardless of their taxonomy (Rikkinen et al. 2002). It might be expected that the thriving of lichens on seashore rocks is enabled by a specific community of photobionts. General knowledge of lichen photobionts from coastal and seashore habitats is scarce and can be briefly summarized as follows. Watanabe et al. (1997) isolated and morphologically identified 13 Trebouxiophyceae photobionts along with one Ulvophycean, *Pseudendoclonioum arthropyreniae*, from lichens from the supralittoral zone. Studies of the littoral zone revealed photobionts from three eukaryotic classes: various "*Dilabifilum*" strains, *Halofilum ramosum*, *Paulbroadya petersii*, and species of *Pseudendoclonium* (Tschermak-Woess 1976, Thüs et al. 2011, Darienko and Proschold 2017, Gasulla et al. 2019) from Ulvophyceae; *Heterococcus caespitosus* (Parra and Redon 1977) from Xanthophyceae, and *Petroderma maculiforme* (Gueidain et al. 2011) from Phaeophyceae, but also cyanobacterial *Rivularia* spp. (Ortiz-Alvarez et al. 2015). In the present study, we focused on the diversity of photobionts of crustose Verrucariaceae lichens from the littoral zone at various sites in Chile. Using DNA sequencing of markers, we identified both the mycobionts and photobionts. Exclusively Ulvophycean photobionts were found, and *Pseudendoclonium submarinum* was the most common. We describe a new genus within the Kornamniaceae, Ulvales, and a filamentous alga from the Ulotrichales not previously known as a lichen photobiont.

2. Materials and Methods

2.1 Sampling

Lichens were collected in February 2019 on the Northern Patagonian Pacific Coast in Chile. Nineteen specimens from nine sites are included in this study (Fig 1, Table S1 in the Supporting Information). Collection data are shown in Table S1. Air-dried lichens were transported to the laboratory in paper bags. Afterward, they were stored in a refrigerator at 4°C until processed.

2.2 Photobiont culturing

About 2 months after the collection, thalli were cut with a sterile razor blade under a stereomicroscope, and about 60-µm pieces that visibly contained photobiont cells were extracted with a sterile needle and placed onto petri dishes with solid artificial sea water (ASW, Starr and Zeikus 1993). Thalli were kept at 16.5°C with 12:12 h light:dark regime. Isolated colonies were then transferred to liquid ASW. The identity of the cultures was confirmed by sequencing nuclear ITS rDNA (see below).

2.3 Sequencing and phylogenetic analyses

DNA was isolated using the CTAB protocol (Cubero et al. 1999), with an additional washing step with 96% ethanol, directly from pieces of the lichen thalli or from cultures. Specific primers were used to amplify the nuclear small subunit (nuSSU) and nuclear large subunit (nuLSU), and mitochondrial small subunit (mtSSU) rDNA genes of the mycobiont and nuclear SSU and ITS rDNA regions, and plastid rbcL gene of the photobiont. The primer pairs used were NS1 and 18L (Hamby et al. 1988) for fungal nuSSU; LR0R and LR6 (Vilgalys and Hester 1990) for fungal nuLSU; mrSSU1 and mrSSU3R (Zoller et al. 1999) for fungal mtSSU; newly designed 18S-Ulvo-F (5'-CCATGCATGT CTAAGTA-3', P. Škaloud, this study) and 18S-Ulvo-R (5'-ACCTTGTTACGACTTCWCCT-3', P. Škaloud, this study) for algal nuSSU; KlebsF (Škaloud and Rindi 2013) and ITS4 (White et al. 1990) for algal nuITS; and rbcL-203F and rbcL991R (Nelsen et al. 2011) for chloroplast rbcL. The PCR conditions are given in Table S2 in the Supporting Information. A negative control was used in each PCR run. PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter) and sequenced by Macrogen Europe, Amsterdam, the Netherlands, using the same primers. GenBank accession numbers of the newly obtained sequences are given in Table 1.

Datasets (see below) were aligned separately for each locus using MAFFT v.7 (Katoh et al. 2017), using the Q-INS-I method and manually checked. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and eliminated. Substitution models were estimated with JModelTest v. 2.1.4 (Darriba et al. 2012) using Bayesian Information Criterion and are given below.

The phylogenetic position of the mycobionts within the Verrucariaceae was verified based on nuSSU rRNA gene, nuLSU, and mtSSU rRNA gene. A dataset was created (Table S3 in the Supporting Information) to include representative taxa of all the main groups and lineages of the family Verrucariaceae following Gueidain et al. (2007), Savić et al. (2008) and Pérez-Ortega et al. (2018). Because, according to BLAST searches, our samples matched either the genus *Hydropunctaria* or the *Wahlenbergiella* group sensu Pérez-Ortega et al. (2010), the following taxa were also added: all nine currently recognized species of *Hydropunctaria* (Orange 2012, Spribille et al. 2020) and all taxa reported to belong to the *Wahlenbergiella* group (Gueidain et al. 2009), including *Mastodia tessellata* and five *Verrucaria* spp. from the Chilean coast (Pérez-Ortega et al. 2010). *Capronia* (Chaetothyriales)

Table 1 List of newly obtained sequences and their GenBank accession numbers

Sample code	Mycobiont	Genbank accessions			Photobiont	Genbank accessions		
		LSU	SSU	mtSSU		ITS	SSU	rbcL
US5022	*Hydropunctaria* group	OL342959	OL342977	OL342987	*Pseudendoclonium submarinum*	OL619283	OL342950	OL684554
US5023	*Hydropunctaria* group	OL342960	OL342978	OL342988	*Pseudendoclonium submarinum*	OL619284	OL342951	OL684555
US5025	*Wahlenbergiella* group	OL342961	-	OL342989	*Pseudendoclonium submarinum*	OL619285	-	-
US5028	*Hydropunctaria* group	OL342962	-	OL342990	*Pseudendoclonium aff. arthropyreniae*	OL619286	-	OL684556
US5029	*Hydropunctaria* group	OL342963	-	-	*Pseudendoclonium submarinum*	OL619287	-	-
US5030	*Hydropunctaria* group	OL342964	-	-	*Pseudendoclonium* sp.	-	OL342952	-
US5092	*Hydropunctaria* group	OL342965	-	-	*Undulifilum symbioticum*	OL619288	-	-
US5093	*Hydropunctaria* group	OL342966	-	OL342991	*Pseudendoclonium submarinum*	OL619289	-	-
US5211	*Wahlenbergiella* group	OL342967	OL342979	OL342992	*Undulifilum symbioticum*	OL619290	-	-
US5215	*Wahlenbergiella* group	OL342968	OL342980	OL342993	*Urospora* sp.	OL619291	OL342953	OL684557
US5216	*Wahlenbergiella* group	OL342969	-	OL342994	*Pseudendoclonium submarinum*	OL619292	OL342954	OL684558
US5220	*Wahlenbergiella* group	OL342970	OL342981	OL342995	*Undulifilum symbioticum*	OL619293	OL342955	-
US5229	*Wahlenbergiella* group	OL342971	OL342982	OL342996	*Pseudendoclonium submarinum*	OL619294	-	-
US5232H	*Wahlenbergiella* group	-	OL342983	OL342997	*Undulifilum symbioticum*	OL619295	-	-
US5232L	*Verrucaria cf. tessellatula*	OL342972	OL342984	OL342998	*Urospora* sp.	OL619296	OL342956	OL684559
US5232V	*Wahlenbergiella* group	OL342973	OL342985	OL342999	*Undulifilum symbioticum*	OL619297	OL342957	-
US5237	*Hydropunctaria* group	OL342974	-	OL343000	*Pseudendoclonium submarinum*	OL619298	-	-
US5242	*Wahlenbergiella* group	OL342975	-	OL343001	*Pseudendoclonium submarinum*	OL619299	-	-
US5244	*Wahlenbergiella* group	OL342976	OL342986	OL343002	*Urospora* sp.	OL619300	OL342958	OL684560

was used as the outgroup. The final concatenated alignment contained 1008 nuSSU rRNA gene positions, of which 200 were variable (V) and 118 parsimony informative (Pi), 930 nuLSU (308 V, 231 Pi) and 641 mtSSU rRNA gene (238 V, 180 Pi) positions, and consisted of 74 taxa, including our specimens. The selected substitution models were TIM1ef+I+G (gamma shape 0.489) for nuSSU rRNA gene, TIM3+I+G (0.641) for nuLSU, and TPM3uf+I+G (0.64) for mtSSU rRNA gene.

BLAST searches of our photobiont sequences matched taxa of either Ulotrichales or Ulvales, so we performed phylogenetic analyses of both orders. Datasets of Škaloud et al. (2018) were simplified so that all families, genera, and main lineages were represented (Tables S4, S5 in the Supporting Information). The phylogeny of Ulotrichales was based on nuclear SSU rRNA gene and ITS and chloroplast tufA, downloaded from GenBank. Additional *Urospora* species were included (Table S4) because BLAST searches of the samples US5215, 5232L, and 5244 matched various species of the

genus. Because the occurrence of *Urospora* within lichens was unexpected, DNA was isolated again from ethanol-surface sterilized pieces of thalli of these samples, and amplification and sequencing were repeated. Each time we obtained chromatograms with single distinct peaks. *Desmochloris molenhaueri* (Chlorocystidales), *Halochlorococcum moorei* (Oltmannsiellopsidales) and *Pseudoneochloris marina* (Ulvales) were used as the outgroup. The final Ulotrichales alignment contained 1729 nuSSU rRNA gene (198 V, 110 Pi), 519 nuITS (277 V, 220 Pi), and 790 tufA (388 V, 238 Pi) positions. Substitution models selected for Ulotrichales were K80+I+G (gamma shape 0.727) for nuSSU rRNA gene, TIM2ef+G (0.760) for ITS1, K80+I for 5.8S, TIM2ef+G (0.639) for ITS2 and F81+G (0.1530), TrN+I+G (0.509), and TIM2+I for the for the first, second, and third codon position of tufA, respectively.

The phylogeny of Ulvales was based on nuclear SSU rDNA, and chloroplast tufA and rbcL genes. *Neoclonium akinetum*, *Ulothrix zonata*, and *Sarcinofilum mucosum* (all Ulotrichales) were used as the outgroup. The final Ulvales alignment consisted of 1729 nuSSU rRNA gene (390 V, 308 Pi), 763 tufA (385 V, 284 Pi), and 1126 rbcL (329 V, 224 Pi) positions, and the substitution models selected were TrNef+I+G (0.501) for nuSSU rRNA gene, TPM3+G (0.250), HKY+G (0.340) and TIM2+G (0.480) for the first, second, and third codon position of the tufA gene, respectively, and JC+I, TPM3uf+I, and TVM+I for the first, second, and third codon position of the rbcL gene, respectively. All of our samples within Ulvales were placed in the family Kornmanniaceae, so an additional analysis of the family, based on nuSSU rRNA gene and nuITS, was performed. All taxa of the family (Darienko and Pröschold 2017, Škaloud et al. 2018) with available DNA sequence data were included. For taxa that are known to be both free-living and lichenized, a sequence from both strains was included, if available (Table S5). *Ctenocladus circinatus* (Ulvales) was used as the outgroup. The concatenated alignment consisted of 1021 nucleotides for the nuSSU rDNA gene (142 V, 86 Pi) and 468 nucleotides for the nuITS region (231 V, 187 Pi), and the substitution models were TrNef+I+G (0.63) for nuSSU rRNA gene, HKY+G (0.928) for nuITS1, K80+I for nu5.8S, and TPM2uf+G (0.627) for nuITS2.

Separate analyses of each marker gave congruent results for all the datasets, so they were concatenated. The phylogenetic trees were inferred by Bayesian Inference (BI) in MrBayes v. 3.2.6 (Ronquist et al. 2012) using partitioned datasets. Two parallel Monte Carlo Markov Chain (MCMC) runs, with one cold and three heated chains, were carried out. Trees and parameters were sampled every 100 generations. Convergence of the chains was verified by the convergent diagnostic of the potential scale reduction factor using the sump option, and it approached 1 in all cases. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). For Verrucariaceae, it was run for 26 million generations (SDSF 0.00211), for Ulotrichales for 5 million generations (SDSF 0.001949), for Ulvales for 6 million generations (SDSF 0.001406), and for Kornmanniaceae for 8 million generations (SDSF 0.00145). The first 25% of the trees were discarded as burn-in in each run. 50% majority rule consensus trees were obtained using the sumt option. Bootstrap analyses were performed by maximum likelihood (ML) using RAxML v. 8.2.12 (Stamatakis 2014). It consisted of 1000 rapid bootstrap inferences with automatic termination. Analyses were run on the CIPRES Science Gateway v. 3.3 web portal (Miller et al. 2010). The resulting trees were visualized using FigTree v. 1.4.3 (Rambaut 2016).

The associations between the mycobiont and photobiont were visualized with phytools::cophylo function in the free software R v. 4.1.0 (R Core Team 2021) using the option to rotate the nodes of both trees to optimize vertical matching of the tips (Revell 2012). For this purpose, simplified ML

trees that included only our samples and an outgroup (*Dermatocarpon miniatum* in the case of the mycobiont and *Desmochloris molenhaueri* in the case of the photobiont) were calculated in GARLI (Zwickl 2006).

3. Results

3.1 Phylogeny of mycobionts

The three loci phylogeny of the family Verrucariaceae (Fig. 2) placed our samples within the genus *Hydropunctaria* or within the *Wahlenbergiella* group. Samples US5022, 5023, 5028, 5029, 5030, 5092, 5093, and 5237 belonged to the genus *Hydropunctaria* with full Bayesian posterior probability (PP) support. The rest of the samples were placed in the *Wahlenbergiella* group with full support, but none matched the genus *Wahlenbergiella*, which has a basal position within the group (in accordance with Pérez-Ortega et al. 2010). Samples US5220, 5232H, 5232V, and 5229 formed a lineage with *Verrucaria cf. serpuloides* MAF-Lich 16296 (Pérez-Ortega et al. 2010), but with low support (PP = 0.64); samples US5211 and 5216 formed a sister lineage to the former, the relationship being fully supported; US5215, 5232L, and 5244 were related to *M. tessellata* with full support; US5242 formed a fully supported lineage with *Verrucaria* sp. MAF-Lich 16297 and *Verrucaria cf. degelii* MAF-Lich 16298 (Pérez-Ortega et al. 2010); and finally, the position of US5025 within the group is not clear (Fig. 2). The taxonomic identity of the mycobionts will be treated in more detail elsewhere.

3.2 Phylogeny of photobionts

The phylogeny of Ulotrichales based on nuSSU rRNA gene, ITS, and tufA (Fig. 3) placed our samples US5215, 5232L, and 5244 within the Acrosiphoniaceae, specifically within *Urospora* with full support. They form a lineage with *Urospora wormskioldii*, but with low support (PP = 0.65). *Urospora wormskioldii* and *Urospora penicilliformis* are virtually indistinguishable based on both ITS and the part of the nuSSU rRNA gene we used for the analysis (Lindstrom and Hanic 2005). The difference between our sequences and *U. wormskioldii* was 0–1 bp in nuSSU rRNA gene and 2–3 bp in ITS; and 1–2 bp in nuSSU rRNA gene and 2– 3 bp in ITS between our sequences and *U. penicilliformis*. We refrain from giving a species name to the *Urospora* photobionts for now.

The phylogeny of Ulvales based on nuSSU rRNA gene, tufA, and rbcL placed our samples within the Kornmanniaceae (Fig. S1 in the Supporting Information) with full support. Further phylogeny of the family based on nuSSU rRNA gene and ITS (Fig. 4) showed that most of our samples belonged in *Pseudendoclonium*. Specifically, samples US5022, 5023, 5025, 5029, 5093, 5216, 5229, 5237, and 5242 belonged to *P. submarinum*; US5030 also belonged to this genus, this position is not clear due to inability to amplify the ITS sequence; sample US5028 is related to *P. arthropyreniae*, with a difference of 17 bp in nuSSU rRNA gene in comparison to *P. arthropyreniae* SAG 467-2. Samples US5092, 5211, 5220, 5232H, and 5232V (Fig. 4) form a completely new lineage within the family, here referred to as *Undulifilum symbioticum*, gen. et sp. nov.

3.3 Photobiont morphology

The photobionts did not present any diagnostic characteristics that would allow identification within the thallus (Fig. 5). Cells of lichenized *Urospora* were irregularly spherical of variable size (6.2 × 5.5–11.5 × 10.1 µm), unevenly scattered within the thallus, forming vertical columns in some parts (Fig. 5a and b). *Pseudendoclonium* photobionts were organized in vertical columns in all specimens examined, a characteristic feature of the lichen genera *Hydropunctaria* and *Wahlenbergiella*

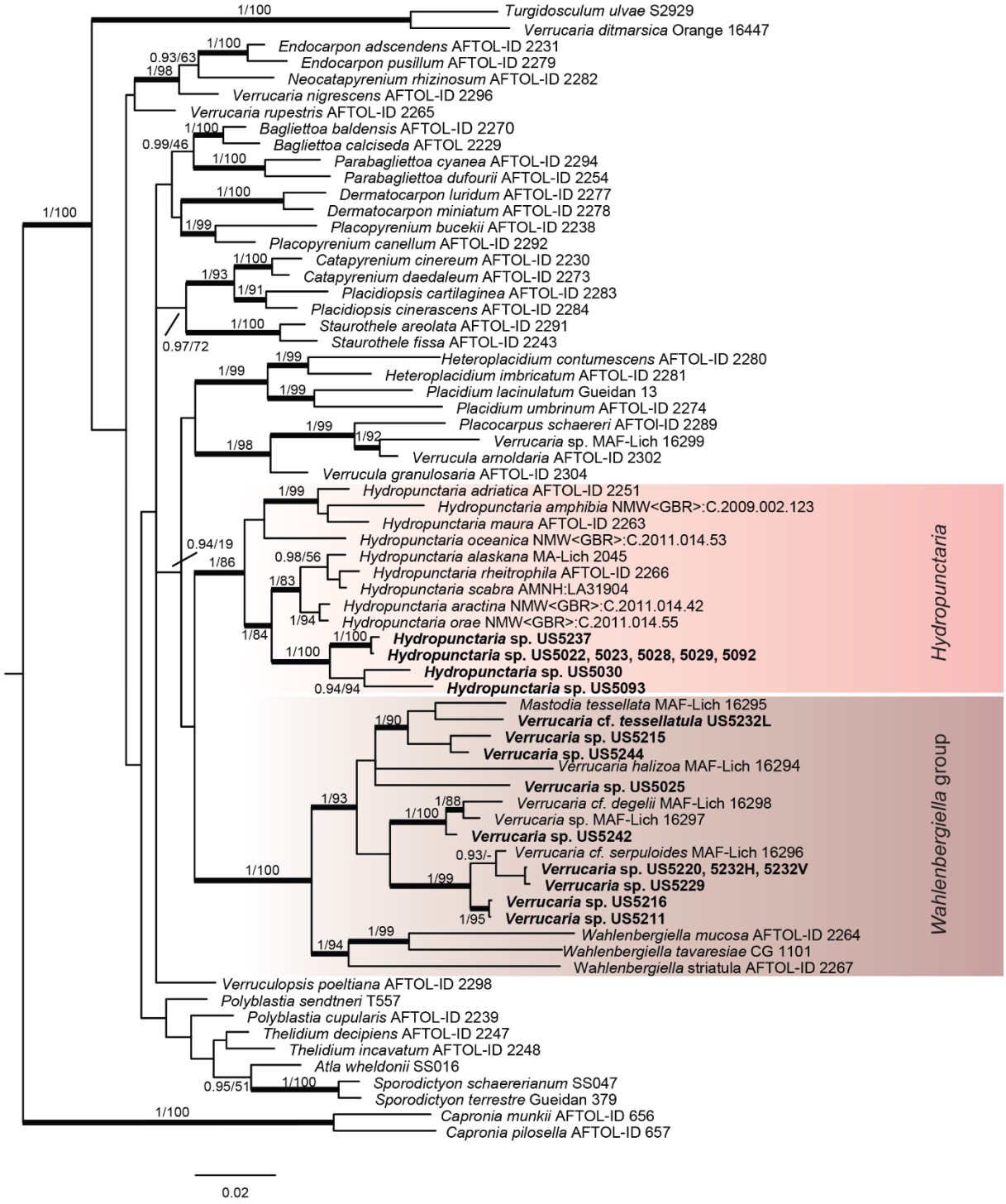

Figure 2 Phylogeny of the family Verrucariaceae obtained by Bayesian Inference of concatenated nuSSU rRNA gene, nuLSU, and mtSSU rRNA gene. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Specimen/voucher numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Tables 1 and S3. Scale bar represents the expected number of substitutions per site.

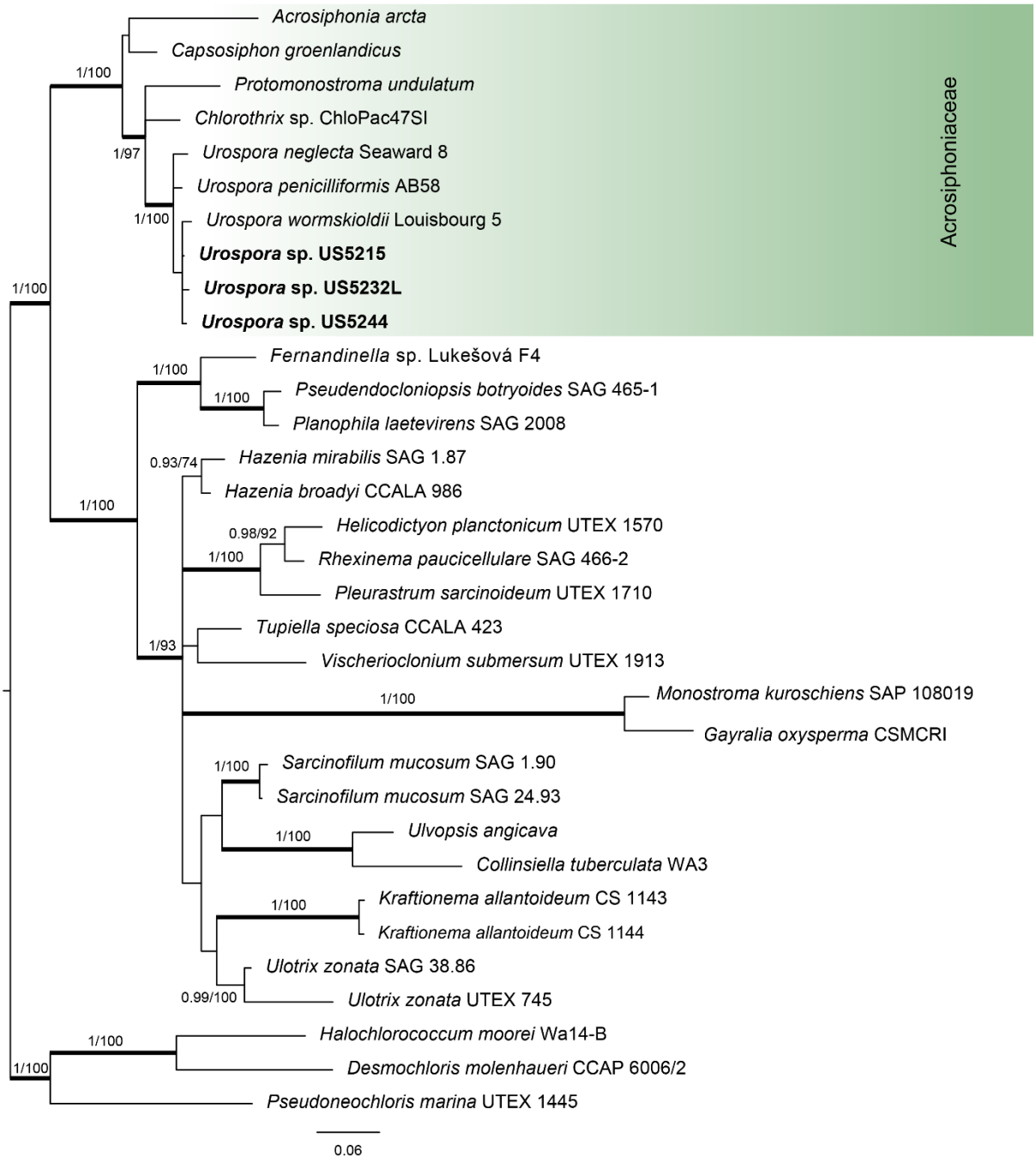

Figure 3 Phylogeny of the order Ulotrichales obtained by Bayesian Inference of concatenated nuSSU rRNA gene, nuITS, and chloroplast tufA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Tables 1 and S4. Scale bar represents the expected number of substitutions per site

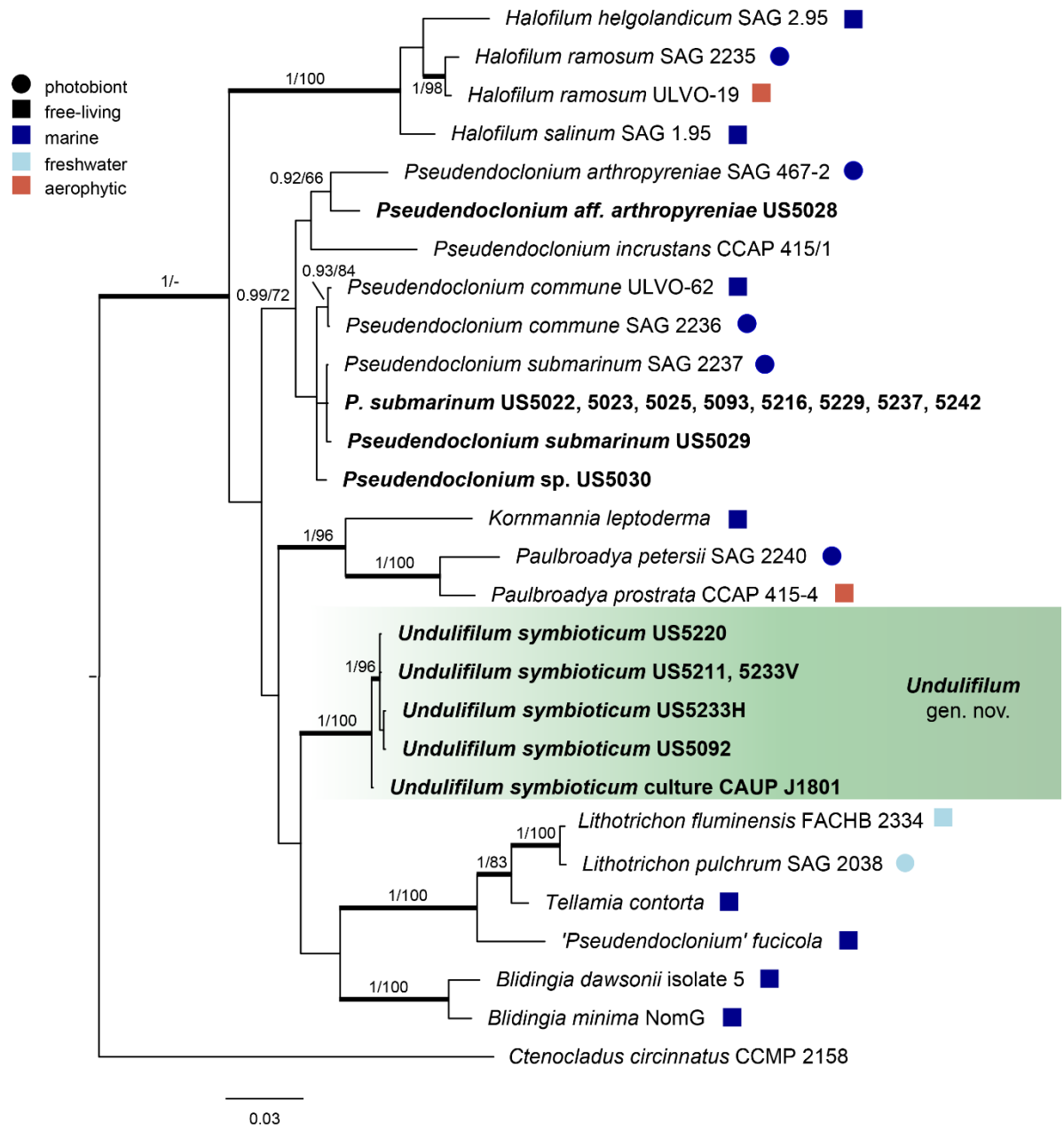

Figure 5 Phylogeny of the family Kornmaniaceae obtained by Bayesian Inference of concatenated nuSSU rRNA gene and ITS. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers, see Table 1 and Table S6 in the Supporting Information. Scale bar represents the expected number of substitutions per site.

(Gueidain et al. 2009). Lichenized *P. submarinum* (Fig. 5c) formed individual cells of 4.5–7 µm. Rarely, two-celled filaments were observed. The lichenized *U. symbioticum* (Fig. 5d) photobionts were found in the form of filaments of up to six elongated cells (6.5–9 × 2.5–5.2 µm), also organized vertically.

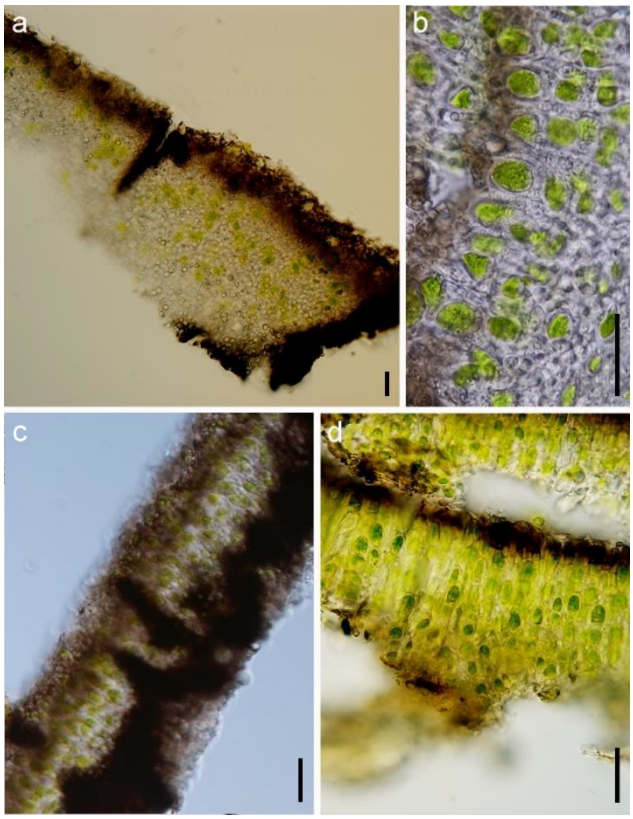

Figure 6 Cross sections of lichen thalli showing different photobionts. **a, b.** Urospora sp. **c.** *Pseudendoclonium submarinum*. **d**. *Undulifilum symbioticum*. Scale bars = 20 µm.

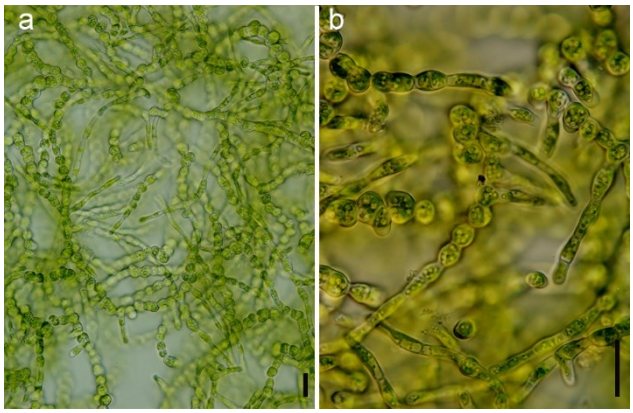

Figure 7 Morphology of *Pseudendoclonium submarinum* in culture. Scale bars = 20 µm

Only three photobiont cultures were obtained, probably due to the delay between collection and isolation, specifically *Pseudendoclonium submarinum* from samples US5216 and 5242 and *Undulifilum symbioticum* from US5220. In the culture of *P. submarinum* in liquid ASW (Fig. 6), cells in the prostrate filaments are close to spherical, 6.4–12 µm in diameter; cells in the erect filaments are cylindrical 8.8– 19 × 2.5–4.5 µm. The chloroplast is parietal with a pyrenoid. For culture detail of *U. symbioticum*, see the description below.

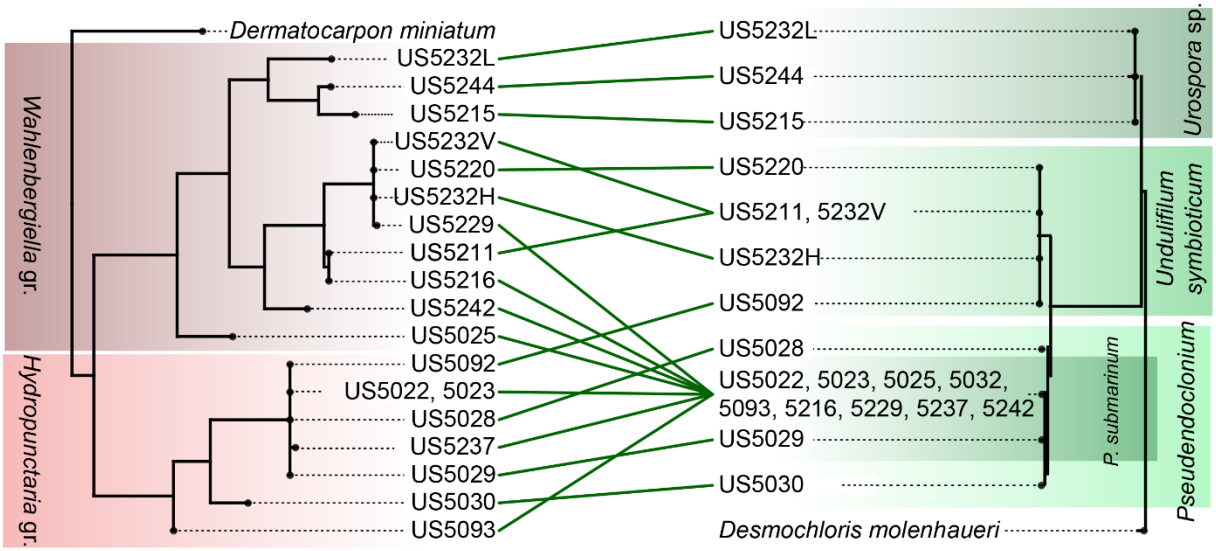

Figure 8 Interaction network between mycobionts and photobionts considering phylogenetic relationships among them.

3.4 Patterns in photobiont occurrence

Pseudendoclonium submarinum was the most common photobiont, regardless of the mycobiont identity (Fig. 7). It was found in nine of 19 samples belonging to various lineages of *Hydropunctaria* (US5022, 5023, 5029, 5093, and 5237) and *Wahlenbergiella* group (US5025, 5216, 5229, and 5242). Its occurrence was not affected by geography, substrate type, orientation, exposure, or vertical position of the lichen (see Table S1). *Undulifilum symbioticum* was found in four *Wahlenbergiella* group samples (US5211, 5220, 5232H, 5232V; Fig. 7), belonging to one lineage within the group (Fig. 2) and also in one *Hydropunctaria* sample (US5092). The four *Wahlenbergiella* samples were collected from horizontal surfaces of schist coastal rocks in distinct zones; US5220 was observed in the upper eulittoral zone (within the barnacle zone), US5232H and US5232V in lower littoral fringe (just above the barnacle zone), and US5211 in the mesic supralittoral zone (at the level of *Mastodia tesselata*), *Hydropunctaria* US5092 was found in the upper littoral fringe of a siliceous seashore. *Urospora* was noted to be the photobiont of three samples related to *M. tesselata* (Figs. 2, 7) within the *Wahlenbergiella* group (US5215, 5232L, and 5244). All of them were found in the littoral fringe, two just above the barnacle zone, one in the black belt (Table S1).

3.5 Taxonomic description

Undulifilum Škaloud, Černajová et Schiefelbein **gen. nov.**

Description: Thallus brush-like and crust-forming, composed of both prostrate and erect filaments. Young thalli consist of a prostrate system of irregularly branched uniseriate filaments, composed of long cylindrical cells possessing a single, parietal, plate-like chloroplast. Most of the filaments are regularly wavy. Lateral branches are formed near the apical part of the cylindrical cells, just below the transverse cell wall. Mature thalli consist of a central prostrate system of densely packed cells

surrounded by numerous branched filaments radiating outwards. The terminal cells of the branches are usually significantly longer than those found close to the thalli center. Usually, a well-developed prostrate system of branched filaments is formed on the central prostrate system. Reproduction occurs by vegetative division. Neither zoospores nor sexual reproduction were observed.

Differs from other genera by 18S rRNA sequences and by a wavy habit of the filaments.

Etymology: 'Unduli' refers to the wavy nature of cells and filaments; 'filum' refers to the filamentous habit.

Type species (designated here): *U. symbioticum* sp. nov.

Undulifilum symbioticum Škaloud, Černajová et Schiefelbein sp. nov. (Figs. 8, 9).

Description: Colonies in both liquid and agarized BBM medium large, up to 0.2(0.3) mm in diameter, consisting of prostrate and erected filaments. Young thalli are formed by branched prostrate filaments with relatively long, cylindrical cells attached to the surface, 3.5–4.5(–6) µm in width and 9–68(–80) µm in length. During filament growth, cells begin to bend axially, leading to the formation of wavy filaments. In mature thalli, basal cells of filaments transform into short, subglobose, densely packeted cells, 3.5–9 µm in diameter. Cell packets up to 14 µm in diameter. These cells may divide in different directions, forming a pseudoparenchymatous mass. The cells possess a single parietal chloroplast with a bulged margin and a single pyrenoid. Asexual reproduction by thallus fragmentation into cell packets, which unipolarly germinate into new filaments. Neither zoospores nor sexual reproduction was observed.

Etymology: The name refers to the symbiotic lifestyle of the species.

Holotype (here designated): PRC 4719.

Ex-type culture: CAUP J 1801 (Culture Collection of Algae of Charles University in Prague).

Habitat: Photobiont of Verrucariaceae lichens on seashore rocks.

Type locality: Chile, Aysen, Puerto Raul Mar ın Balmaceda, Rada Del Palena, S43.74625 W72.99108333.

GenBank accession numbers: OL343003 for ITS and OL343004 for nuSSU rDNA.

Additional material examined: US5092, US5211, US5232H, 5232V.

4. Discussion

Coastal rock habitats host about 700 lichen species (Hawksworth 2000), however, only a small portion of them is able to survive in the littoral zone. They have been primarily studied in temperate regions of the Northern hemisphere, but are still understudied (Hawksworth 2000). Therefore, it is not surprising that only one of our Chilean samples could be identified as a known lichen species. Our results suggest that the real diversity of black Verrucariaceae from the littoral zone substantially exceeds the diversity currently described. At higher taxonomic ranks, lichen mycobiont–photobiont associations are quite stable, where whole lichen families or even orders have preferences for specific genera of photobionts (Miadlikowska et al. 2006). The most common, by far, is *Trebouxia*

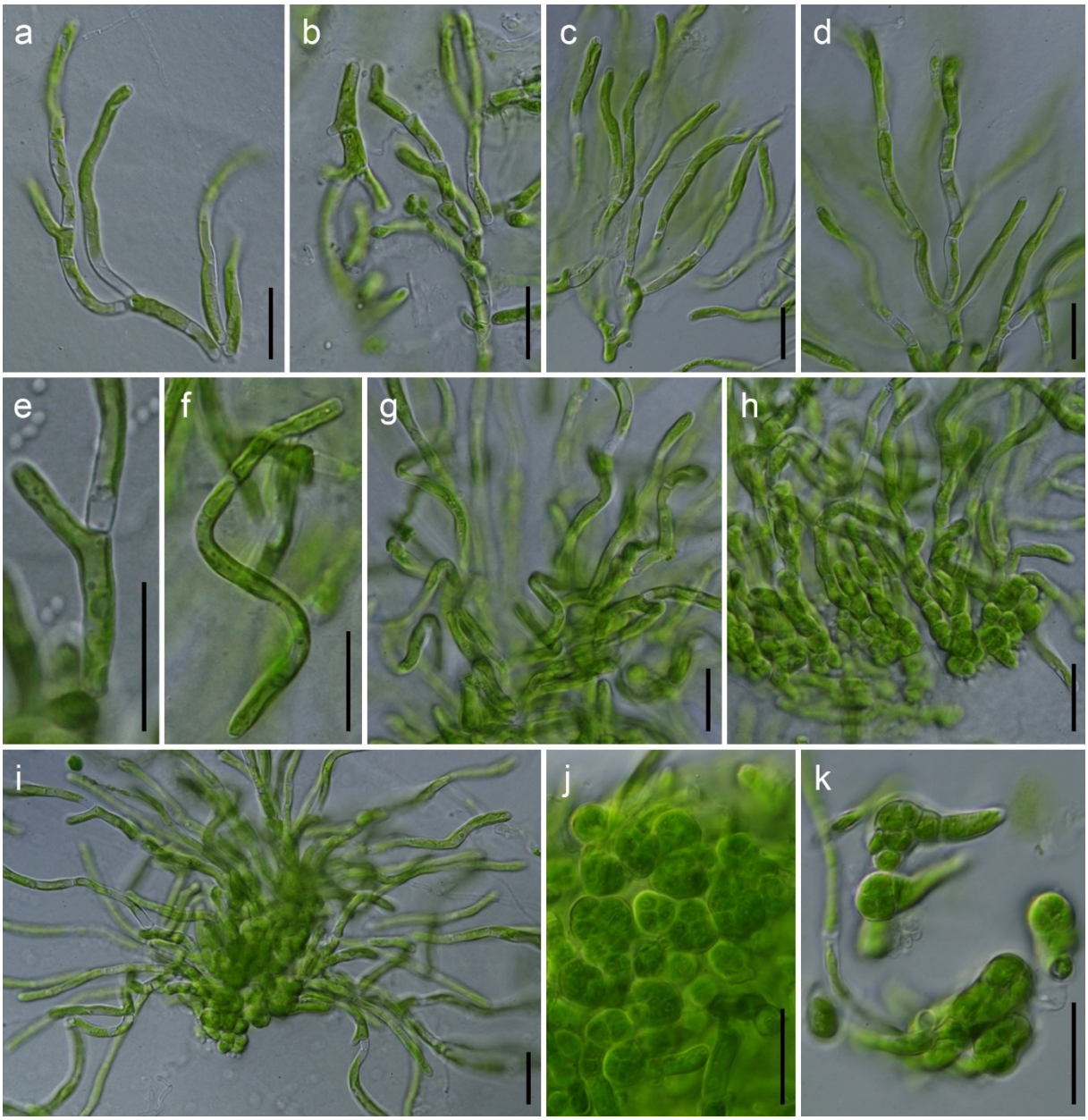

Figure 9 Morphology of *Undulifilum symbioticum*, gen. et sp. nov. **a**. Young filaments consisting of elongated, cylindrical cells. **b**. Production of side branches. **c**. Growth of branches into elongated cells. **d**. Formation of wavy filaments. **e**. Branching cell with a well-visible pyrenoid. **f**. Axially curved cell. **g**. Mature filaments formed by significantly curved cells. **h**. Transformation of basal cells into short, subglobose, densely packeted cells. **i**. Overall view of a mature thallus consisting of a central prostrate system of densely packed cells surrounded by numerous branched filaments radiating outward. **j**. Close-up view of the central pseudoparenchymatous mass of cells. **k**. Germination of cell packets into new filaments. Scale bars = 20 µm.

(Trebouxiophyceae), followed by *Nostoc* (Cyanobacteria), *Trentepohlia* (Ulvophyceae), and *Asterochloris* (Trebouxiophyceae; dePriest 2004, Miadlikowska et al. 2006). However, these photobionts are rarely found in Verrucariaceae, a family in which most of the amphibious lichens (both freshwater and marine) belong. Instead, Verrucariaceae host a plentitude of other algae, as

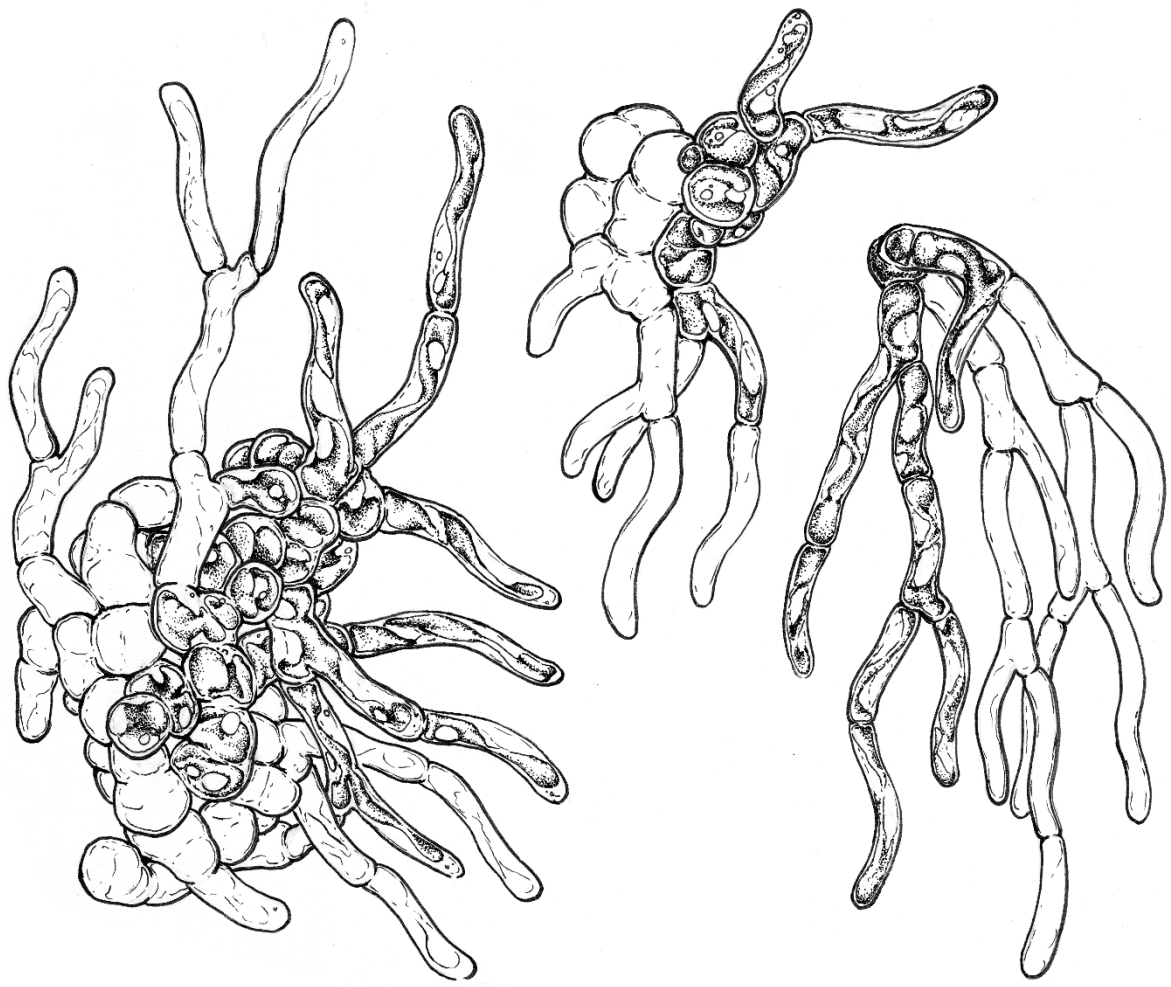

Figure 10 Illustration of *Undulifilum symbioticum*, gen. et sp. nov.

summarized by Tschermak-Woess (1989), Thüs et al. (2011), and Sanders and Masumoto (2021).

Members of the Ulvophycean family Kornmaniaceae are the most often reported photobionts of amphibious lichens. Formerly, it was the genus *Dilabifilum* (Binz and Vischer 1956, Tschermak-Woess 1976, 1989, Thüs et al. 2011) whose taxonomy was resolved recently by Darienko and Pröschold (2017). They synonymized *Dilabifilum* with *Pseudendoclonium* and placed some of its species into novel, closely related genera *Halofilum*, *Lithotrichon*, and *Paulbroadya*. A majority of amphibious Verrucariaceae photobionts identified as *Dilabifilum* strains by Thüs et al. (2011) actually also belong to *Pseudendoclonium* and *Halofilum*, according to BLAST searches of available nuSSU rRNA gene sequences (not shown). Sixteen of 19 photobionts in our samples belonged to the Kornmanniaceae, *P. submarinum* being the most common species (nine samples). Thus, the family Kornmanniaceae itself is rich in lichen photobionts (Fig. 4), especially of amphibious Verrucariaceae. In addition to the above-mentioned genera and *Undulifilum symbioticum* described herein, *Blidingia minima* occasionally associates with *Turgidosculum ulvae* (Pérez-Ortega et al. 2018) forming one of the few

known peculiar borderline lichens with inverted thallus structure, where the photobiont forms the major part of it, and the mycobiont is the inhabitant (Kohlmeyer et al. 2004).

Although the family is predominantly marine, there are also freshwater and aerophytic taxa (Fig. 4). The genera *Kornmannia* and *Tellamia* are exclusively marine, while *Lithotrichon* is exclusively freshwater (Darienko and Pröschold 2017, Liu et al. 2019) and other genera show wider ecological amplitudes. The two closely related species of *Paulbroadya*, *P. prostrata* and *P. petersii* are an aerophytic alga and marine lichen photobiont, respectively. *Pseudendoclonium* is mainly marine, but *Ps. incrustans* is a photobiont of freshwater *Verrucaria aquatilis* (Darienko and Pröschold 2017). At the species level, for example, the marine *Ps. submarinum*, *Blidingia minima*, and *Banksia marginata* can also be found in brackish estuaries, the *Blidingia* species occasionally even in freshwater habitats (Wille 1901, Škaloud et al. 2018). The coastal photobiont *Ps. arthropyreniae* is also known as free-living, terrestrial aerophytic on various substrates (Škaloud et al. 2018). However, in these cases, DNA sequence evidence that the different eco-forms actually represent the same species has not been generated. On the other hand, *Halofilum ramosum*, known as the photobiont of marine lichens, has also been isolated from the green biofilm on a wall of ruins, its identity verified by DNA sequence data (Darienko and Pröschold 2017). Physiological experiments found distinct osmoregulatory responses between strains isolated from lichens from different vertical zones on the seashore and the hypervariable chloroplast RPL10A region sequence data suggested that the eco-forms might actually represent young sister species (Gasulla et al. 2019).

Taken together, there are multiple transitions from marine to freshwater or aerophytic lifestyles at various levels within the Kornmanniaceae. This capacity may be the reason why Kornmanniaceae are the most common photobionts of lichens in the littoral zone. Although salinity at a site is more or less constant, lichen thalli deal with huge fluctuations in both salinity and water content, causing considerable changes in osmotic pressure. They are submerged by the sea or washed by waves, and then as the water falls, the lichen dries, and a layer of salt is left on the lichen surface, then if it rains the salt is washed off, and the lichen absorbs fresh water (Dobson 2014). Thus, the flexibility in osmoregulation of the photobiont represents a clear advantage, if not a necessity, for seashore lichens.

No lichen photobiont has previously been reported among the Ulotrichales. In the present study, we observed *Urospora* in association with three related mycobionts (*Mastodia* lineage of the *Wahlenbergiella* group, Table 1, Figs. 2, 7) from three different localities (Fig. 1, Table S1). It was also confirmed by repeated DNA isolation, amplification, and sequencing. Thus, although unexpected, we consider this finding reliable. The photobionts cannot be assigned any species name because the DNA sequence markers used do not allow for a clear distinction between *Urospora wormskioldii* and *U. penicilliformis* (Lindstrom and Hanic 2005). It might be another, yet unknown, closely related species.

Urospora belongs to Acrosiphoniaceae, the most ancestral lineage within Ulotrichales (Škaloud et al. 2018). *Urospora* species are macroscopic filamentous algae growing in intertidal zones of cold seas (Lindstrom and Hanic 2005). Acquisition of a locally adapted photobiont is reasonable from the mycobiont's point of view, but it is not clear as to what would be the advantage of the lichenized state for an alga, which is successful in the same habitat on its own. *Petroderma maculiforme* (Phaeophyceae), which usually forms free-living crustose thalli, is also known as the photobiont of *Wahlenbergiella tavaresiae*. Where the two forms coexist, the latter also inhabits upper parts of the

intertidal zone, while the former is limited to the lower and mid-intertidal zones (Sanders et al. 2004). *Urospora wormskioldii* and *U. penicilliformis* occur in the upper and lower littoral zones, respectively (Hanic 2005). Thus, for now, it cannot be concluded whether the switch to the lichenized state enables ecological niche widening in the case of *Urospora*.

The fact that most of the seashore lichen species seem to be specific toward their photobiont may be the result of lack of data. There is also evidence that the photobiont choice depends on the ecology of a lichen (Ortiz-Álvarez et al. 2015). Gasulla et al. (2019) suggested that the zonation of lichens on the seashore is, at least partly, driven by photobiont physiology. That would concur with the hypothesis of photobiont mediated guilds (Rikkinen et al. 2002), which expects lichens with the same environmental preferences to share a set of well-adapted photobionts. However, conclusions cannot be drawn before the diversity of seashore photobionts is generally recognized. Even the very limited sampling of this study revealed novel seashore lichen photobionts, indicating we might be only at the beginning of uncovering their diversity.

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References

Beckett R. P., Kranner I. & Minibayeva F. V. (2008) Stress physiology and the symbiosis. In: Nash III. T. H. (Ed.) Lichen Biology. Cambridge University Press, New York, USA, pp. 134-151.

Binz A. & Vischer W. (1956) Zur Flora des Rheinlaufes bei Basel. Verhandlungen der Naturforschenden Gesellschaft in Basel 67: 195-217.

Brodo I. M. & Slone N. A. (2004) Lichen zonation on coastal rocks in Gwaii Haanas National Park Reserve, Haida Gwaii (Queen Charlotte Islands), British Columbia. The Canadian Field-Naturalist 118: 405-424.

Castresana J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540-552.

Cubero O. F., Crespo A., Fatehi J. & Bridge P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243-249.

Darienko T. & Pröschold T. (2017) Toward a monograph of non-marine Ulvophyceae using an integrative approach (Molecular phylogeny and systematics of terrestrial Ulvophyceae II.). Phytotaxa 324: 1-41.

Darriba T., Taboada G. L., Doallo R. & Posada D. (2012) jModelTest2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

DePriest P. T. (2004) Early molecular investigations of lichen-forming symbionts: 1986–2001. Annual Review of Microbiology 58: 273-301.

Dobson F. S. (2014) A field key to the coastal and seashore lichens. New Malden, UK, 100 pp.

Ertz D., Guzow-Krzemińska B., Thor G., Łubek A. & Kukwa M. (2018) Photobiont switching causes changes in the reproduction strategy and phenotypic dimorphism in the Arthoniomycetes. Scientific Reports 8: 4952.

Fletcher A. (1973) The ecology of marine (littoral) lichens on some rocky shores of Anglesey. Lichenologist 5: 368-400.

Gasulla F., Guéra A., de los Ríos A. & Pérez-Ortega S. (2019) Differential responses to salt concentrations of lichen photobiont strains isolated from lichens occuring in different littoral zones. Plant and Fungal Systematics 64: 149-162.

Garrido-Benavent I., de los Ríos A., Fernández-Mendoza F. & Pérez-Ortega S. (2018) No need for stepping stones: Direct, joint dispersal of the lichen-forming fungus *Mastodia tessellata* (Ascomycota) and its photobiont explains their bipolar distribution. Journal of Biogeography 45: 21-224.

Gueidain C., Roux C. & Lutzoni F. (2007) Using a multigene phylogenetic analysis to assess generic delineation and character evolution in Verrucariaceae (Verrucariales, Ascomycota). Mycological Research 111: 1145-1168.

Gueidan C., Savić S., Thüs H., Roux C., Keller C., Tibell L., Prieto M., Heidmarsson S., Breuss O., Orange A., Fröberg L., Wynns A. A., Navarro-Rosinés P., Krzewicka B., Pykälä J., Grube M. & Luztoni F. (2009) Generic classification of the Verrucariaceae (Ascomycota) based on molecular and morphological evidence: Recent progress and remaining challenges. Taxon 58: 184-208.

Gueidain C., Thüs H. & Pérez-Ortega S. (2011) Phylogenetic position of the brown algae-associated lichenized fungus *Verrucaria tavaresiae* (Verrucariaceae). Bryologist 114: 563-569.

Hamby R. K., Sims L. E., Issle L. E. & Zimmer E. A. (1988) Direct RNA sequencing: optimization of extraction and sequencing techniques for work with higher plants. Plant Molecular Biology Reporter 6: 179-197.

Hanic L. A. (2005) Taxonomy, gamete morphology, and mating types of *Urospora* (Ulotrichales, Chlorophyta) in North America. Phycologia 44: 183-193.

Hawksworth D. L. (2000) Freshwater and marine lichen-forming fungi. In: Hyde K. D., Ho W. H. & Pointing S. B. (Eds.) Aquatic Mycology across the Millennium. Fungal Diversity 5: 1-7.

Helms G. (2003) Taxonomy and symbiosis in associations of Physciaceae and *Trebouxia.* PhD dissertation, Univerisity of Göttingen, 156 pp.

Katoh K., Rozewicki J. & Yamada K. D. (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefiengs in Bioinformatics 18: 1-7.

Kohlmeyer J., Hawksworth D. L. & Volkmann-Kohlmeyer B. (2004) Observations on two marine and maritime “borderline” lichens: *Mastodia tessellata* and *Collemopsidium pelvetiae*. Mycological Progress 3: 51-56.

Lindstrom S. C. & Hanic L. A. (2005) The phylogeny of North American *Urospora* (Ulotrichales, Chlorophyta) based on sequence analysis of nuclear ribosomal genes, introns and spacers. Phycologia 44: 194-201.

Liu B., Wang Q., Li S., Fang J., Liu G. & Hu Z. (2019) Taxonomic transfer of *Gongrosira fluminensis* Fritsch (Chaetophorales, Chlorophyceae) to *Lithotrichon* Darienko et Pröschold (Ulvales, Ulvophyceae) based on morphological observation and phylogenetic analyses. Fottea 19: 25-32.

Miadlikowska J., Kauff F., Hofstetter V., Fraker E., Grube M., Hafellner J., Reeb V., Hodkinson B. P., Kukwa M., Lücking R., Hestmark G., Garcia Otalora M., Rauhut A., Büdel B., Scheidegger C., Timdal E., Stenroos S., Brodo I., Perlmutter G., Ertz D., Diederich P., Lendemer J. C., May P., Schoch C. L., Arnold A. E., Gueidan C., Tripp E., Yahr R., Robertson C. & Lutzoni F. (2006) New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA- and two protein-coding genes. Mycologia 98: 1088-1103.

Miller M. A., Pfeiffer W. & Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop. 14 Nov. 2010: 1-8.

Nelsen M. P., Rivas Plata E., Andrew C. J., Lücking R. & Lumbsch H. T. (2011) Phylogenetic diversity of trentepohlialean algae associated with lichen-forming fungi. Journal of Phycology 47: 282-290.

Orange A. (2012) Semi-cryptic marine species of *Hydropunctaria* (Verrucariaceae, lichenized Ascomycota) from north-west Europe. Lichenologist 44: 299-320.

Ortiz-Álvarez R., de los Ríos A., Fernández-Mendoza F., Torralba-Burrial A. & Pérez-Ortega S. (2015) Ecological specialization of two photobiont-specific maritime cynolichen species of the genus *Lichina*. PLoS ONE 10: e0132718.

Parra O. O. & Redon J. (1977) Aislamento de *Heterococcus caespitosus* Vischer ficobionte de *Verrucaria maura* Wahlenb. Boletin de la Sociedad de Biología de Concepción 51: 219.

Peksa O. & Škaloud P. (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). Molecular Ecology 20: 3936-3948.

Pérez-Ortega S, de los Ríos A., Crespo A. & Sancho L. G. (2010) Symbiotic lifestyle and phylogenetic relationships of the bionts of *Mastodia tessellata* (Ascomycota, incertae sedis). American Journal of Botany 97: 738-752.

Pérez-Ortega S., Miller K. A. & de los Ríos A. (2018) Challenging the lichen concept: *Turgidosculum ulvae* (Verrucariaceae) represents an independent photobiont shift to a multicellular blade-like alga. Lichenologist 50: 341-356.

R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/.

Rambaut A. (2016) FigTree. Tree Figure Drawing Tool. University of Edinburgh. Available at: http://tree.bio.ed.ac.uk/.

Revell L. J. (2012) phytools: An R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3: 217-223.

Rikkinen J., Oksanen I. & Lohtander K. (2002) Lichen guilds share related cyanobacterial symbionts. Science 297: 357.

Rolshausen G., Dal Grande F., Sadowska-Deś A. D., Otte J. & Schmitt I. (2018) Quantifying the climatic niche of symbiont partners in a lichen symbiosis indicates mutualist-mediated niche expansions. Ecography 41: 1380-1392.

Ronquist F., Teslenko M., van der Mark P., Ayres D., Darling A., Hohna S., Larget B., Liu L., Suchard M. A. & Huelsenbeck J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539-542.

Sanders W. B. & Masumoto H. (2021) Lichen algae: the photosynthetic partners in lichen symbioses. Lichenologist 53: 347-393.

Sanders W. B., Moe R. L. & Ascaso C. (2004) The intertidal marine lichen formed by the Pyrenomycete fungus *Verrucaria tavaresiae* (Ascomycotina) and the brown alga *Petroderma maculiforme* (Phaeophyceae): thallus organization and symbiont interaction. American Journal of Botany 91: 511-52.

Savić S., Tibell L., Gueidan C. & Lutzoni F. (2008) Molecular phylogeny and systematics of *Polyblastia* (Verrucariaceae, Eurotiomycetes) and allied genera. Mycological Research 112: 1307-1318.

Škaloud P. & Rindi F. (2013) Ecological differentiation of cryptic species within an asexual protist morphospecies: A case study of filamentous green alga *Klebsormidium* (Streptophyta). Journal of Eukaryotic Microbiology 60: 350-362.

Škaloud P., Rindi F., Boedeker C. & Leliaert F. (2018) Freshwater Flora of Central Europe, Vol 13: Chlorophyta: Ulvophyceae. Springer Spektrum, Berlin, 289 pp.

Spribille T., Fryday A., Pérez-Ortega S., Svensson M., Tønsberg T., Ekman S., Holien H., Resl P., Schneider K., Stabentheiner E., Thüs H., Vondrák J. & Sharman L. 2020. Lichens and associated fungi from Glacier Bay National Park, Alaska. Lichenologist 52: 61-181.

Stamatakis A. (2014) RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. Bioinformatics 10.1093/bioinformatics/btu033.

Starr R. C. & Zeikus J. A. (1993) UTEX The Culture Collection of Algae at the University of Texas at Austin. Journa of Phycology 29 (suppl.): 1-106.

Thüs H., Muggia L., Pérez-Ortega S., Favero-Longo S. E., Joneson S., O'Brien H., Nelsen M. P., Duque-Thüs R., Grube M., Friedl T., Brodie J., Andrew C. J. Lücking R., Lutzoni F. & Gueidan C. (2011) Revisiting photobiont diversity in the lichen family Verrucariaceae (Ascomycota). European Journal of Phycology 46: 399-415.

Thüs H. & Schultz M. (2009) Freshwater Flora of Central Europe, Vol 21: Fungi. Part 1: Lichens. Springer Spektrum, Berlin, 223 pp.

Tschermak-Woess E. (1976) Algal taxonomy and the taxonomy of lichens: the phycobiont of *Verrucaria adriatica*. In: Brosn D. H., Hawksworth D. L. & Bailey R. H. (Eds.) Lichenology: Progress and Problems Academic Press, New York, pp. 79-88.

Tschermak-Woess E. (1989) The algal partner. In: Galun M. (Ed.) CRC Handbook of Lichenology, Volume I. CRC Press, Boca Raton, Florida, pp. 39-92.

Vančurová L., Muggia L., Peksa O., Řídká T. & Škaloud P. (2018) The complexity of symbiotic interactions influences the ecological amplitude of the host: a case study in Stereocaulon (lichenized Ascomycota). Molecular Ecology 27: 3016-3033.

Vilgalys R. & Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238-4246.

Watanabe S., Nakano T. & Deguchi H. (1997) Photobionts from maritime lichens. Journal of Marine Biotechnology 5: 103-112.

White T. J., Bruns T., Lee S. & Taylor J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand D. H., Sninsky J. J. & White T. J. (Eds.) PCR protocols: a guide to methods and applications. Academic Press, San Diego, USA, pp 315-322.

Wickham H. (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wille N. (1901) Studien über Chlorophyceen I-VII. Skrifter udgivne af Videnskabsselskabet i Christiania. Mathematisk-Naturvidenskaelig Klasse 6: 1-46.

Zoller S., Scheidegger C. & Sperisen C. (1999) PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming Ascomycetes. Lichenologist 31: 511-516.

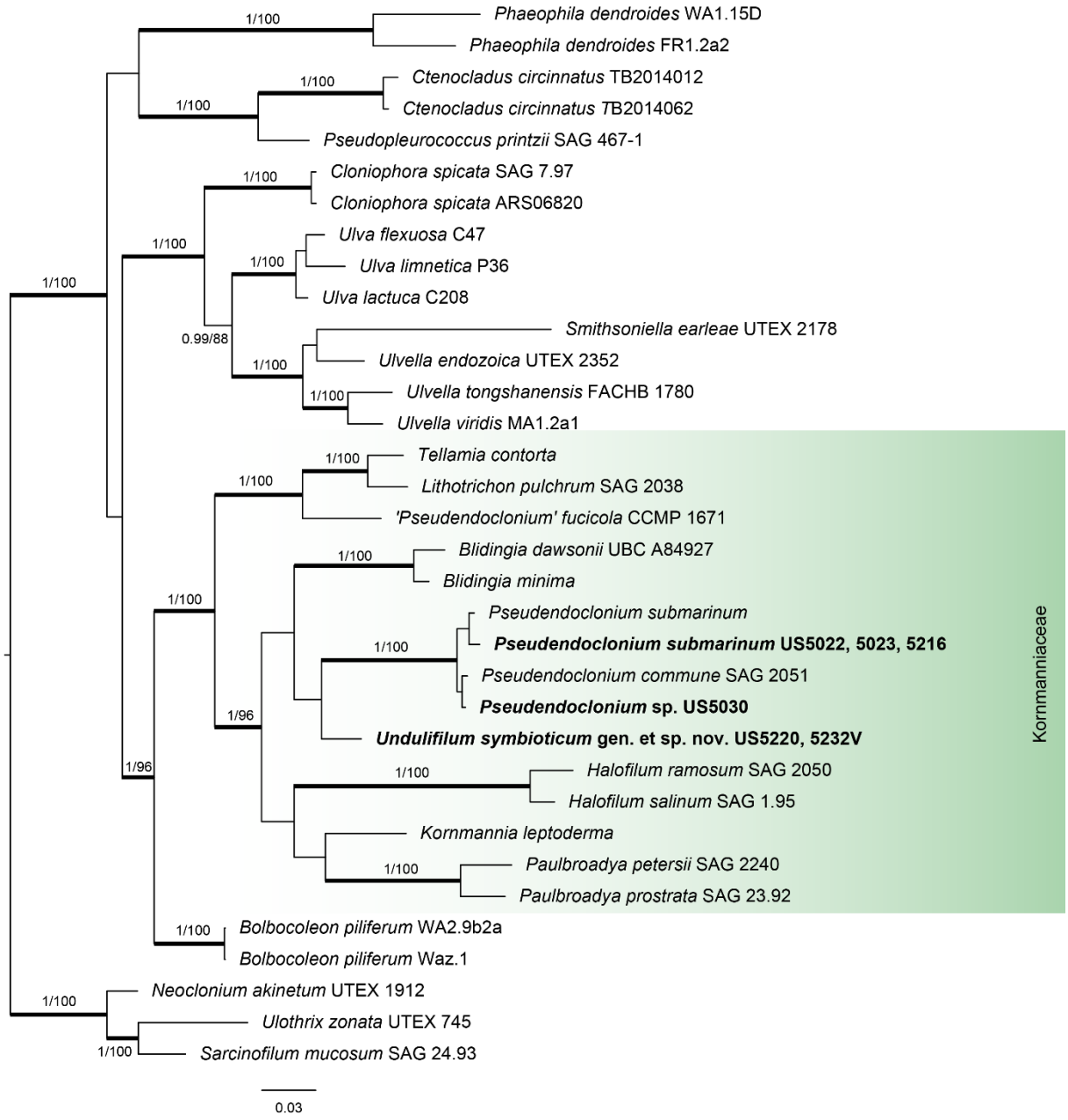

Figure S1 Phylogeny of the order Ulvales obtained by Bayesian Inference concatenated nuSSU and chloroplast tufA and rbcL. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Only statistical supports with posterior probability higher than 0.9 are shown. Thick branches represent nodes with full PP support. Strain numbers (where available) are provided for each taxon. Newly obtained sequences are in bold. For GenBank accession numbers see Table 1 and Table S5. Scale bar represents the expected number of substitutions per site.

Table S1 Herbarium codes and collection data.

Code[a]	Locality	GPS coordinates	Habitat, biological zone and notes	Bedrock
US5022	Chile, Aysén, Ventisquero fjord, Puyuhuapi village	S44.32641667 W72.57041667	stony beech with boulders, mesic-supralittoral zone, on boulder facing the sea, zone with white crustose lichens	volcanic
US5023			stony beech with boulders, submesic-supralittoral zone (lower border), on boulder facing the sea, lower part of the zone with foliose lichens	
US5025	Chile, Aysén, Ventisquero fjord, Puyuhuapi village	S44.32658333 W72.56958333	stony beech with boulders, submesic-supralittoral zone, on boulder facing away from the sea, zone with foliose lichens	volcanic
US5028	Chile, Aysén, Queulat fjord, Puyuhuapi	S44.51905556 W72.54577778	stony beech with boulders, submesic-supralittoral zone (lower border), in the upper part of the beach, shady place	granite
US5029				
US5030			stony beech with boulders, upper littoral fringe, in the lower part of the beach, shady place	
US5092	Chile, Aysén, Caleta Tortel	S47.80561111 W73.54391667	coastal rocks, upper littoral fringe, rock facing the sea, in upper part of rock, shady place	siliceous
US5093			coastal rocks, upper littoral fringe, top of the rock	
US5211	Chile, Aysén, Puerto Raúl Marín Balmaceda, Estero Los Patos	S43.75391667 W72.95066667	coastal rocks, mesic-supralittoral zone, on horizontal surface, at altitute of *Mastodia tesselata*	schist
US5215	Chile, Aysén, Puerto Raúl Marín Balmaceda, Rada Del Palena	S43.74625 W72.99108333	coastal rocks, lower littoral fringe, on horizontal surface, in lower part of the rock, above the barnacle zone	schist
US5216			coastal rocks, lower littoral fringe, on horizontal surface, in upper part of the rock	
US5220 = PRC 4719			coastal rocks, upper eulittoral, on horizontal surface, in lower part of the rock, in the barnacle zone	
US5229	Chile, Los Lagos, Chaitén, Santa Bárbara	S42.853 S72.80105556	coastal rocks, upper eulittoral, on steep face, in the algae zone	schist
US5232H US5232L US5232V			coastal rocks, lower littoral fringe, on horizontal surface, in lower part of the rock, above the barnacle zone	
US5237	Chile, Los Lagos, Caleta Gonzalo, Fiordo Reñihue	S42.55852778 W72.605138889	coastal rocks, lower littoral fringe, steep face covered by forests	schist
US5242	Chile, Los Lagos, Contao, Seno de Reloncaví	S41.78355556 W72.696972222	boulder beach, mesic-supralittoral zone, together with *Lecanora* and *Caloplaca*	schist
US5244			boulder beach, upper littoral fringe, black belt	

[a]All specimens are stored in the personal herbarium of U. Schiefelbein, except US5220 which is deposited in PRC, Charles University, Prague (PRC 4719).

Table S2 PCR conditions

	Mycobiont			Photobiont		
	nuSSU	nuLSU	mtSSU	nuSSU	nuITS	rbcL
initial denaturation	95°C 2 min	95°C 3 min	95°C 5 min	95°C 2 min	95°C 2 min	95°C 2 min
denaturation	95°C 45 s	95°C 30 s	95°C 30 s	95°C 30 s	95°C 30 s	95°C 1 min
annealing	52°C 40 s	55°C 30 s	56°C - 54°C - 50°C 30 s	48°C 45 s	62°C 30 s	50°C 1 min
extension	72°C 3 min	72°C 1 min	72°C 30s	72°C 1 min 45 s	72°C 45 min	72°C 1 min
number of cycles	30	35	5 - 10 - 20	40	35	35
final extension	72°C 5 min	72°C 2 min	72°C 7 min	72°C 7 min	72°C 5 min	72°C 7 min

Table S3. GenBank accession numbers of the sequences used in the phylogenetic analyses of the Verrucariaceae.

Genus	Species	voucher/isolate	nuLSU	nuSSU	mtSSU
Atla	*wheldonii*	SS016	EU598728	-	-
Bagliettoa	*baldensis*	AFTOL-ID 2270	EF643786	EF689823	FJ225666
Bagliettoa	*calciseda*	AFTOL 2229	EF643788	EF689828	FJ225667
Capronia	*munkii*	AFTOL-ID 656	EF413604	EF413603	FJ225723
Capronia	*pilosella*	AFTOL-ID 657	DQ823099	DQ823106	FJ225725
Catapyrenium	*cinereum*	AFTOL-ID 2230	EF643747	EF689829	FJ225671
Catapyrenium	*daedaleum*	AFTOL-ID 2273	EF643748	EF689830	FJ225672
Dermatocarpon	*luridum*	AFTOL-ID 2277	EF643750	EF689833	-
Dermatocarpon	*miniatum*	AFTOL-ID 2278	EF469160	EF689834	-
Endocarpon	*adscendens*	AFTOL-ID 2231	EF643751	EF689835	FJ225673
Endocarpon	*pusillum*	AFTOL-ID 2279	EF643754	EF689837	FJ225677
Heteroplacidium	*contumescens*	AFTOL-ID 2280	EF643755	EF689838	-
Heteroplacidium	*imbricatum*	AFTOL-ID 2281	EF643756	EF689839	FJ225679
Hydropunctaria	*adriatica*	AFTOL-ID 2251	EF643783	EF689862	FJ225680
Hydropunctaria	*alaskana*	MA-Lich 2045	-	-	MN508286
Hydropunctaria	*amphibia*	NMW<GBR>:C.2009.002.123	JN638252	-	-
Hydropunctaria	*aractina*	NMW<GBR>:C.2011.014.42	JN638255	-	JN638290
Hydropunctaria	*maura*	AFTOL-ID 2263	EF643801	EF689876	FJ225681
Hydropunctaria	*oceanica*	NMW<GBR>:C.2011.014.53	JN638279	-	JN638299
Hydropunctaria	*orae*	NMW<GBR>:C.2011.014.55	JN638284	-	JN638295
Hydropunctaria	*rheitrophila*	AFTOL-ID 2266	EF643808	EF689881	-
Hydropunctaria	*scabra*	AMNH:LA31904	KY773251	-	FJ225682
Mastodia	*tessellata*	MAF-Lich 16295	FN668948	FN668947	-
Neocatapyrenium	*rhizinosum*	AFTOL-ID 2282	EF643757	EF689840	FJ225683
Parabagliettoa	*cyanea*	AFTOL-ID 2294	EF643790	EF689866	-
Parabagliettoa	*dufourii*	AFTOL-ID 2254	EF643792	EF689868	FJ225684
Placidiopsis	*cartilaginea*	AFTOL-ID 2283	EF643758	EF689841	FJ225685
Placidiopsis	*cinerascens*	AFTOL-ID 2284	EF643759	EF689842	FJ225686
Placidium	*lacinulatum*	Gueidan 13	EF469158	EF689847	FJ225688
Placidium	*umbrinum*	AFTOL-ID 2274	EF643749	EF689831	FJ225691
Placocarpus	*schaereri*	AFTOl-ID 2289	EF643766	EF689850	-
Placopyrenium	*bucekii*	AFTOL-ID 2238	EF643768	EF689852	FJ225693
Placopyrenium	*canellum*	AFTOL-ID 2292	EF643785	EF689864	FJ225695
Polyblastia	*cupularis*	AFTOL-ID 2239	EF643769	EF689853	-
Polyblastia	*sendtneri*	T557	EU598696	-	-
Sporodictyon	*schaererianum*	SS047	EU598721	-	-
Sporodictyon	*terrestre*	Gueidan 379	EU364561	-	FJ225698
Staurothele	*areolata*	AFTOL-ID 2291	EF643772	EF689856	FJ225699
Staurothele	*fissa*	AFTOL-ID 2243	EF643775	EF689858	FJ225701
Thelidium	*decipiens*	AFTOL-ID 2247	EF643779	EF689859	-
Thelidium	*incavatum*	AFTOL-ID 2248	EF643780	EF689860	-
Turgidosculum	*ulvae*	S2929	MF970433	MF970431	MF970437
Verrucaria	*cf. degelii*	MAF-Lich 16298	FN668954	-	-
Verrucaria	*ditmarsica*	Orange 16447	FJ664846	-	-
Verrucaria	*halizoa*	MAF-Lich 16294	FN668956	FN668955	-

Verrucaria	*nigrescens*	AFTOL-ID 2296	EF643804	EF689879	-
Verrucaria	*rupestris*	AFTOL-ID 2265	EU598724	-	FJ225708
Verrucaria	*cf. serpuloides*	MAF-Lich 16296	FN668953	FN668952	-
Verrucaria	*sp.*	MAF-Lich 16297	FN668951	FN668950	-
Verrucaria	*sp.*	MAF-Lich 16299	-	FN668949	-
Verrucula	*arnoldaria*	AFTOL-ID 2302	EF643816	EF689886	FJ225713
Verrucula	*granulosaria*	AFTOL-ID 2304	EF643818	EF689889	FJ225715
Verruculopsis	*poeltiana*	AFTOL-ID 2298	EF643822	EF689880	FJ225719
Wahlenbergiella	*mucosa*	AFTOL-ID 2264	EF643802	EF689877	FJ225720
Wahlenbergiella	*striatula*	AFTOL-ID 2267	EF643810	EF689882	FJ225721
Wahlenbergiella	*tavaresiae*	CG 1101	HQ822059	HQ822058	HQ822057

Table S4 GenBank accession numbers of the sequences used in the phylogenetic analyses of the Ulotrichales.

Genus	Species	Strain	SSU	ITS	tufA
Acrosiphonia	*arcta*		AY303600	-	AY454423
Capsosiphon	*groenlandicus*		DQ821514	DQ821514	-
Collinsiella	*tuberculata*	WA3	AY198125	AY198125	-
Desmochloris	*molenhaueri*	CCAP 6006/2	FM882217	FM882217	-
Fernandinella	*sp.*	Lukešová F4	MF000562	MF000577	MF000596
Gayralia	*oxysperma*	CSMCRI	-	JF918550	JF918549
Halochlorococcum	*moorei*	Wa14-B	AY198122	-	AY454417
Hazenia	*broadyi*	CCALA 986	HF570951	HF570954	-
Hazenia	*mirabilis*	SAG 1.87	AF387156	HF570953	-
Helicodictyon	*planctonicum*	UTEX 1570	KM464720	HE575892	-
Chlorothrix	*sp.*	ChloPac47SI	AY476827	AY476827	-
Kraftionema	*allantoideum*	CS-1143	KU862658	-	KX268524
Kraftionema	*allantoideum*	CS-1144	KU862659	-	-
Monostroma	*kuroschiense*	SAP108019	GU062568	GU062561	-
Planophila	*laetevirens*	SAG 2008	AJ416102	MF034638	-
Pleurastrum	*sarcinoideum*	UTEX 1710	Z47998	Z47998	-
Protomonostroma	*undulatum*		DQ821517	DQ821517	-
Pseudendocloniopsis	*botryoides*	SAG 465-1	AJ416103	FR865755	-
Pseudoneochloris	*marina*	UTEX 1445	U41102	-	AY454422
Rhexinema	*paucicellulare*	SAG 466-2	MF000569	MF000587	MF000600
Sarcinofilum	*mucosum*	SAG 4.90	AM109906	MF000581	-
Sarcinofilum	*mucosum*	SAG 24.93	KM020139	MF000582	MF000597
Tupiella	*speciosa*	CCALA 423	MF000567	MF000585	MF000598
Ulotrix	*zonata*	SAG 38.86	Z47999	Z47999	-
Ulotrix	*zonata*	UTEX 745	AY278217	-	AY454424
Ulvopsis	*angicava*		KT180156	KT180156	-
Urospora	*penicilliformis*	AB58	AY476812	AY476812	-
Urospora	*neglecta*	Seaward 8	AY476821	AY476821	-
Urospora	*wormskioldii*	Louisbourg 5	AY476816	AY476816	-
Vischerioclonium	*submersum*	UTEX 1913	MF000568	MF000586	MF000599

Table S5 GenBank accession numbers of the sequences used in the phylogenetic analyses of the Ulvales.

Genus	Species	Strain	SSU	tufA	rbcL
Blidingia	*dawsonii*	UBC A84927	DQ001138	-	-
Blidingia	*minima*		AF499659	-	-
Bolbocoleon	*piliferum*	WA2-9b2a	AY303598	AY454421	-
Bolbocoleon	*piliferum*	Waz.1	AY303596	AY454418	-
Cloniophora	*spicata*	SAG 7.97	JF680949	JF680963	-
Cloniophora	*spicata*	ARS06820	KM677010	-	-
Ctenocladus	*circinnatus*	TB2014012	KU362724	-	-
Ctenocladus	*circinnatus*	TB2014062	KU362725	-	-
Halofilum	*ramosum*	SAG 2050	MF000571	MF000589	-
Halofilum	*salinum*	SAG 1.95	AF124337	-	-
Kornmannia	*leptoderma*		AF499661	-	AF499677
Lithotrichon	*pulchrum*	SAG 2038	MF034614	-	-
Neoclonium	*akinetum*	UTEX 1912	DQ011230	AY835431	AY835431
Paulbroadya	*petersii*	SAG 2240	MF034620	-	-
Paulbroadya	*prostrata*	SAG 23.92	FR865752	MF000590	-
Phaeophila	*dendroides*	FR1.2a2	AY454430	AY454415	-
Phaeophila	*dendroides*	WA1.15D	AY454432	AY454414	-
Pseudendoclonium	*commune*	SAG 2051	MF000572	MF000591	-
"Pseudendoclonium"	*fucicola*	CCMP 1671	AF499662	-	AF499678
Pseudendoclonium	*submarinum*		EF591129	-	-
Pseudopleurococcus	*printzii*	SAG 467-1	MF000573	MF000592	-
Sarcinofilum	*mucosum*	SAG 24.93	KM020139	MF000597	-
Smithsoniella	*earleae*	UTEX 2178	JF680958	-	-
Tellamia	*contorta*		AF499663	-	AF499679
Ulothrix	*zonata*	UTEX 745	KU865575	AY454424	-
Ulva	*flexuosa*	C47	AB425963	-	-
Ulva	*lactuca*	C208	AB425960	-	-
Ulva	*limnetica*	P36	AB425959	-	AB425968
Ulvella	*endozoica*	UTEX 2352	AY205327	AY454412	-
Ulvella	*tongshanensis*	FACHB 1780	KM226211	KM226208	KM226206
Ulvella	*viridis*	MA1.2a1	AY303594	AY454407	-

Table 6 GenBank accession numbers of the sequences used in the phylogenetic analysis of the Kornmanniaceae.

Genus	Species	Isolate	SSU	ITS
Blidingia	*dawsonii*	isolate 5		DQ001138
Blidingia	*minima*	NomG	-	AF163110
Ctenocladus	*circinnatus*	CCMP 2158		MF034603
Halofilum	*helgolandicum*	SAG 2.95		MF034635
Halofilum	*ramosum*	SAG 2235		MF034617
Halofilum	*ramosum*	ULVO-19		MF034621
Halofilum	*salinum*	SAG 1.95		MF034634
Kornmannia	*leptoderma*		AF499661	AF415168
Lithotrichon	*pulchrum*	SAG 2038		MF034614
Paulbroadya	*prostrata*	CCAP 415-4		MF034612
Paulbroadya	*petersii*	SAG 2240		MF034620
Pseudendoclonium	*arthropyreniae*	SAG 467-2		KM020041
Pseudendoclonium	*commune*	ULVO-62		MF034626
Pseudendoclonium	*commune*	SAG 2236		MF034618
Pseudendoclonium	*fucicola*		AF499662	-
Pseudendoclonium	*incrustans*	CCAP 415/1		MF034610
Pseudendoclonium	*submarinum*	SAG 2237		MF034619
Tellamia	*contorta*		AF499663	-

Paper 4

The curious case of *Cladonia luteoalba*: no support for its distinction

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Abstract

Cladonia luteoalba shows a specific pattern in chemical variability. Its chemotype coincides with that of the associated *Cladonia* thalli. This has led to the formation of various hypotheses, but its true nature has never been clarified. We collected *C. luteoalba* in Central Europe and Norway. The chemotypes were detected by TLC and the mycobionts and photobionts were identified by Sanger sequencing of ITS rDNA. Mycobiont cultures were obtained and Illumina metabarcoding of the fungal ITS1 rDNA region was performed targeting minor mycobionts within the thalli. None of the methods supported *C. luteoalba* as a distinct *Cladonia* species. In phylogenetic analyses, it was placed in *C. straminea* and the *C. coccifera* agg., following the pattern in chemistry. No minor *Cladonia* were detected by metabarcoding or cultivation. Thus, *C. luteoalba* remains enigmatic as our data did not support its distinction as a separate *Cladonia* species.

Key words: *Asterochloris*, chemotypes, lichen, metabarcoding, mycobiont culture, phylogeny, Sanger sequencing

1. Introduction

Cladonia luteoalba A. Wilson & Wheldon (Wheldon & Wilson 1907) is a recognizable species, characterized by large primary squamules with a yellow cottony-arachnoid lower surface, particularly conspicuous when they dry and recurve. It only rarely forms podetia, which are escyphose or very narrowly scyphose, covered with a yellow cottony hyphal layer (Stenroos 1990). It is usually found on soil or rocks, growing among other *Cladonia* species, particularly from the clade *Erythrocarpae* (former section *Cocciferae*; Stenroos *et al*. 2019), sometimes even on their podetia. *Cladonia luteoalba* is rare but distributed worldwide, from Southern Patagonia to Svalbard, mainly with an arctic boreal distribution in Eurasia and North America (e.g., Stenroos 1990, Elvebakk & Hertel 1996, Ahti et al. 2013).

Cladonia luteoalba was called enigmatic by Stenroos (1990) due to a peculiar pattern in its chemical variability. Remarkably, its chemotype corresponds to the chemotype of the associated *Cladonia* species. Thalli associated with *C. coccifera* (L.) Willd. and related species produce zeorin (with

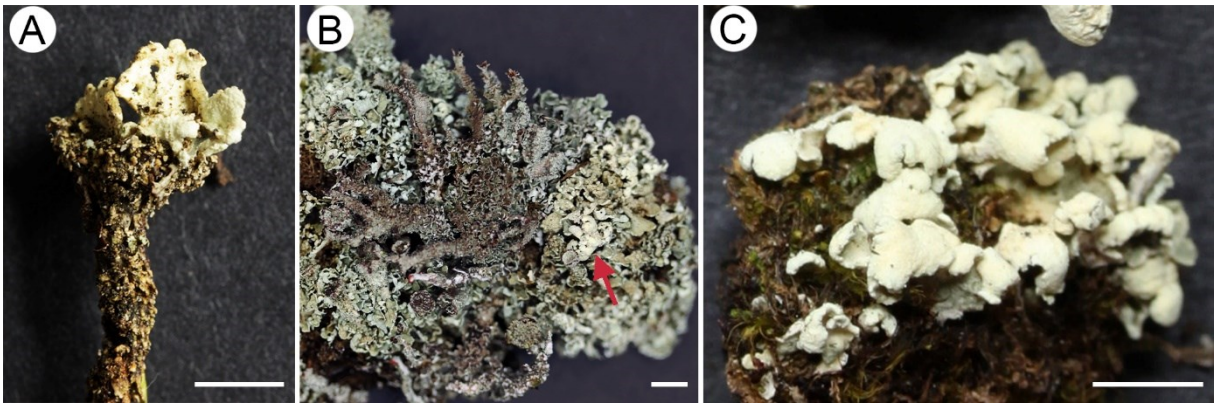

Figure 1 *Cladonia luteoalba* thalli. **A**, C. luteoalba squamule on a podetium of *C. coccifera*. **B**, *C. luteoalba* (arrow) growing among other *Cladonia* species. **C**, C. luteoalba with no associated *Cladonia* thalli. Scales = 5 mm.

accessory compounds, chemotype 1), thalli associated with *C. straminea* (Sommerf.) Flörke. produce squamatic acid (with accessory compounds, chemotype 2) and those associated with *C. borealis* S. Stenroos produce barbatic acid with accessory compounds (chemotype 3). Stenroos suggested three possible explanations (Stenroos 1990): mechanical hybridization, a commensalistic symbiosis system of two mycobionts with one photobiont, and a disease that induces morphological changes to other *Cladonia* species, considering the second option the most plausible. In that scenario, initially lichenicolous *C. luteoalba* parasitizes an existing *Cladonia* thallus, then acquires its photobiont and forms a symbiotic thallus of its own. Based on this hypothesis, *C. luteoalba* has been used as an example of a lichen that obtains its photobiont through theft (Nelsen & Gargas 2009, Dal Grande et al. 2012, Williams et al. 2017).

The status of *C. luteoalba* was doubted, for example by Sandstede (1938) who regarded it as a form of *C. digitata* (L.) Hoffm. The lectotype in BM was revised by Ahti who considered it a synonym of *C. sulphurina* (Michaux) Fr. (as *C. gonecha* (Ach.) Asahina) but further field collections convinced him that *C. luteoalba* was a good species (Ahti 1965). Although the species is generally accepted, the necessity of further studies has been noted (Burgaz et al. 2020, Pino-Bodas et al. 2021).

Sequences from a single specimen of *C. luteoalba* are available in GenBank. A more detailed revision using DNA sequence data could usefully resolve the unknowns. For instance, do the different chemotypes represent a single *C. luteoalba* species? Is it a well-supported *Cladonia* species (i.e. not a morphological change induced by external factors)? Is there any evidence for mechanical hybridization? What photobionts does it associate with? Is its photobiont shared with the associated *Cladonia* thalli?

The aim of this study was to address these questions using multiple approaches. First, we identified the chemotypes of the collected thalli. Second, mycobionts and photobionts of *C. luteoalba* and its associated *Cladonia* thalli were characterized by Sanger sequencing. Third, Illumina metabarcoding and mycobiont cultivation were performed in order to reveal minor mycobionts or possible mechanical hybrids.

2. Materials and Methods

2.1 Sampling

Altogether 38 *Cladonia luteoalba* thalli (Fig. 1) were collected at 21 sites, 29 in Norway (14 collection sites), eight in Czechia (six sites) and one in Poland. Twenty-five specimens were growing in close contact with thalli of other *Cladonia* species (Fig. 1B), and two grew directly on the top of *C. coccifera* podetia (Fig. 1A). In Norway, *C. luteoalba* was found mostly on acidic soil in open habitats or on the upper horizontal surfaces of large boulders. A single epiphytic specimen was found on the trunk of a pine tree. In Central Europe, it was found exclusively in boulder screes in mountain areas. The most closely associated *Cladonia* thalli, together with other *Cladonia* species at certain localities, not in

direct contact with *C. luteoalba* but at a maximum of 30 cm away, were also collected. Three of them were used as controls for metabarcoding (see below). *Cladonia luteoalba* specimens are encoded LUTxx, the associated *Cladonia* thalli LUTxx-A and control thalli LUTxx-C. All collection data are presented in Supplementary Material Table S1 (available online); specimens are deposited in PRC.

2.2 Secondary chemistry

To determine chemotypes of *C. luteoalba* thalli, standard thin-layer chromatography (TLC) in solvent systems A, B and C was performed following Orange et al. (2010).

2.3 Sanger sequencing: DNA extraction, amplification and sequence analyses

DNA was extracted using the CTAB protocol (Cubero et al. 1999), with freezing prolonged to 30 min after isopropanol precipitation and an additional washing step with 96% ethanol. A single *Cladonia* squamule was used for each extraction. Fungal nuclear ITS rDNA was amplified using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Algal nuITS rDNA was amplified using the forward primers Zeleny_F2 (Moya et al. 2018) or nr-SSU-1780 (Piercey-Normore & DePriest 2001) with the reverse primer ITS4. The PCR conditions were as in Škvorová et al. (2022). PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). GenBank Accession numbers of the newly obtained sequences are given in Table 1 and Supplementary Material Table S2 (available online).

Mycobiont ITS sequences newly obtained from *C. luteoalba* and their associated *Cladonia* thalli (Table 1) were aligned with the sequences of related species. These were selected based on BLAST searches and included *C. straminea* and zeorin-containing red-fruited *Cladonia* species (Steinová et al. 2013), viz. *C. coccifera*, *C. deformis* (L.) Hoffm., *C. diversa* Asperges. and *C. pleurota* (Flörke) Schaer., referred to here as the *C. coccifera* aggregate. The sequences were downloaded from GenBank and additional *C. straminea* sequences were produced (see Supplementary Material Table S2). The dataset was aligned with MAFFT v. 7 (Katoh *et al*. 2019), using the Q-INS-I method and manually checked. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and eliminated. The final alignment consisted of 529 positions and 48 unique sequences, including *C. divaricata* used as the outgroup. Substitution models estimated with jModelTest v. 2.1.4 (Darriba et al. 2012) using Bayesian Information Criterion were K80 for ITS1, JC for 5.8S and K80 for ITS2.

The newly obtained ITS photobiont sequences were aligned with *Asterochloris* Tschermak-Woess sequences downloaded from GenBank (Supplementary Material Table S3, available online), based on

Table 1 Mycobiont and photobiont identification of *Cladonia luteoalba* and their associated *Cladonia* thalli. Samples codes are listed with GenBank Accession numbers of the newly obtained ITS sequences, and respective chemotype and locality data (for more collection data see Supplementary Material Table S1 (available online). *Cladonia luteoalba* specimens are encoded LUTxx, the associated *Cladonia* thalli LUTxx-A and control thalli LUTxx-C.

	Mycobiont			Photobiont		
Sample	lineage[a]	GB[b] Acc. no.	Chemo-type[c]	species	GB[b] Acc. no.	Locality (site code)
LUT19/1	*C. coccifera* agg. lin. 1	OM914247	1 (C-P)	*A. aff. italiana*	OM914199	Norway, Østfold, Lilleby (NO-01)
LUT19/1-A	*C. coccifera* agg. lin. 1	OM914248	1 (C-P)	*A. aff. italiana*	OM914200	Norway, Østfold, Lilleby (NO-01)
LUT19/2	*C. coccifera* agg. lin. 1	OM914249	1 (C-P)	*A. leprarii*	OM914201	Norway, Østfold, Lilleby (NO-01)
LUT19/2-A	*C. coccifera* agg. lin. 1	OM914250	1 (C-P)	n/a		Norway, Østfold, Lilleby (NO-01)
LUT19/3	*C. straminea*	OM914251	2	*A. irregularis*	OM914202	Norway, Østfold, Lilleby (NO-02)
LUT19/4	*C. straminea*	OM914252	2	*A. glomerata*	OM914203	Norway, Østfold, Lilleby (NO-02)
LUT19/5	*C. coccifera* agg. lin. 1	OM914253	1 (P)	*A. italiana*	OM914204	Norway, Østfold, Lilleby (NO-01)
LUT19/6	*C. coccifera* agg. lin. 1	OM914254	1 (C-P)	*A. stereocaulonicola*	OM914205	Norway, Rogaland, Vikeså (NO-03)
LUT19/7	*C. coccifera* agg. lin. 1	OM914255	1 (P)	*A. italiana*	OM914206	Norway, Rogaland, Vikeså (NO-04)
LUT19/8	*C. coccifera* agg. lin. 1	OM914256	1 (C-P)	*A. italiana*	OM914207	Norway, Rogaland, Vikeså (NO-04)
LUT19/8-A	*C. coccifera* agg. lin. 1	OM914257	1 (C-P)	*A. italiana*	OM914208	Norway, Rogaland, Vikeså (NO-04)
LUT19/9	*C. coccifera* agg. lin. 1	OM914258	1 (C-P)	*A. italiana*	OM914209	Norway, Rogaland, Vikeså (NO-04)
LUT19/10	*C. coccifera* agg. lin. 1	OM914259	1 (C-P)	*A. italiana*	OM914210	Norway, Rogaland, Vikeså (NO-04)
LUT19/10-A	*C. coccifera* agg. lin. 1	OM914260	1 (P)	*A. italiana*	OM914211	Norway, Rogaland, Vikeså (NO-04)
LUT19/11	*C. coccifera* agg. lin. 1	OM914261	1 (C-P)	*Asterochloris* sp. StA3	OM914212	Norway, Rogaland, Vikeså (NO-05)
LUT19/12	*C. coccifera* agg. lin. 1	OM914262	1 (C-P)	*A. italiana*	OM914213	Norway, Rogaland, Lyngaland (NO-06)
LUT19/12-C	*C. coccifera* agg. lin. 2	OM914263	1	n/a		Norway, Rogaland, Lyngaland (NO-06)
LUT19/14	*C. coccifera* agg. lin. 2	OM914264	1 (P)	*Asterochloris* sp. StA3	OM914214	Norway, Rogaland, Paddevatnet (NO-07)
LUT19/15	*C. coccifera* agg. lin. 2	OM914265	1 (C-P)	*Asterochloris* sp. StA3	OM914215	Norway, Rogaland, Paddevatnet (NO-07)
LUT19/16	*C. coccifera* agg. lin. 2	OM914266	1 (C-P)	*A. italiana*	OM914216	Norway, Rogaland, Paddevatnet (NO-07)
LUT19/17	C. *coccifera* agg. lin. 2	OM914267	1	*A. irregularis*	OM914217	Norway, Rogaland, Blåsjø (NO-08)
LUT19/17-C1	*C. borealis*	OM914268	3	*A. irregularis*	OM914218	Norway, Rogaland, Blåsjø (NO-08)
LUT19/17-C2	C. *coccifera* agg. lin. 2	OM914269	1	*A. irregularis*	OM914219	Norway, Rogaland, Blåsjø (NO-08)
LUT19/19	C. *coccifera* agg. lin. 2	OM914270	1 (P)	*A. glomerata*	OM914220	Norway, Rogaland, Blåsjø (NO-09)
LUT19/19-A	C. *coccifera* agg. lin. 2	OM914271	1 (P)	*A. glomerata*	OM914221	Norway, Rogaland, Blåsjø (NO-09)
LUT19/20	C. *coccifera* agg. lin. 2	OM914272	1 (P)	*A. irregularis*	OM914222	Norway, Rogaland, Blåsjø (NO-09)
LUT19/20-A	*C. borealis*	OM914273	3	*A. irregularis*	OM914223	Norway, Rogaland, Blåsjø (NO-09)
LUT19/21	C. *coccifera* agg. lin. 2	OM914274	1 (P)	*A. irregularis*	OM914224	Norway, Rogaland, Blåsjø (NO-10)

LUT19/21-A	C. *coccifera* agg. lin. 2	OM914275		1	*A. irregularis*	OM914225	Norway, Rogaland, Blåsjø (NO-10)
LUT19/22	C. *coccifera* agg. lin. 2	OM914276		1	*A. glomerata*	OM914226	Norway, Rogaland, Blåsjø (NO-10)
LUT19/23	C. *coccifera* agg. lin. 2	OM914277		1	*A. irregularis*	OM914227	Norway, Rogaland, Birkelandsvegen (NO-11)
LUT19/24	C. *coccifera* agg. lin. 2	OM914278		1	*A. irregularis*	OM914228	Norway, Rogaland, Birkelandsvegen (NO-11)
LUT19/24-A	C. *coccifera* agg. lin. 2	OM914279		1	*A. irregularis*	OM914229	Norway, Rogaland, Birkelandsvegen (NO-11)
LUT19/25	C. *coccifera* agg. lin. 2	OM914280		1	*A. irregularis*	OM914230	Norway, Rogaland, Birkelandsvegen (NO-11)
LUT19/26	C. *coccifera* agg. lin. 2	OM914281		1	*A. irregularis*	OM914231	Norway, Rogaland, Birkelandsvegen (NO-11)
LUT19/28	C. *coccifera* agg. lin. 1	OM914282		1	*A. irregularis*	OM914232	Norway, Hordaland, Dyrskar (NO-12)
LUT19/29	C. *coccifera* agg. lin. 1	OM914283		1	*A. irregularis*	OM914233	Norway, Hordaland, Dyrskar (NO-12)
LUT19/30	C. *coccifera* agg. lin. 2	OM914284		1	*A. irregularis*	OM914234	Norway, Hordaland, Dyrskar (NO-12)
LUT19/31	C. *coccifera* agg. lin. 2	OM914285		1	*A. irregularis*	OM914235	Norway, Rogaland, Øvre Moen (NO-13)
LUT19/33	C. *coccifera* agg. lin. 1	OM914286	1 (P)		*A. irregularis*	OM914236	Norway, Telemark, Froland-Døkki (NO-14)
LUT19/34	C. *coccifera* agg. lin. 2	OM914287		1	*A. irregularis*	OM914237	Czechia, Krkonoše, Sněžka (CZ-01)
LUT19/35	C. *coccifera* agg. lin. 2	OM914288		1	*A. irregularis*	OM914238	Czechia, Krkonoše, Sněžka (CZ-01)
LUT19/36	C. *coccifera* agg. lin. 2	OM914289		1	*A. irregularis*	OM914239	Czechia, Krkonoše, Sněžka (CZ-01)
LUT19/37	*C. straminea*	OM914290		2	*A. glomerata*	OM914240	Czechia, Šumava, Povydří (CZ-02)
LUT19/37-A	*C. straminea*	OM914291		2	*A. irregularis*	OM914241	Czechia, Šumava, Povydří (CZ-02)
LUT20/1	*C. straminea*	OM914292		2	*A. glomerata*	OM914242	Czechia, Šumava, Povydří (CZ-03)
LUT21/1	*C. straminea*	OM914293		2	*A. irregularis*	OM914243	Czechia, Šumava, Obří hrad (CZ-04)
LUT21/2	*C. straminea*	OM914294		2	*A. irregularis*	OM914244	Czechia, Šumava, Buzošná (CZ-05)
LUT-JS863	C. *coccifera* agg. lin. 2	OM914295		1	*A. irregularis*	OM914245	Czechia, Krkonoše, Luční hora (CZ-06)
LUT-JS864	C. *coccifera* agg. lin. 2	OM914296		1	*A. irregularis*	OM914246	Poland, Karkonosze, Mały Szyszak (PL-01)

[a] see Fig. 2; [b] GenBank; [c] chemotype 1 = zeorin with accessory porphyrillic (P) and conporpyrillic (C) acid, chemotype 2 = squamatic and didymic acid, chemotype 3 = barbatic acid.

the datasets of Škaloud & Peksa (2010), Kim et al. (2020) and Vančurová et al. (2018, 2020). To increase phylogenetic resolution, actin type I sequences were also downloaded from GenBank and processed as above. The two markers gave congruent topologies so were concatenated. *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner was used as the outgroup. The alignment was processed as above and finally consisted of 71 unique sequences, and 498 ITS and 516 actin positions. The estimated substitution models were K80 + G for ITS1, JC for 5.8S, TrNef + G for ITS2 and K80 + I + G, TrNef + G and K80 + G for the first, second and third actin positions, respectively.

The phylogenetic trees were inferred by Bayesian inference in MrBayes v. 3.2.6 (Ronquist et al. 2012) using partitioned datasets. Two parallel MCMC runs, with one cold and three heated chains, were carried out, sampling the trees and parameters every 100 generations. Convergence of the chains was verified by the convergent diagnostic of the potential scale reduction factor (PSFR) using the sump option, and it approached 1 in all cases. Convergence of the two cold chains was assessed by the average standard deviation of split frequencies (SDSF). It was 0.005 and 0.001 after 15 million generations for the photobiont and mycobiont, respectively. The first 25% of the trees was discarded as burn-in in each run. A 50% majority-rule consensus tree was obtained using the sumt option. Bootstrap analyses were performed by maximum likelihood (ML) using GARLI v. 2 (Zwickl 2006) on partitioned datasets, specified as above, consisting of 500 rapid bootstrap inferences with automatic termination. Other GARLI parameters were set to default.

Interaction networks were created using the package *bipartite* (Dormann et al. 2009) in the free software R v. 4.1.0 (R Core Team 2021).

2.4 Illumina metabarcoding and bioinformatics

To reveal possible multiple *Cladonia* mycobionts in *C. luteoalba* thalli, Illumina metabarcoding of the fungal ITS1 rDNA region was carried out. Six *C. luteoalba* samples and three control *Cladonia* samples of various chemotypes and from different localities (Supplementary Material Tables S1 & S4) were included. Amplicons for Illumina MiSeq sequencing were generated using the newly designed barcoded primers ITS1_NGS_Cladonia_forward (5'-barcode-TGC GGA AGG ATC ATT AAT GAG-3') and ITS1_NGS_Cladonia_reverse (5'-barcode-AGA TCC GTT GTT GAA AGT TTT-3'). These fungal primers were primarily designed to discriminate in favour of *Cladonia*. In a pilot study (data not shown), they did not amplify *Cladonia* exclusively but they effectively increased the ratio of *Cladonia* sequences compared to other fungi. Therefore, the composition of the fungal community obtained using these primers is highly biased and the results obtained mainly serve the purpose of seeking *Cladonia* sequences.

PCRs were performed in a volume of 20 µl, each reaction containing 10 µl of Q5 High-Fidelity DNA polymerase (BioLabs Inc.), 5 µl of sterile water, 1.5 µl of each primer and 2 µl of DNA. Each sample was run in three replicates and three PCR negative controls (PNC) were included. PCR conditions were as follows: initial denaturation at 98 °C for 30 s, 35 cycles of 98 °C denaturation for 10 s, 52° C amplification for 45 s and 72 °C elongation for 1 min, with a final 72 °C extension for 2 min. The PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter), pooled equimolarly and sent for library preparation and sequencing to Fasteris (Plan-les-Ouates, Switzerland). Sequencing was performed on the Illumina MiSeq platform with paired end mode (2 × 300 bp).

Quality control analysis of the Illumina MiSeq paired-end reads was performed using FastQC v. 0.11.8 (Andrews 2010). Raw reads were processed according to the pipeline published by Báilint et al. (2014), including quality filtering, paired-end assembly, removing primer artifacts, extracting reads by barcodes, reorienting reads to 5'-3', demultiplexing, dereplicating, OTU clustering (this step carried out using Swarm v. 2 (Mahé et al. 2015), with denoising set to d = 3) and chimera filtering. Only the OTUs that had more than 100 reads in at least two of the three replicates were considered further. Fungal OTUs were identified by BLAST searches (excluding uncultured/environmental sample sequences) in SEED2 (Větrovský et al. 2018).

2.5 Mycobiont culturing

In order to capture possible multiple mycobionts in *C. luteoalba* thalli, isolates for culturing were prepared from selected specimens of both chemotypes. Under a stereomicroscope, tiny pieces of either medulla or the arachnoid lower surface were extracted with a sterile needle and placed onto cultivation media. Sabouraud 2% medium (SAB), malt-yeast extract medium (MYA) and Bold's Basal Medium (BBM) with 1% glucose were prepared following the instructions in Stocker-Wörgötter & Hager (2008). Fifty plates were inoculated per thallus and were incubated at 16.5 °C with a 12 h of light/dark regime. After six weeks, the plates were checked and morphologically identified mycobiont isolates were reinoculated onto fresh media. Their identity was subsequently confirmed by obtaining nuITS rDNA sequences as described above. Three to ten isolates were obtained per thallus, with the exception of LUT-JS863 from which we obtained 21 mycobiont isolates. Twelve of the LUT-JS863 cultures were selected for sequencing, while all the cultures were sequenced from the other specimens.

3. Results

3.1 Chemistry

The *Cladonia luteoalba* specimens belonged to two chemotypes (Table 1): chemotype 1 containing zeorin was found in 32 specimens, 17 of which contained porphyrillic acid and 10 also conporphyrillic acid, with the majority also containing an unidentified accessory compound; six specimens were of chemotype 2 containing squamatic and didymic acids. All samples contained usnic acid. The chemotype of the most closely associated *Cladonia* thallus was the same in all cases examined, with one exception (LUT19/20 contained zeorin and the associated thallus barbatic acid, i.e. chemotype 3). No geographical pattern in chemotype occurrence was observed (longitude, latitude or altitude; data not shown).

3.2 Mycobionts

No unique sequence belonging to *C. luteoalba* that would distinguish it from related *Cladonia* species was obtained. The ITS rDNA sequences obtained by Sanger sequencing were identical to and grouped with the corresponding *Cladonia* species or species complex defined by the chemotypes (Fig. 2); specifically, squamatic acid-containing specimens belonged to *C. straminea*, and zeorin-containing specimens were placed in various lineages of the *Cladonia coccifera* agg., which includes the morphospecies *C. coccifera*, *C. deformis*, *C. diversa* and *C. pleurota* that are indistinguishable based on DNA sequence data, as shown previously (Steinová et al. 2013).

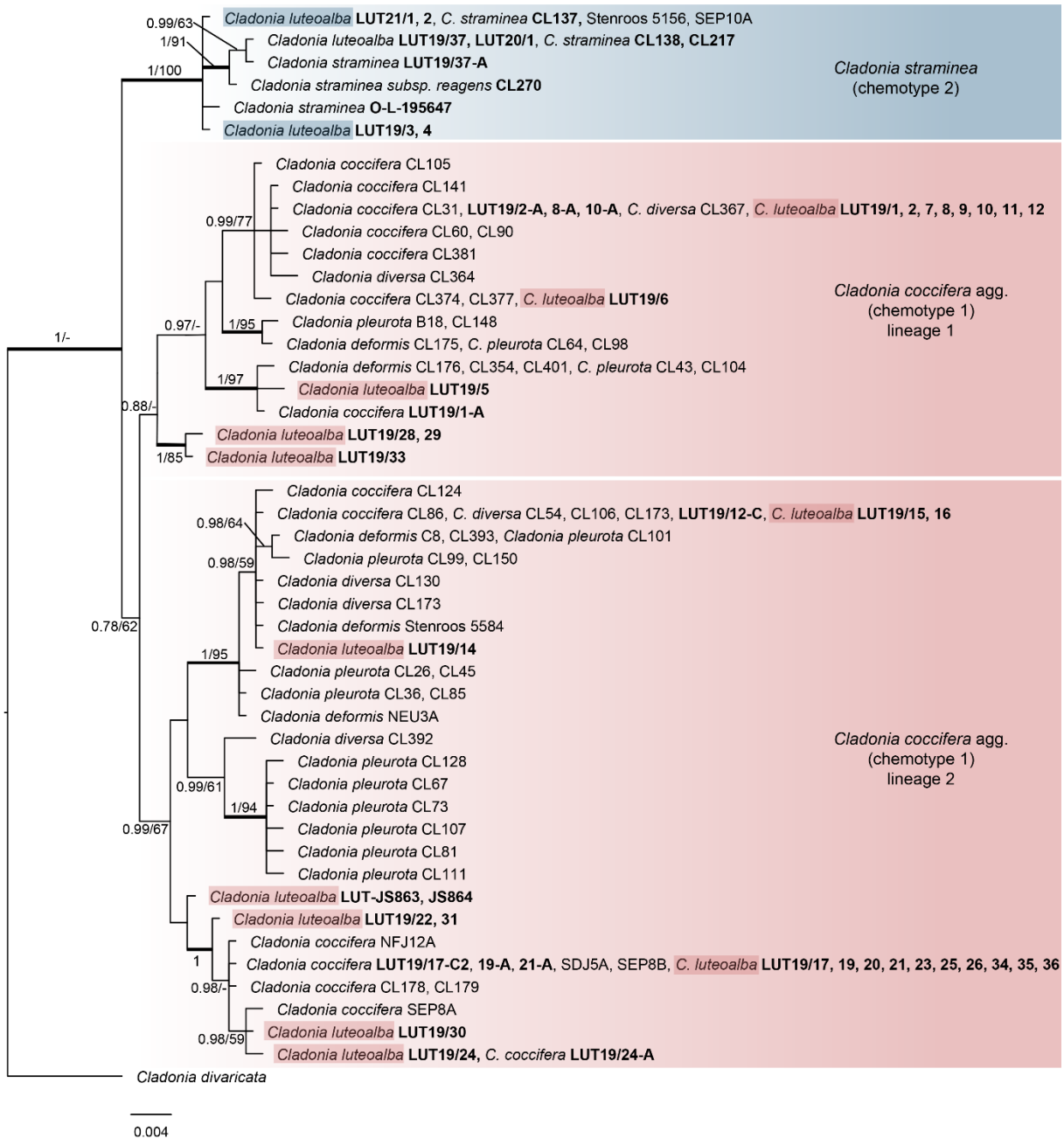

Figure 2 Phylogenetic relationship of *Cladonia luteoalba* (highlighted) and related taxa obtained by Bayesian inference of ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/maximum likelihood bootstrap. Only statistical supports with PP > 0.75 are shown. Thick branches represent nodes with full PP support. Newly obtained sequences are in bold. Shaded areas indicate chemotype and lineage information. Cladonia divaricata is the outgroup. Cladonia luteoalba specimens are encoded LUTxx, the associated Cladonia thalli LUTxx-A and control thalli LUTxx-C. For GenBank Accession numbers see Table 1 and Supplementary Material Table S2

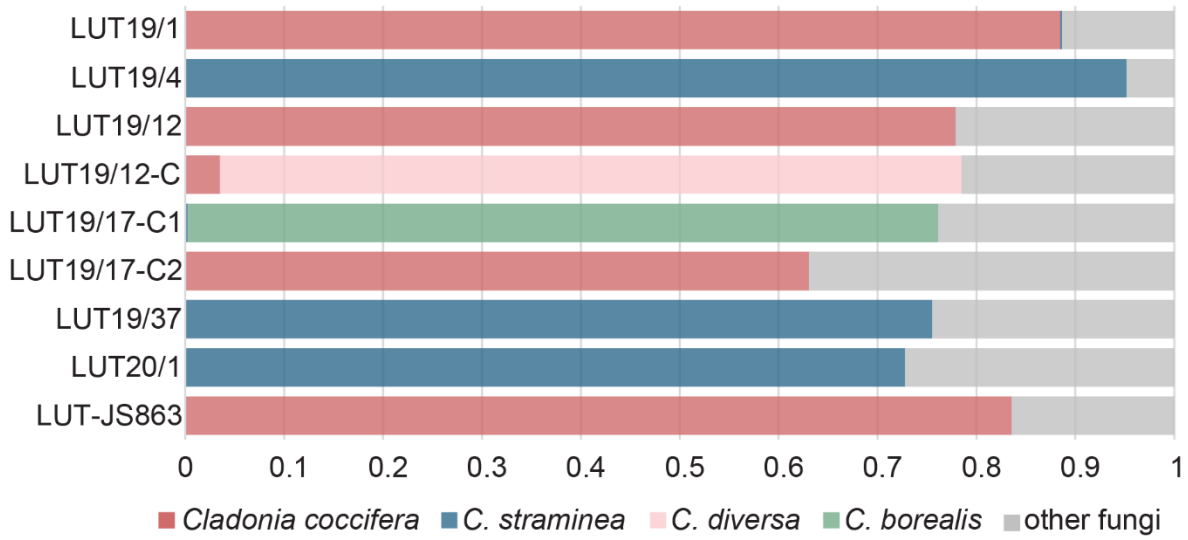

Figure 3 Relative abundances of mycobiont sequences in *Cladonia* thalli as revealed by Illumina metabarcoding. For further details see Supplementary Material Table S4.

Culturing of mycobionts did not result in unique *C. luteoalba* cultures either. We successfully obtained *Cladonia* cultures from six zeorin-containing specimens (LUT19/1, 12, 19, 24, 30 and JS863). Multiple mycobiont cultures obtained from one thallus were always identical in their ITS sequence, which was also always identical to the sequence obtained by Sanger sequencing directly from the lichen thallus.

Illumina metabarcoding did not support the hypothesis that *C. luteoalba* is the result of mechanical hybridization of more *Cladonia* species. A total of 7 582 820 reads passed demultiplexing and subsequently 5 814 251 reads passed filtering. Finally, 132 OTUs passed the criterion of occurrence of more than 100 reads in at least two of the triplicates of a sample, and they represented more than 50 genera (Supplementary Material Table S4, available online). The majority of the OTUs were found in one sample only (101 OTUs). The proportion of *Cladonia* OTUs in each sample is shown in Fig. 3. For each sample, the dominant sequence corresponded to the sequence obtained by Sanger sequencing. An additional *Cladonia* sequence was detected in seven samples. These were at least one or two orders of magnitude lower in abundance than the dominant mycobiont and they were also found in the PCR negative controls, so they should be considered cross-contaminations. Besides *Cladonia*, the most frequent OTU (OTU5, found in the three control samples and three out of six *C. luteoalba* samples) matched an unknown fungus isolated from *Quercus montana* leaf litter (KX908501, 98.7% similarity) and an uncultured fungus from alpine soil (LS958441, 100% similarity).

All the OTUs that gave relevant BLAST search results belonged to *Ascomycetes*, with two *Basidiomycete* exceptions: OTU220 (76.5% similarity to *Erythrobasidium* sp. LC272890) from a control, *C. coccifera* CLZ1; and OTU9 (93.1% similarity to *Tremella diploschistina* Millanes et al., JN790587), recovered from Czech *C. luteoalba* JS863, LUT19/37 and LUT20/1. Other lichenicolous taxa recovered were *Cryptodiscus galaninae* Zhurb. & Pino-Bodas (OTU68; 98.7% similarity to KY661636, in LUT20/1), *Epithamnolia xanthoriae* (Brackel) Diederich & Suija (OTU19; 99.3% similarity

to MT028049, in JS863 and CLZ2) and *Lichenosticta alcicornaria* (Linds.) D. Hawksw. (OTU7; 97.1% similarity to KY661621, in CLZ1). Also, sequences belonging to various lichen species commonly co-occurring in *C. luteoalba* habitats were detected (see Supplementary Material Table S4).

3.3 Photobionts

Photobionts belonging to seven lineages of *Asterochloris* were identified (Fig. 4, Table 1): *A. irregularis* (Hildreth & Ahmadjian) Skaloud & Peksa (24 samples), *A. italiana* (P. A. Archibald) Skaloud & Peksa (9 samples), *A. glomerata* (Waren) Skaloud & Peksa (6 samples), *A. leprarii* Skaloud & Peksa (1 sample), *A. stereocaulonicola* Y. J. Kim *et al*. (1 sample), and two undescribed lineages *Asterochloris* sp. StA3 (3 samples) and *Asterochloris* aff. *italiana* (1 sample), both *sensu* Vančurová et al. (2018).

Cladonia luteoalba shared its photobiont with the most closely associated *Cladonia* thalli in all cases examined, with one exception (*A. glomerata* vs *A. irregularis* in LUT19/37 and the associated *Cladonia* thallus, respectively).

Chemotype 1 of *C. luteoalba* associated with all seven *Asterochloris* species found. Chemotype 2 (*C. straminea*) associated only with *A. irregularis* and *A. glomerata. Asterochloris irregularis, A. italiana* and *Asterochloris* sp. StA3 were shared by both *C. coccifera* agg. lineages. Additionally, *C. coccifera* agg. lineage 1 also associated with *A.* aff. *italiana*, *A. leprarii* and *A. stereocaulonicola*; whereas *C. coccifera* agg. lineage 2 also associated with *A. glomerata* (Fig. 5). This pattern could not be explained by geography, altitude or substratum type (see Supplementary Material Table S1).

4. Discussion

In the genus *Cladonia*, species delimitation and taxonomy are particularly problematic. Phenotypic variability within species is wide and similarity to closely related species high, making it difficult to set boundaries. While phylogenetic studies have been beneficial in some taxa delimitations (e.g., Pino-Bodas et al. 2010*a*, Kanz et al. 2015, Stenroos et al. 2015) they have produced ambivalent results in others. Many taxa have proved to be polyphyletic; however, the authors often discuss the processes underlying low phylogenetic resolution and discrepancies in the molecular data, such as incomplete lineage sorting, unrecognized paralogs, introgression, homoplasy or horizontal gene transfer, and consider their data insufficient to draw taxonomic conclusions (e.g., Piercey-Normore et al. 2010, Steinová et al. 2013, Pino-Bodas et al. 2015). In other cases, phenotypically recognizable taxa were synonymized based on molecular revisions, and differences were attributed to effects of environmental conditions, for example, *C. pocillum* with *C. pyxidata* (L.) Hoffm. (Kotelko & Piercey-Normore 2010) and *C. convoluta* (Lamkey) Anders. with *C. foliacea* (Huds.) Willd. (Pino-Bodas et al. 2010b). The taxonomic value of lichen secondary metabolites is also inconsistent (e.g., Pino-Bodas et al. (2010a) vs Pino-Bodas et al. (2015)).

We collected two out of the four known chemotypes of *Cladonia luteoalba* (Stenroos 1990). These chemotypes correspond to the chemotypes of *C. straminea* (didymic and squamatic acids) and the *C. coccifera* agg. (zeorin and accessory (con-)porphyrillic acid). *Cladonia straminea* is a well-defined monophyletic species (see Fig. 2), while *C. coccifera* agg. includes four morphological species that are indistinguishable based on ITS rDNA and β-tubulin sequence data (Steinová et al. 2013). The phylogenetic placement of the *C. luteoalba* samples coincided with their chemistry. Therefore, not

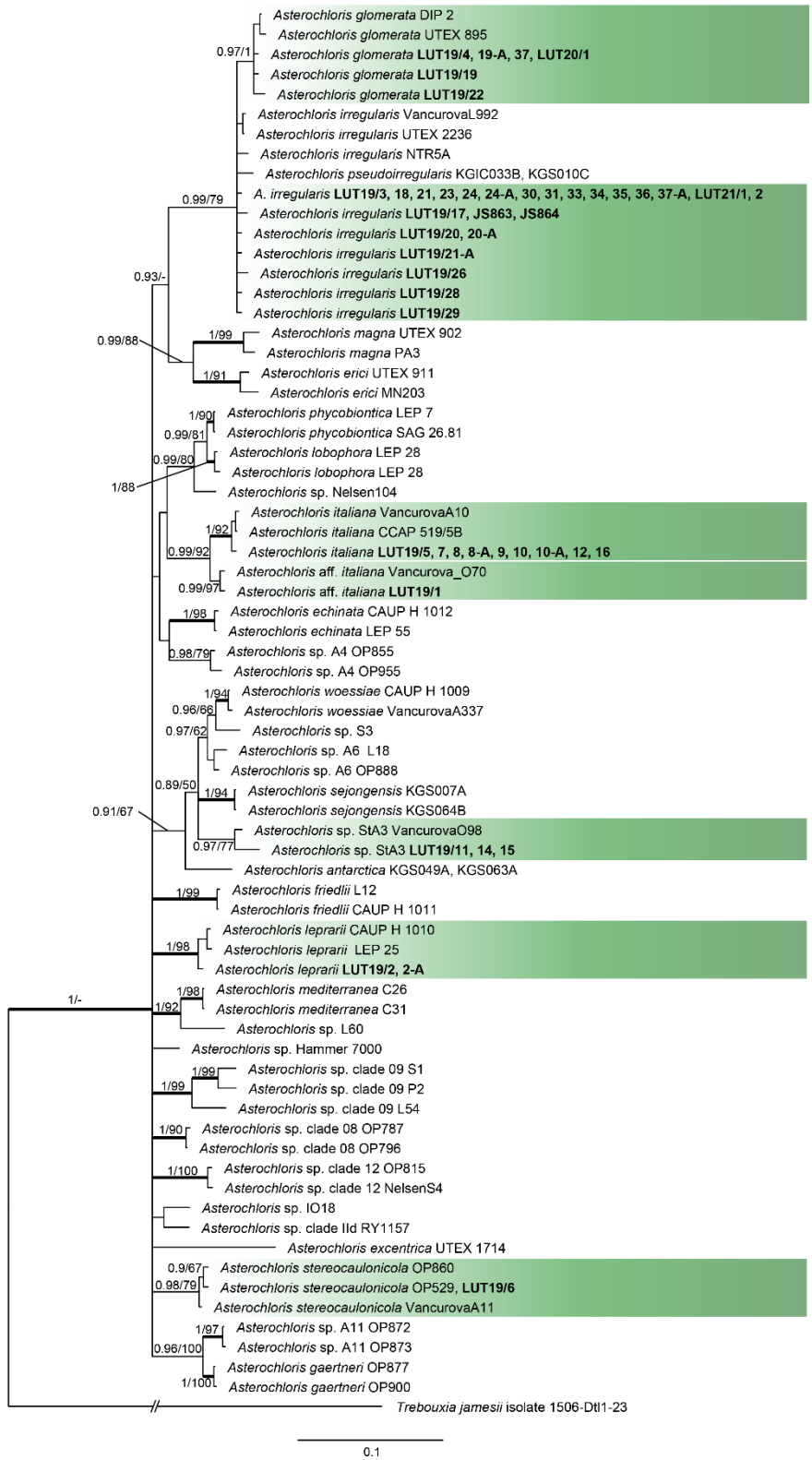

Figure 4 Phylogeny of *Asterochloris* obtained by Bayesian inference of concatenated nuclear ITS rDNA and actin type I. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/maximum likelihood bootstrap. Only statistical support with PP > 0.75 is shown. Thick branches represent nodes with full PP support. Lineages with *C. luteoalba* photobionts are highlighted. Newly obtained sequences are in bold. *Trebouxia jamesii* is the outgroup. *Cladonia luteoalba* specimens are encoded LUTxx, the associated *Cladonia* thalli LUTxx-A and control thalli LUTxx-C. For GenBank Accession numbers see Table 1 and Supplementary Material Table S3.

only is *C. luteoalba* polyphyletic, it also appears to be conspecific with *Cladonia* species of the corresponding chemotypes. It also indistinguishable from the most closely associated *Cladonia* thallus. Commonly, the widely accepted fungal barcode marker ITS rDNA provides poor phylogenetic resolution in the genus *Cladonia* and alternative candidate markers have been suggested (Pino-Bodas et al. 2013). However, within the clade Erythrocarpae even additional markers might not resolve morphologically well-defined species (*C. coccifera* agg., two loci in Steinová et al. (2013), *C. coccifer*a agg. and *C. macilenta-C. floerkeana* agg., five loci in Stenroos et al. (2019), and *C. bellidiflora-C. polydactyla-C. umbricola* complex, five loci in Steinová et al. (2022)), possibly due to low genetic differentiation resulting from recent speciations (Stenroos et al. 2019). Advanced methods, such as microsatellite and RADseq data, are helpful in discriminating closely related species (e.g., *Usnea antarctica* Du Rietz. and *U. auratiacoatra* (Jacq.) Bory; Grewe et al. 2018, Lagostina et al. 2018) and will be essential in building a robust well-resolved phylogeny including a wide sampling of the Erythrocarpae clade that should be the basis for future studies. However, the fact that the *C. luteoalba* phenotype is found in different, not closely related lineages strictly following the pattern in chemotypes makes it unlikely that involvement of such methods would support its existence as a distinctive species.

Other reasons why morphologically well-distinguishable lichens are not supported by molecular data have been reported. Velmala et al. (2009), for example, showed that *Bryoria fremontii* (Tuck.) Brodo & D. Hawksw. and *B. tortuosa* (G. Merr.) Brodo & Hawksw., distinguished by the production of secondary metabolites and thus also colour, are conspecific and the difference between them was later attributed to the presence of associated fungi, specifically *Cystobasidiomycete* yeast (Spribille et al. 2016). Even more striking conspecificity was shown between *Lecanographa amylacea* (Ehrh. ex Pers.) Egea & Torrente (*Arthoniomycetes*) and *Buellia violaceofusca* G. Thor & Muhr (previously placed in *Lecanoromycetes*) and was explained by photobiont switching between *Trentepohlia* and *Trebouxia* (Ertz et al. 2018).

The distinctiveness of *C. luteoalba* was also not supported by mycobiont culturing and DNA metabarcoding. They did not support the hypothesis that *C. luteoalba* is the result of mechanical hybridization and did not reveal any fungal taxon always associated with *C. luteoalba* and never with the other related lichens. However, the possibility that the morphotype is caused by a fungal infection still cannot be ruled out. The primers we used were designed to favour *Cladonia* sequences, thus the PCR bias here is great and the fungal spectrum we obtained cannot be considered representative. Lichenicolous fungi commonly cause morphological changes in the thallus, most conspicuously discolorations or necrotic patches formed by, for example, *Lichenoconium* species (Hawksworth 1977) or colour change of whole *Cladonia* squamules by *Arthrorhaphis aeruginosa* R. Sant. & Tønsberg (Santesson & Tønsberg 1994), and by galls induced by, for example, *Tremella* species (Millanes *et al*. 2012, 2015, Zamora et al. 2018). However, in those cases, the parasite mycelia are visible in cross-sections of the host thalli if fruiting bodies are absent. Galls on lichens are also provoked by invertebrates such as nematodes (Siddiqi & Hawksworth 1982) or mites (Gerson 973). The increased production of usnic acid that causes the yellow colour of the squamule underside suggests a parasite might be involved, since antibiotic, antiviral, antifungal, anti-insect, antiherbivore and other effects of usnic acid have been shown (reviewed by, e.g., Ingólfsdóttir (2002)). Therefore, DNA metabarcoding studies targeting a wide range of organisms (i.e., fungi and bacteria, but also viruses) should be the next step in resolving this enigma.

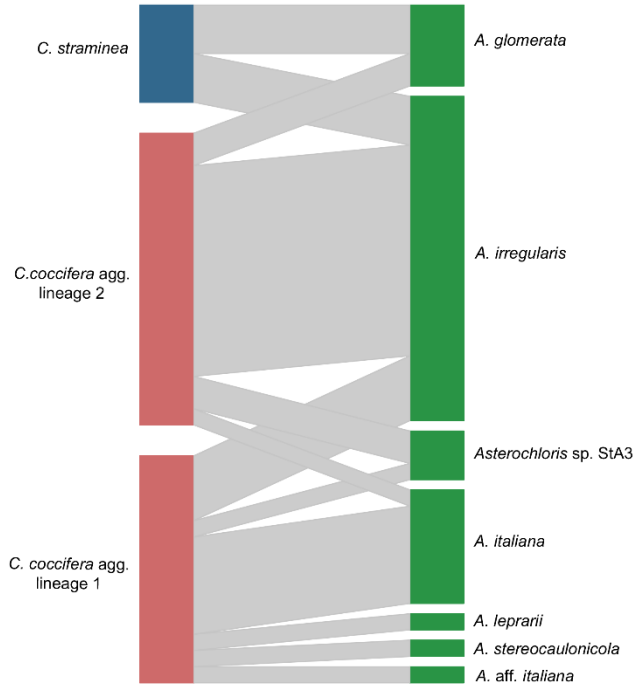

Figure 5 Association network between *Cladonia* mycobiont lineages and *Asterochloris* photobiont species. Link widths are proportional to the number of samples in the association.

The *C. luteoalba* morphotype is obviously not linked to photobiont switching. It shares its photobiont with the closely associated *Cladonia* thalli. Our *C. luteoalba* samples can be divided into two groups based on the *Asterochloris* species they associate with (Fig. 5). The first group included the *C. straminea* genotypes and several representatives of the *C. coccifera* agg.; it associated with *A. glomerata* and *A. irregularis* which are the typical *Cladonia* photobionts of colder climates and acidic substrata, according to Škvorová et al. (2022: module 2 therein). All Central European samples from higher altitudes and more than half of the Norwegian samples belonged to this group (Table 1). The second group included *C. coccifera* agg. representatives, which associated with the other five *Asterochloris* species (see 'Results'). Among them, only two were included in the study of Škvorová et al. (2022): compared to the first group, *A. italiana* represents a photobiont of warmer, wetter and more nutrient-rich habitats, while *A.* aff. *italiana* is of warmer and drier habitats with higher substratum pH (modules 4 and 1, respectively, in Škvorová et al. (2022)). Given the acidic bedrocks and relative climatic uniformity of our Norwegian collection sites, we suggest that microclimatic differences or minor pH variations, caused, for example, by surrounding vegetation, may also play a role in photobiont choice. In any case, no clear patterns between the mycobiont phylogenetic lineages and their associated photobionts were observed in *C. luteoalba.*

In conclusion, our data do not support the existence of *C. luteoalba* as a separate *Cladonia* species. However, neither the holotype (NMW 0000803) nor the lectotype (BM 000006761) contain identifiable associated *Cladonia* species with which *C. luteoalba* could be synonymized. The lectotype contains zeorin (Østhagen 1972) but the taxonomy of the zeorin-containing species of the *C. coccifera* agg. is unclear and requires further revision. Consequently, *C. luteoalba* remains a valid name for now.

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References

Ahti T. (1965) Some notes on British *Cladoniae*. Lichenologist 3: 84-88.

Ahti T., Stenroos S. & Moberg R. (2013) Nordic Lichen Flora Vol. 5 Cladoniaceae. Museum of Evolution, Uppsala University, 117 pp.

Andrews S. (2010) FastQC: a quality control tool for high throughput sequence data. URL https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Báilint M., Schmidt P.-A., Sharma R., Thines M. & Schmitt I. (2014) An Illumina metabarcoding pipeline for fungi. Ecology and Evolution 4: 2642-2653.

Burgaz A. R., Ahti T. & Bino-Bodas R. (2020) Mediterranean Cladoniaceae. Spanish Lichen Society, Madrid, 117 pp.

Castresana J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540-552.

Cubero O. F., Crespo A., Fatehi J. & Bridge P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243-9.

Dal Grande F., Widmer I., Wagner H. H. & Scheidegger C. (2012) Vertical and horizontal photobiont transmission within populations of a lichen symbiosis. Molecular Ecology 21: 3159-3172.

Darriba T., Taboada G. L., Doallo R. & Posada D. (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Dormann C. F., Frueund J., Bluethgen N. & Gruber B. (2009) Indices, graphs and null models: analyzing bipartite ecological networks. Open Ecology Journal 2: 7-24.

Elvebakk A. & Hertel H. (1996) Lichens. In: Elvebakk A. & Prestrud P. (Eds.) A Catalogue of Svalbard Plants, Fungi, Algae and Cyanobacteria. Norsk Polarinstitutt Skrifter, Oslo, pp. 271-359.

Ertz D., Guzow-Krzemińska B., Thor G., Łubek A. & Kukwa M. (2018) Photobiont switching causes changes in the reproduction strategy and phenotypic dimorphism in the Arthoniomycetes. Scientific Reports 8: 4952.

Gardes M. & Bruns T. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Gerson U. (1973) Lichen-arthropod associations. Lichenologist 5: 434-443.

Grewe F., Lagostina E., Wu H., Printzen C. & Lumbsch H. T. (2018) Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*. MycoKeys 43: 91-113.

Hawksworth D. L. (1977) Taxonomic and biological observations on the genus *Lichenoconium* (*Sphaeropsidales*). Persoonia 9: 159-198.

Ingólfsdóttir K. (2002) Usnic acid. Phytochemistry 61: 729-736.

Kanz B., von Brackel W., Cezanne R., Eichler M., Hohmann M.-L., Teuber D. & Printzen C. (2015) Molekulargenetische Untersuchung zum Vorkommen der Rentierflechte *Cladonia stygia* in Hessen. Botanik und Naturschutz in Hessen 28: 5-20.

Katoh K., Rozewicki J. & Yamada K. D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20: 1160-1166.

Kim J. I., Kim Y. J., Nam S. W., So J. E., Hong S. G., Choi H.-G. & Shin W. (2020) Taxonomic study of three new Antarctic *Asterochloris* (*Trebouxiophyceae*) based on morphological and molecular data. Algae 35: 17-32.

Kotelko R. & Piercey-Normore M. D. (2010) *Cladonia pyxidata* and *C. pocillum*; genetic evidence to regard them as conspecific. Mycologia 102: 534-545.

Lagostina E., Dal Grande F., Andreev M. & Printzen C. (2018) The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*. Mycologia 110: 1047-1057.

Mahé F., Rognes T., Quince C., de Vargas C. & Dunthorn M. (2015) Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ 3: e1420.

Millanes A. M., Westberg M., Wedin M. & Diederich P. (2012) *Tremella diploschistina* (Tremellomycetes, Basidiomycota, Fungi), a new lichenicolous species growing on *Diploschistes*. Lichenologist 44: 321-332.

Millanes A. M., Diederich P., Westberg M., Pippola E. & Wedin M. (2015) *Tremella cetrariellae* (Tremellales, Basidiomycota, Fungi), a new lichenicolous fungus on *Cetrariella delisei*. Lichenologist 47: 359-368.

Moya P., Chiva S., Molins A., Jadrná I., Škaloud P., Peksa O. & Barreno E. (2018) *Myrmecia israeliensis* as the primary symbiotic microalga in squamulose lichens growing in European and Canarian terricolous communities. Fottea 18: 72-85.

Nelsen M. P. & Gargas A. (2009) Symbiont flexibility in *Thamnolia vermicularis* (Pertusariales: Icmadophilaceae). Bryologist 112: 404-417.

Orange A., James P. W. & White F. J. (2010) Microchemical Methods for the Identification of Lichens. British Lichen Society, London, 101 pp.

Østhagen H. (1972) The chemical strains in *Cladonia luteoalba* Wils. et Wheld. and their distribution. Norwegian Journal of Botany 19: 37-41.

Piercey-Normore M. D. & DePriest P. T. (2001) Algal switching among lichen symbioses. American Journal of Botany 88: 1490-1498.

Piercey-Normore M. D., Ahti T. & Goward T. (2010) Phylogenetic and haplotype analyses of four segregates within *Cladonia arbuscula* s.l. Botany 88: 397-408.

Pino-Bodas R., Burgaz A. R. & Martín M. P. (2010)a Elucidating the taxonomic rank of *Cladonia subulata* versus *C. rei* (*Cladoniaceae*). Mycotaxon 113: 311-326.

Pino-Bodas R., Martín M. P. & Burgaz A. R. (2010)b Insight into the *Cladonia convoluta-C. foliacea* (Cladoniaceae, Ascomycota) complex and related species, revealed through morphological, biochemical and phylogenetic analyses. Systematics and Biodiversity 8: 575-586.

Pino-Bodas R., Martín M. P., Burgaz A. R. & Lumbsch T. (2013) Species delimitation in *Cladonia* (Ascomycota): a challenge to the DNA barcoding philosophy. Molecular Ecology Resources 13: 1058-1068.

Pino-Bodas R., Burgaz A. R., Martín M. P., Ahti T., Stenroos S., Wedin M. & Lumbsch H. T. (2015) The phenotypic features used for distinguishing species within the *Cladonia furcata* complex are highly homoplasious. Lichenologist 47: 287-303.

Pino-Bodas R., Sanderson N., Cannon P., Aptroot A., Coppins B., Orange A. & Simkin J. (2021) Lecanorales: Cladoniaceae, including the genera *Cladonia*, *Pilophorus* and *Pycnothelia*. Revisions of British and Irish Lichens 19: 1-45.

R Core Team (2021) R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ronquist F., Teslenko M., van der Mark P., Ayres D., Darling A., Hohna S., Larget B., Liu L., Suchard M. A. & Huelsenbeck J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539-542.

Sandstede H. (1938) Erganzungen zu Wainios Monographia Cladoniarum Universalis, unter besonderer Beriicksichtigung des Verhaltens zu Asahinas Diaminprobe. Repertorium Specierum Novarum Regni Vegetabilis 103: 1-103.

Santesson R. & Tønsberg T. (1994) *Arthrorhaphis aeruginosa* and *A. olivaceae*, two new lichenicolous fungi. Lichenologist 26: 295-299.

Siddiqi M. R. & Hawksworth D. L. (1982) Nematodes associated with galls on *Cladonia glauca*, including two new species. Lichenologist 14: 175-184.

Škaloud P. & Peksa O. (2010) Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta). Molecular Phylogenetics and Evolution 54: 36-46.

Škvorová Z., Černajová I., Steinová J., Peksa O., Moya P. & Škaloud P. (2022) Promiscuity in lichens follows clear rules: partner switching in *Cladonia* is regulated by climatic factors and soil chemistry. Frontiers in Microbiology 12: 781585.

Spribille T., Tuovinen V., Resl P., Vanderpool D., Wolinski H., Aime M. C., Schneider K., Stabentheiner E., Toome-Heller M., Thor G., Mayrhofer H., Johannesson H. & McCutcheon J. P. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353: 488-492

Steinová J., Stenroos S., Grube M. & Škaloud P. (2013) Genetic diversity and species delimitation of the zeorin-containing red-fruited *Cladonia* species (lichenized Ascomycota) assessed with ITS rDNA and β-tubulin data. Lichenologist 45: 665-684.

Steinová J., Holien H., Košuthová A. & Škaloud P. (2022) An exception to the rule? Could photobiont identity be a better predictor of lichen phenotype than mycobiont identity? Journal of Fungi 8: 275.

Stenroos S. (1990) *Cladonia luteoalba* – an enigmatic *Cladonia*. Karstenia 30: 27-32.

Stenroos S., Pino-Bodas R., Weckman D. & Ahti T. (2015) Phylogeny of *Cladonia uncialis* (*Cladoniaceae*, *Lecanoromycetes*) and its allies. Lichenologist 47: 215-231.

Stenroos S., Pino-Bodas R., Hyvönen J., Lumbsch H. T. & Ahti T. (2019) Phylogeny of the family *Cladoniaceae* (*Lecanoromycetes*, *Ascomycota*) based on sequences of multiple loci. Cladistics 35: 351-384.

Stocker-Wörgötter E. & Hager A. (2008) Appendix: culture methods for lichens and lichen symbionts. In: Nash III. T. H. (Ed.) Lichen Biology, 2nd edition. Cambridge University Press, New York, pp. 353-363.

Vančurová L., Muggia L., Peksa O., Řídká T. & Škaloud P. (2018) The complexity of symbiotic interactions influences the ecological amplitude of the host: a case study in *Stereocaulon* (lichenized *Ascomycota*). Molecular Ecology 27: 3016-3033.

Vančurová L., Kalníková V., Peksa O., Škvorová Z., Malíček J., Moya P., Chytrý K., Černajová I. & Škaloud P. (2020) Symbiosis between river and dry lands: phycobiont dynamics on river gravel bars. Algal Research 51: 102062.

Velmala S., Myllys L., Halonen P., Goward T. & Ahti T. (2009) Molecular data show that *Bryoria fremontii* and *B. tortuosa* (*Parmeliaceae*) are conspecific. Lichenologist 41: 231-242.

Větrovský T., Baldrian P. & Morais D. (2018) SEED 2: a user-friendly platform for amplicon high-throughput sequencing data analyses. Bioinformatics 34: 2292-2294.

Wheldon J. A. & Wilson A. (1907) The Flora of West Lancashire. Henry Young and Sons, Liverpool, 558 pp.

White T. J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (Eds.) PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, pp. 315-322.

Williams L., Colesie C., Ullmann A., Westberg M., Wedin M. & Büdel B. (2017) Lichen acclimation to changing environments: photobiont switching vs. climate-specific uniqueness in *Psora decipiens*. Ecology and Evolution 7: 2560-2574.

Zamora J. C., Millanes A. M., Etayo J. & Wedin M. (2018) *Tremella mayrhoferi*, a new lichenicolous species on *Lecanora allophana*. Herzogia 31: 666-676.

Zwickl D. J. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. thesis, University of Texas at Austin.

Supplementary material

Table S1 Collection data.

Code	Herbarium number	Locality	GPS coordinates	Altitude	Date	Collectors
LUT19/1	PRC 4875	Norway, Østfold, Lilleby	N59.1987467 E11.249266665	119 m	20 Jul 2019	I. Černajová & J. Steinová
LUT19/2	PRC 4876	Norway, Østfold, Lilleby	N59.1987467 E11.249266665	119 m	20 Jul 2019	I. Černajová & J. Steinová
LUT19/3	PRC 4877	Norway, Østfold, Lilleby	N59.1994292 E11.249102222	115 m	20 Jul 2019	I. Černajová & J. Steinová
LUT19/4	PRC 4878	Norway, Østfold, Lilleby	N59.1994292 E11.249102222	115 m	20 Jul 2019	I. Černajová & J. Steinová
LUT19/5	PRC 4879	Norway, Østfold, Lilleby	N59.1987467 E11.249266665	119 m	20 Jul 2019	I. Černajová & J. Steinová
LUT19/6	PRC 4880	Norway, Rogaland, Vikeså	N58.60825394 E6.11208313	178 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/7	PRC 4881	Norway, Rogaland, Vikeså	N58.608446 E6.112822	178 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/8	PRC 4882	Norway, Rogaland, Vikeså	N58.608446 E6.112822	178 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/9	PRC 4883	Norway, Rogaland, Vikeså	N58.608446 E6.112822	178 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/10	PRC 4884	Norway, Rogaland, Vikeså	N58.608446 E6.112822	178 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/11	PRC 4885	Norway, Rogaland, Vikeså	N58.6095017 E6.11464638889	220 m	23 Jul 2019	I. Černajová & J. Steinová
LUT19/12	PRC 4886	Norway, Rogaland, Lyngaland	N58.7200290 E5.7876219	118 m	24 Jul 2019	I. Černajová & J. Steinová
LUT19/12-C	PRC 4887	Norway, Rogaland, Lyngaland	N58.7200290 E5.7876219	118 m	24 Jul 2019	I. Černajová & J. Steinová
LUT19/14	PRC 4889	Norway, Rogaland, Paddevatnet	N59.1188222 E6.1157297	286 m	25 Jul 2019	I. Černajová & J. Steinová
LUT19/15	PRC 4890	Norway, Rogaland, Paddevatnet	N59.1188222 E6.1157297	286 m	25 Jul 2019	I. Černajová & J. Steinová
LUT19/16	PRC 4891	Norway, Rogaland, Paddevatnet	N59.1188222 E6.1157297	286 m	25 Jul 2019	I. Černajová & J. Steinová
LUT19/17	PRC 4892	Norway, Rogaland, Blåsjø	N59.3776779 E6.7444848	1060 m	26 Jul 2019	I. Černajová & J. Steinová
LUT19/17-C1	PRC 4893	Norway, Rogaland, Blåsjø	N59.3776779 E6.7444848	1060 m	26 Jul 2019	I. Černajová & J. Steinová
LUT19/17-C2	PRC 4894	Norway, Rogaland, Blåsjø	N59.3776779 E6.7444848	1060 m	26 Jul 2019	I. Černajová & J. Steinová
LUT19/19	PRC 4895	Norway, Rogaland, Blåsjø	N59.3777353 E6.7439621	1060 m	26 Jul 2019	I. Černajová & J. Steinová
LUT19/20	PRC 4896	Norway, Rogaland, Blåsjø	N59.3777353 E6.7439621	1060 m	26 Jul 2019	I. Černajová & J. Steinová
LUT19/21	PRC 4897	Norway, Rogaland, Blåsjø	N59.3774048 E6.7430468	1060 m	26 Jul 2019	J. Steinová
LUT19/22	PRC 4898	Norway, Rogaland, Blåsjø	N59.3774048 E6.7430468	1060 m	26 Jul 2019	J. Steinová
LUT19/23	PRC 4899	Norway, Rogaland, Birkelandsvegen	N59.7072831 E6.5386003	540 m	27 Jul 2019	I. Černajová & J. Steinová
LUT19/24	PRC 4900	Norway, Rogaland, Birkelandsvegen	N59.7072831 E6.5386003	540 m	27 Jul 2019	I. Černajová & J. Steinová

LUT19/25	PRC 4901	Norway, Rogaland, Birkelandsvegen	N59.7072831 E6.5386003	540 m	27 Jul 2019	I. Černajová & J. Steinová
LUT19/26	PRC 4902	Norway, Rogaland, Birkelandsvegen	N59.7072831 E6.5386003	540 m	27 Jul 2019	I. Černajová & J. Steinová
LUT19/28	PRC 4904	Norway, Hordaland, Dyrskar	N59.8443171 E7.0571492	1070 m	27 Jul 2019	I. Černajová & J. Steinová
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LUT19/30	PRC 4906	Norway, Hordaland, Dyrskar	N59.8443171 E7.0571492	1070 m	27 Jul 2019	I. Černajová & J. Steinová
LUT19/31	PRC 4907	Norway, Rogaland, Øvre Moen	N59.4163850 E6.7518234	850 m	26 Jul 2019	J. Steinová
LUT19/33	PRC 4909	Norway, Telemark, Froland-Døkki	N59.447797 E7.7795969	578 m	28 Jul 2019	J. Steinová
LUT19/34	Steinová 1009a (PRC)	Czechia, Krkonoše, Sněžka	N50.7355022 E15.7383630	1560 m	15 Sept 2019	J. Steinová
LUT19/35	Steinová 1009b (PRC)	Czechia, Krkonoše, Sněžka	N50.7355022 E15.7383630	1560 m	15 Sept 2019	J. Steinová
LUT19/36	Steinová 1009c (PRC)	Czechia, Krkonoše, Sněžka	N50.7355022 E15.7383630	1560 m	15 Sept 2019	J. Steinová
LUT19/37	PRC 4910	Czechia, Šumava, Povydří	49.0841350N, 13.5113311E	800 m	9 Oct 2019	L. Syrovátková, I. Černajová & J. Steinová
LUT20/1	PRC 4911	Czechia, Šumava, Povydří	49.0843475N, 13.5111381E	830 m	17 Jun 2020	L. Syrovátková, F. Bouda, O. Peksa
LUT21/1	Steinová 1054 (PRC)	Czechia, Šumava, Obří hrad	N49.1017587 E13.5938229	945 m	19 Jun 2021	J. Steinová
LUT21/2	Steinová 1055 (PRC)	Czechia, Šumava, Buzošná	49.1065628N, 13.5863078E	803 m	19 Jun 2021	J. Steinová
LUT-JS863	Steinová 863	Czechia, Krkonoše, Luční hora	50.7311611, 15.6698039	1440 m	12 Sept 2020	J. Steinová
LUT-JS864	Steinová 864	Poland, Karkonosze, Mały Szyszak	N50.7612367 E15.6489511	1370 m	12 Aug 2020	J. Steinová

Table 7 List of taxa used in the phylogenetic analysis of *Cladonia*, their GenBank accession numbers and countries of collection. Newly obtained sequences are given in bold.

Cladonia species	DNA isolation code/voucher	ITS GenBank accession	country
C. coccifera	CL31/Hafellner 66608 (GZU)	HE611155	Austria
C. coccifera	CL60/Peksa 359 (PL)	HE611159	Czechia
C. coccifera	CL86/Steinová 97 (PRC)	KU053046	Czechia
C. coccifera	CL90/Steinová 43 (PRC)	HE611160	Czechia
C. coccifera	CL105/Steinová 401 (PRC)	HE611162	Spain
C. coccifera	CL124/Steinová 160 (PRC)	KU053015	Czechia
C. coccifera	CL141/Steinová 242 (PRC)	HE611163	Austria
C. coccifera	CL178/Steinová 332 (PRC)	HE611171	Norway
C. coccifera	CL179/Steinová 334 (PRC)	HE611172	Finland
C. coccifera	CL374/Steinová 464 (PRC)	KU053021	Norway
C. coccifera	CL377/Steinová 528 (PRC)	KU053022	Wales, UK
C. coccifera	CL381/Orange 20406 (NMW)	KU053011	Wales, UK
C. coccifera	NFJ12A/PRC 4793	OL605180	Norway
C. coccifera	SDJ5A/PRC 4912	OL605381	Sweden
C. coccifera	SEP8A/PRC 4155	OL605400	Sweden
C. coccifera	SEP8B/PRC 4155	OL605401	Sweden
C. deformis	C8/Peksa 918 (PL)	HE611205	Czechia
C. deformis	CL175/Steinová 330 (PRC)	HE611190	Finland
C. deformis	CL176/Steinová 336 (PRC)	HE611186	Finland
C. deformis	CL354/Pentti Alanko 150786 (H)	KU053019	Finland
C. deformis	CL393/Steinová 644 (PRC)	KU053028	Czechia
C. deformis	CL401/Søchting 10. IX. 2013 (C)	KU053031	Denmark
C. deformis	NEU3A/PRC 4182	OL605160	Germany
C. deformis	Stenroos 5584 (TUR)	AF454448	Finland
C. divaricata	Stenroos 4999 (TUR)	AF457910	Brazil
C. diversa	CL54/Bouda 777	HE611164	Czechia
C. diversa	CL106/Steinová 400 (PRC)	HE611165	Portugal
C. diversa	CL173/Steinová 352 (PRC)	HE611168	Belgium
C. diversa	CL364/Steinová 596 (PRC)	KU053013	Germany
C. diversa	CL367/Steinová 635 (PRC)	KU053035	Spain
C. diversa	CL392/Steinová 616 (PRC)	KU053014	Czechia
C. pleurota	B18/Peksa 820 (PL)	HE611191	Slovakia
C. pleurota	CL26/Palice 11305 (PRA)	HE611193	Czechia
C. pleurota	CL36/Hafellner 65635 (GZU)	HE611194	Austria
C. pleurota	CL43/Peksa 562 (PL)	HE611182	Czechia
C. pleurota	CL45/Peksa 563 (PL)	HE611195	Czechia
C. pleurota	CL64/Vondrák 3631 (CBFS)	HE611187	Romania
C. pleurota	CL67/Vondrák 2868 (CBFS)	HE611173	Czechia
C. pleurota	CL73/Peksa 574 (PL)	HE611174	Czechia
C. pleurota	CL81/Lendemer 7139 (NY)	HE611175	USA
C. pleurota	CL85/Steinová 103 (PRC)	HE611196	Czechia
C. pleurota	CL98/Steinová 45 (PRC)	HE611188	Czechia
C. pleurota	CL99/Steinová 99 (PRC)	HE611202	Czechia
C. pleurota	CL101/Steinová 108 (PRC)	HE611203	Czechia

C. pleurota	CL104/Steinová 126 (PRC)	HE611185	Czechia
C. pleurota	CL107/Harris 51548 (NY)	HE611177	USA
C. pleurota	CL111/Harris 52433 (NY)	HE611179	USA
C. pleurota	CL128/Steinová 164 (PRC)	HE611180	Czechia
C. pleurota	CL148/Steinová 241 (PRC)	HE611189	Austria
C. pleurota	CL150/Steinová 187 (PRC)	HE611204	Finland
C. straminea	CL137/Steinová 228 (PRC)	**OM914297**	Austria
C. straminea	CL138/Steinová 278 (PRC)	**OM914298**	Austria
C. straminea	CL217/Steinová 409 (PRC)	**OM914299**	Finland
C. straminea (subsp. reagens)	CL270/Tonsberg 40148 (BG)	**OM914300**	Norway
C. straminea	O-L-195647 (O)	**OM914301**	Norway
C. straminea	SEP10A/PRC 4493	OL605387	Sweden
C. straminea	Stenroos 5156 (TUR)	AF453705	Finland

Table S3 GenBank accession numbers of the taxa used in the phylogeny of *Asterochloris*.

Species	strain/voucher	ITS	actin	Notes
Asterochloris antarctica	KGS063A	MT036574	MT073208	
Asterochloris antarctica	KGS049A	MT036573	MT073207	
Asterochloris echinata	CAUP H 1012/OP186	AM905992	AM906017	
Asterochloris echinata	LEP 55/OP551	FM955667	FM955671	
Asterochloris erici	UTEX 911	AF345440	AM906018	
Asterochloris erici	MN203/Normore 375	AF345442	--	
Asterochloris excentrica	UTEX 1714	AM905993	AM906019	
Asterochloris friedlii	L12/Nelsen 3960	EU008675	EU008704	
Asterochloris friedlii	CAUP H 1011/OP 235	AM905995	AM906021	
Asterochloris gaertneri	OP877	FM955668	FM955672	
Asterochloris gaertneri	OP900	FM955669	FM955673	
Asterochloris glomerata	DIP 2/OP498	AM905998	AM906026	
Asterochloris glomerata	UTEX 895	AF345382	AM906024	
Asterochloris irregularis	VancurovaL992	MH415370	MH382143	
Asterochloris irregularis	UTEX 2236	AF345411	AM906027	
Asterochloris irregularis	NTR5A	OL620560	--	
Asterochloris italiana	VancurovaA10	MH415217	MH382121	
Asterochloris italiana	CCAP 219/5B	AM906001	AM906030	
Asterochloris leprarii	CAUP H 1010/OP183	AM906002	AM906031	
Asterochloris leprarii	LEP 25/OP204	AM906004	AM906033	
Asterochloris lobophora	LEP 28/OP166	AM906010	AM906039	
Asterochloris lobophora	OP866	FN556044	KP318679	
Asterochloris magna	UTEX 902	AM906012	AM906041	
Asterochloris magna	PA3	KP318675	--	
Asterochloris mediterranea	C26	KP257391	KP257358	
Asterochloris mediterranea	C31	KP257396	KP257363	
Asterochloris phycobiontica	LEP 7	AM906013	AM906044	
Asterochloris phycobiontica	SAG 26.81	AM900490	AM906042	
Asterochloris pseudoirregularis	KGS010C	MT036565	MT073199	
Asterochloris pseudoirregularis	KGIC033	MT036564	MT073198	
Asterochloris sejongensis	KGS007A	KX051235	KX051239	
Asterochloris sejongensis	KGS064B	KX051236	KX051240	
Asterochloris stereocaulonicola	OP860	FN556035	FN556048	
Asterochloris stereocaulonicola	OP529	FN556036	--	
Asterochloris stereocaulonicola	VancurovaA11	MH415218	MH382122	
Asterochloris woessiae	CAUP H 1009/Bayerová 3401	AM900492	AM906045	
Asterochloris woessiae	VancurovaA337	MH415238	MH382127	
Asterochloris aff. italiana	VancurovaO70	MH415422	MH382147	1
Asterochloris sp. A4	OP855	FN556031	FN556047	2
Asterochloris sp. A4	OP955	FN556032	--	2
Asterochloris sp. A6	L18/Nelsen 2166a	EU008687	EU008714	2
Asterochloris sp. A6	OP888	FN556033	--	2
Asterochloris sp. A11	OP872	FN556041	FN556050	2
Asterochloris sp. A11	OP873	FN556042	FN556051	2
Asterochloris sp. clade IId	RY1157	DQ482677	--	3

Asterochloris sp. clade 08	OP787	FM945380	FM955675	2
Asterochloris sp. clade 08	OP796	FM945358	FM955674	2
Asterochloris sp. clade 09	S1/Nelsen 2181b	DQ229884	DQ229896	2
Asterochloris sp. clade 09	P2/Nelsen 2233f	DQ229883	DQ229895	2
Asterochloris sp. clade 09	L54/Nelsen 2211a	EU008684	EU008711	2
Asterochloris sp. clade 12	OP815	FM945359	FM955676	2
Asterochloris sp. clade 12	S4/Talbot 12 101	DQ229887	DQ229891	
Asterochloris sp. StA3	VancurovaO98	MH415438	MH382150	1
Asterochloris sp.	Hammer 7000	AF345437	--	
Asterochloris sp.	IO18/Oksanen 186	AF345428	--	
Asterochloris sp.	Nelsen 104/Talbot 400	DQ229882	DQ229893	
Asterochloris sp.	L60/Nelsen 2585	EU008690	EU008715	
Asterochloris sp.	S3/Talbot KIS 187	DQ229886	DQ229897	
Trebouxia jamesii	1506_Dtl1_23	GQ375329	HM573599	4

[1] sensu Vančurová et al. 2018, [2] sensu Peksa & Škaloud 2011, [3] sensu Yahr et al. 2006, [4] outgroup

Table S4 is available online at https://doi.org/10.1017/S002428292200024X.

Paper 5

Niche expansion through photobiont switch: contrary evidence from highly-selective seashore lichen communities

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A manuscript

Abstract

The upper intertidal zone of rocky seashore habitats is commonly dominated by crustose lichens. The nature of the symbiotic interactions that shape these communities is largely unknown. We sampled seashore Verrucariaceae lichens along a salinity gradient in Northern Europe and identified both mycobionts and photobionts based on DNA sequence data. Free-living algal communities from the lichens' proximity were screened by Illumina metabarcoding. The photobionts belonged to Kornmanniaceae, Ulvales and *Urospora*, Ultorichales. The photobionts were not dominant, but always present also in the free-living algal communities. The lichens were highly selective in their photobiont choice, considering the available algal pool. Individual mycobiont species varied in their specificity level. *Hydropunctaria maura*, a cosmopolitan lichen occupying wide ecological niche, showed low specificity but high selectivity. Our data suggest that environmental factors are not the main drivers of the symbiont pairing in seashore lichens; and also contradict the niche widening via photobiont switch.

Keywords: free-living algae, *Hydropunctaria*, symbiotic interactions, metabarcoding, *Pseudendoclonium*, *Urospora*, *Verrucaria*

1. Introduction

Lichens inhabit virtually any terrestrial ecosystem and habitat on the planet – from tropical to polar regions, from lowlands to highest mountains, from rainforests to deserts, from the ground to the highest treetops, from natural substrates to rusty tractors and thrown-away rubber. They often represent early colonizers and serve numerous ecosystem services, for example, soil stabilization (reviewed e.g., by Rosentreter et al. 2016), rock weathering, pedogenesis and related biogeochemical processes (reviewed e.g., by Jones 1988), modification of ecosystem composition through filtering

plant seedlings during primary succession (Asplund and Wardle 2016) and food and/or shelter provision (e.g., Baur et al. 1994, Lalley et al. 2006) and finally, they provide substrate for microscopic organisms of diverse functions (Nash III. 2012). The significance of these roles presumably rises in lichen-dominated habitats.

Some lichens, mainly within Verrucariaceae (Eurotiomycetes) are capable of life in semi-aquatic or even aquatic habitats. Although they often dominate rocky seashores (Dobson 2014), they are seriously understudied. The diversity of the symbionts, the dynamics of the symbiotic interaction and the effects of environmental conditions on them are still largely unknown. Fundamental questions, such as where the symbiotic algae are recruited from or how strict is the specificity among the symbionts have never been studied in aquatic and amphibious lichens.

Seashore habitats are one of the most threatened by the global climate change (Strandmark et al. 2015). Addressing the above issues is crucial for our understanding of the future development of seashore lichen communities. Does their symbiotic nature make them extremely vulnerable, as is the case of corals mirrored in coral bleaching (Abrego et al. 2008)? Or does the flexibility (Osyczka et al. 2020) of their symbiotic nature make them more resistant with the potential to mitigate the effects of climate change on the shore ecosystems?

In the present paper, we focused on the littoral fringe within the intertidal zone. It is characterized as the zone occasionally submerged by the incoming tide and frequently subject to waves and heavy spray (Dobson 2014). It has also been referred to as the geolittoral zone (Nordheim and Boedeker 1998). Organisms thriving in this zone are exposed to large fluctuations in salinity; the lichen thallus absorbs the seawater, then as it dries a layer of salt is left on its surface, later it absorbs freshwater from the rain, etc.; resulting in rapid changes in the osmotic pressure within the thalli (Dobson 2014).

The presence of lichens in a specific habitat is delimited by or reflected in the choice of their associated photobionts (Helms 2003, Peksa and Škaloud 2011). It has been repeatedly demonstrated that lichens in certain habitats share a specific set of photobionts, a phenomenon referred to as lichen photobiont guilds (Rikkinen 2003). The very few studies that focused on or at least included lichens from the intertidal zone indicate that among green algae it is mainly the Ulvophycean family Kornmanniaceae, Ulvales (Tschermak-Woess 1976, Watanabe et al. 1997, Thüs et al. 2011, Darienko and Pröschold 2017, Gasulla et al. 2019, Černajová et al. 2022a) that relates with this lifestyle. Additionally, *Urospora*, Acrosiphoniaceae, Ulotrichales was recently reported from Verrucariaceae lichens from the Patagonian shore (Černajová et al. 2022a).

Here, we focused on the lichen mycobiont – photobiont interactions, their variation along an environmental gradient and relation to the pool of available free-living algae. Specifically, we aimed to answer the following questions: 1) What is the diversity of lichen photobionts in the habitat in focus? 2) Does it change with the salinity level? 3) Is the pool of the available free-living photobionts reflected in the lichen photobiont choice? 4) Are the mycobionts selective towards their photobionts or do they associate with the most abundant (the best adapted) algae in the environment? We sampled crustose lichens and free-living microscopic algal communities from the littoral fringe of the shores of the Baltic Sea, Kattegat and the North Sea.

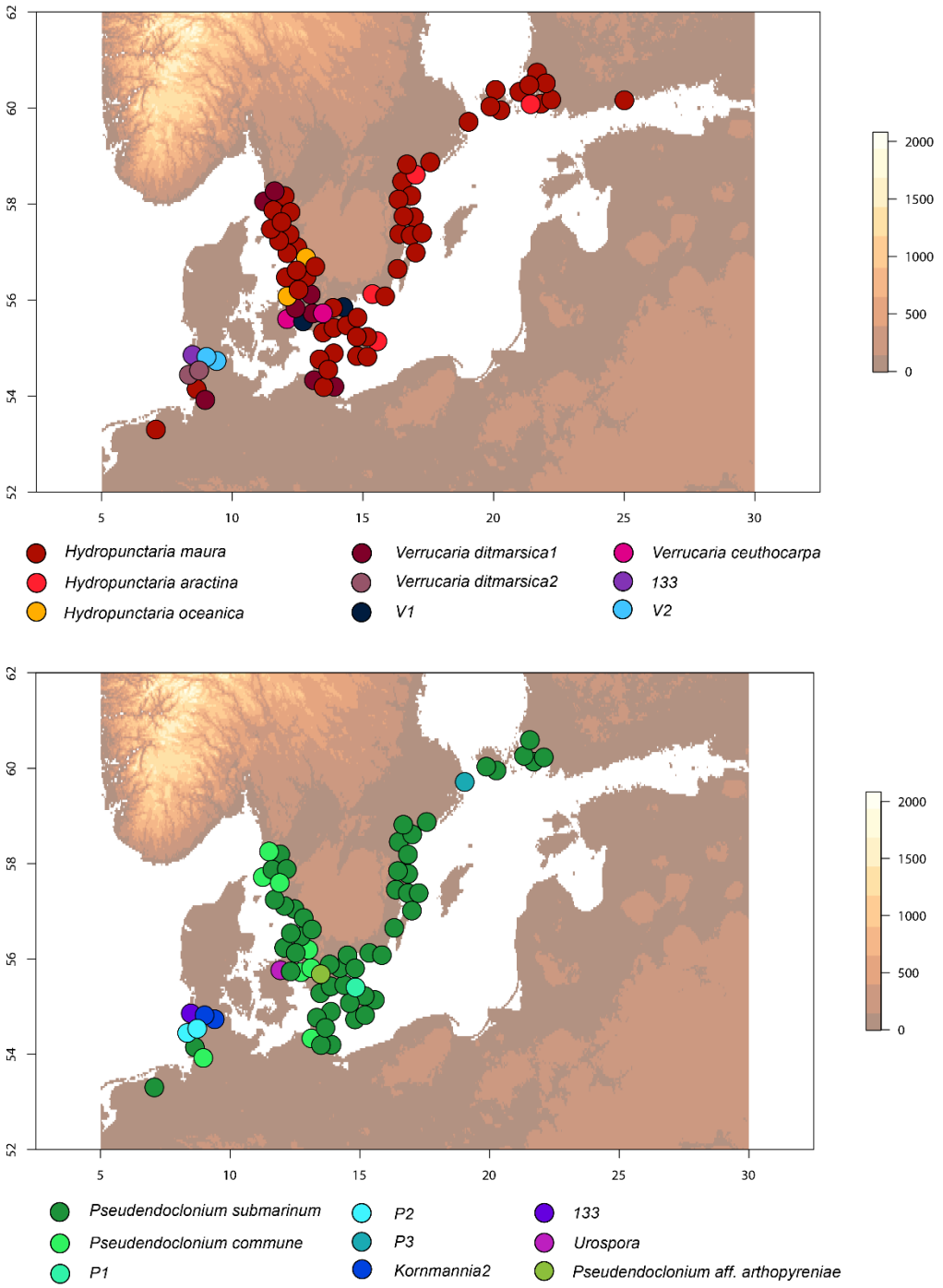

Figure 1 Maps of the sampling sites showing diversity of **A** the mycobionts and **B** photobionts.

2. Materials and Methods

2.1 Sampling

From 2019 to 2021 we sampled the shores of the Baltic Sea, Kattegat and the North Sea; from Helsinki through Turku Archipelago and Åland Islands, the southern Swedish coast from Norrtälje Municipality to Tjörn Municipality, Bornholm and Rügen Islands and Syddjurs Municipality in eastern Denmark to the north-west coast of Germany (Fig. 1). The three regions represent a salinity gradient from 5 – 8 PSU in the Baltic Sea, to 8 – 26 PSU in Kattegat and 26 – 35 PSU in the North Sea (Geburzi et al. 2021, Olofsson et al. 2020, Paavola et al. 2005). We collected Verrucariaceae lichens in the littoral fringe (see above). All species found at each site were collected. In order to capture all the photobiont diversity at the sites where free-living algal communities were sampled (see below), several thalli of the same lichen, or in case of large continuous thalli of *H. maura*, several pieces of the same crust, were sampled. Collection data are given in Table 1. Air-dried thalli were stored at 4°C until processed. Morphologically, the lichens were identified as *Hydropunctaria maura*, *Hydropunctaria* sp., *Verrucaria ceuthocarpa* and *V. ditmarsica* (Table 1).

At seven collection sites (Table 1), free-living rock-inhabiting microscopic algal communities (available pool of photobionts) were also sampled. Rock surface that was close to the collected lichens but free of any visible lichen thalli or other organism biofilms was scraped at ten spots with a sterile spoon directly into an Eppendorf tube. The samples were immediately frozen and stored at -20°C until processed.

2.2 Sanger sequencing and phylogenetic analyses

DNA from the lichen thalli was isolated using the CTAB protocol (Cubero et al. 1999) with minor modifications as in Černajová et al. (2022b). Nuclear ITS rDNA of the mycobiont was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). To identify the photobionts, nuclear SSU rDNA was primarily amplified using 18S-Ulvo-F and 18S-Ulvo-R (Černajová et al. 2022a). However, because SSU seemed insufficient to distinguish *Pseudendoclonium submarinum* and *P. commune*, two frequently recovered and closely related photobiont species, the algal ITS was also amplified for selected specimens with the KlebsF (Škaloud and Rindi 2013)/newly designed ZelenyF1 (5‘-CCG CCC GTC GCT CCT ACC GA-3’) and ITS4 primers. Additionally, algal ITS was also amplified for selected specimens containing *Urospora* in order to strengthen the Ulotrichales phylogeny. The PCR conditions were as in Černajová et al. (2022a). The PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter) and sequenced by Macrogen Europe, Amsterdam, the Netherlands. For the GenBank accession numbers of the newly obtained sequences see Table 1.

The sequences were aligned with relevant sequences downloaded from GenBank (see below) separately for each marker using MAFFT v.7 (Katoh et al. 2019), applying the G-INS-I method and manually checked. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and eliminated. Substitution models, estimated with JModelTest v. 2.1.4 (Darriba et al. 2012) using Bayesian Information Criterion, are given below.

Verrucariaceae is the third largest lichen family (Lücking et al. 2017) with many yet undescibed/undiscovered taxa and many unresolved phylogenetic relationships (e.g., Savić et al. 2008, Orange and Chhetri 2022). The BLAST searches of the obtained mycobiont sequences supported their affiliation to three different groups (as identified morphologically) within the family. Because the ITS alignment across whole Verrucariaceae is very challenging, the three lineages were approached separately in the phylogenetic analyses.

Table 8 Collection data, symbiont identity and GenBank accession numbers. Samples from localities where free-living algal communities were also sampled are highlighted in blue.

sample code	Locality	GPS coordinates	mycobiont species/lineage	GB accession	photobiont species/lineage	GB accession (SSU/ITS/culture ITS)
1	Denmark, Bornholm, Nexo	55°04'51.6"N 15°09'21.5"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/xx
1-2			*Hydropunctaria aractina*	xx	*Pseudendoclonium submarinum*	-/xx/-
1-4			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
2	Denmark, Bornholm, Balka	55°02'32.6"N 15°06'57.2"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/xx
2-2			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
2-4			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
2-5			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
3-1	Denmark, Bornholm, Grisby	55°07'10.8"N 15°08'49.7"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/xx
5	Denmark, Bornholm, Osand Bugt	55°17'29.0"N 14°46'40.1"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/xx
6-2			*Hydropunctaria maura*	xx	*Pseudendoclonium* sp. P1	xx/-/-
7-1	Sweden, Skåne, Kullaberg	56°17'27.9"N 12°28'34.7"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
7-2			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
7-5			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
7-6			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
8	Sweden, Skåne, Kullaberg	56°17'58.6"N 12°28'58.0"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
8-1			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
8-2			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
8-3			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
8-4			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
11	Sweden, Halland, Laxvik	56°35'51.2"N 12°55'16.2"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
11-1			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
11-2			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
11-4			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
13-4	Sweden, Västra Götaland, Fiskebäck	57°38'59.5"N 11°50'42.3"E	*Hydropunctaria maura*	xx	*Pseudendoclonium commune*	xx/-/-
13-1			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
14	Sweden, Skåne, Barsebäckshamn	55º45'11.6"N 12º54'08.7"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	-/xx/-
15			*Verrucaria* sp. V1	xx	*Pseudendoclonium commune*	xx/-/-
16-1-1	Sweden, Skåne, Barsebäckshamn	55°45'11.2"N 12°54'08.6"E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/xx/-

16-1-3			*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
16-1-4			*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
					Pseudendoclonium submarinum	-/xx/-
16-2-1			*Verrucaria ceuthocarpa*	xx	*Uropsora* sp.	-/xx/-
					Pseudendoclonium aff. arthropyreniae	-/-/xx
16-2-2			*Verrucaria ceuthocarpa*	xx	*Uropsora* sp.	-/xx/-
					Pseudendoclonium aff. arthropyreniae	-/-/xx
16-2-3			*Verrucaria ceuthocarpa*	xx	*Uropsora* sp.	-/xx/-
					Pseudendoclonium aff. arthropyreniae	-/-/xx
17			*Verrucaria ceuthocarpa*	xx	*Uropsora* sp.	-/xx/-
28	Germany, Mecklenburg-Vorpommern, Rügen Island	54°20'26.4''N 13°31'33.1''E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/xx/-
29			*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
30A	Germany, Mecklenburg-Vorpommern, Rügen Island	54°20'26.3''N 13°31'23.8''E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
30B					*Pseudendoclonium submarinum*	xx/-/-
39	Germany, Mecklenburg-Vorpommern, Rügen Island	54°20'26.7''N 13°31'28.4''E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
31	Germany, Mecklenburg-Vorpommern, Rügen Island	54º23'55.1''N 13º37'32.9''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
32	Germany, Mecklenburg-Vorpommern, Rügen Island	54°35'06.4''N 13°37'01.6''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
33			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
37	Germany, Mecklenburg-Vorpommern, Rügen Island	54º40'54.4''N 13º22'08.8''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
38	Germany, Mecklenburg-Vorpommern, Rügen Island	54º40'55.4''N 13º22'16.1''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
46	Finland, Varsinais-Suomi, Kyrklandet Island	60°09'59.1''N 21°41'29.5''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
48	Finland, Varsinais-Suomi, Mossala Island	60°17'19.8''N 21°26'23.1''E	*Hydropunctaria aractina*	xx	*Pseudendoclonium submarinum*	xx/xx/-
49	Finland, Varsinais-Suomi, Lillandet Island	60°13'16.0''N 22°05'43.5''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-

50	Finland, Varsinais-Suomi, Mussalo Island	60°32'03.4''N 21°32'16.2''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
55	Finland, Åland, Herrö	59°58'10.0''N 20°10'38.6''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
59	Finland, Ålandy, Järsö Island	60°00'41.5''N 19°59'07.8''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
60	Sweden, Stockholm, Kapellskär	59°42'39.6''N 19°03'02.9''E	*Hydropunctaria maura*	xx	*Pseudendoclonium sp.*	xx/-/-
64	Sweden, Södermanland, Trosa	58°52'16.2''N 17°34'35.3''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
65	Sweden, Södermanland, Nävekvarn	58°37'26.0''N 16°47'32.6''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
66	Sweden, Södermanland, Nävekvarn	58°37'23.6''N 16°47'45.2''E	*Hydropunctaria aractina*	xx	*Pseudendoclonium submarinum*	xx/xx/-
67	Sweden, Östergötland, Östra Husby	58°37'43.0''N 16°35'42.7''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
70	Sweden, Östergötland, Gryt	58°10'16.3''N 16°50'55.4''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
72	Sweden, Kalmar, Västervik	57°44'02.6''N 16°40'30.0''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
73	Sweden, Kalmar, Björnhuvudsfjärden	57°38'03.6''N 16°30'14.9''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
75	Sweden, Kalmar, Västervik	57°43'34.5''N 16°31'30.5''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
76	Sweden, Kalmar,Öland, Byxelkrok	57°20'17.8''N 17°00'52.3''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
78	Sweden, Kalmar, Öland, Trollskogen	57°21'23.6''N 17°07'28.5''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
79	Sweden, Kalmar, Öland, Vikegård	57°04'40.3''N 16°58'30.7''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
80	Sweden, Kalmar, Stora Rör	56°45'26.3''N 16°31'34.6''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
81	Sweden, Kalmar, Stensö	56°38'52.2''N 16°19'07.7''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
82	Germany, Mecklenburg-Vorpommern, Rügen Island	54°34'54.6''N 13°38'35.9''E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
83	Sweden, Skåne, Abbekås	55º23'13.4"N 13º34'30.5"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
84	Sweden, Skåne, Svarte	55º25'34.9"N 13º42'42.2"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
87	Sweden, Skåne, Brantevik	55º30'56.5"N 14º20'54.1"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/xx/-
88	Sweden, Skåne, Åhus	55º48'37.6"N 14º12'41.4"E	*Verrucaria* sp. V1	xx	*Pseudendoclonium submarinum*	xx/-/-
91	Sweden, Blekinge, Kugebodda	56º07'25.1"N 15º21'06.2"E	*Hydropunctaria aractina*	xx	*Pseudendoclonium submarinum*	xx/-/-
92	Sweden, Blekinge, Torhamn	56º04'40.6"N 15º50'22.1"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
93	Sweden, Skåne, Barsebäckshamn	55º45'11.6"N 12º54'08.7"E	*Verrucaria ceuthocarpa*	xx	*Urospora sPseudendoclonium*	xx/xx/-
95	Sweden, Skåne, Kullaberg	56º18'00.6"N 12º28'36.5"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
96			*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
97			*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-

98			*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/-/-
99	Sweden, Skåne, Kullaberg	56º17'27.7"N 12º28'28.1"E	*Hydropunctaria oceanica*	xx	-	-/-/-
101	Sweden, Halland, Tylösand	56º38'31.3"N 12º44'05.8"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
102	Sweden, Halland, Grimsholmen	56º50'23.7"N 12º33'14.5"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
103			*Hydropunctaria oceanica*	xx	*Pseudendoclonium submarinum*	xx/xx/-
104	Sweden, Halland, Träslövsläge	57º02'52.0"N 12º16'41.8"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
110	Sweden, Västra Götaland, Kärna	57º47'33.1"N 11º43'51.4"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	
112	Sweden, Västra Götaland, Tjuvkil	57º53'35.1"N 11º42'11.6"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	
116	Sweden, Västra Götaland, Klädesholmen Island	57º56'41.4"N 11º32'05.0"E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	
117	Sweden, Västra Götaland, Tjörn Island	58º00'22.5"N 11º33'05.7"E	*Verrucaria ditmarsica* 1	xx	*Pseudendoclonium commune*	xx/xx/-
123	Sweden, Västra Götaland, Kärna	57º47'33.1"N 11º43'51.4"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	xx/-/-
129	Germany, Schleswig-Holstein, Pellworm	54º29'55"N 8º35'43"E	*Hydropunctaria maura*	xx	*Pseudendoclonium submarinum*	
133	Germany, Schleswig-Holstein, Pellworm	54º31'29.2"N 8º35'16.2"E	*Verrucaria* sp.	xx	Kornmanniaceae sp.	xx/xx/-
134	Germany, Schleswig-Holstein,Hamburger Hallig	54°36'01''N 08°48'38.9''E	*Verrucaria* sp. V2	xx	*Kornmannia* sp. 2	xx/-/-
135			*Verrucaria ditmarsica* 2	xx	*Pseudendoclonium* sp. P2	xx/-/-
137	Germany, Schleswig-Holstein,Hamburger Hallig	54°36'13.7''N 08°48'31.5''E	*Verrucaria* sp. V2	xx	*Kornmannia* sp. 2	xx/-/-
138			*Verrucaria ditmarsica* 2	xx	*Pseudendoclonium* sp. P2	xx/-/-
140	Germany, Schleswig-Holstein, Friedrichskoog	54º01'25.5"N 8º48'39.0"E	*Verrucaria ditmarsica 1*	xx	*Pseudendoclonium commune*	xx/-/-

For the genus *Hydropunctaria*, the dataset included all the nine currently described species (Orange 2012, Spribille et al. 2020) and representatives of a yet-undescribed lineage from the Patagonian coast (Černajová et al. 2022a) and *Wahlenbergiella mucosa* as the outgroup. The final alignment contained 48 unique sequences and 519 positions, of which 251 were variable (V) and 185 parsimony informative (Pi). The selected substitution models were TPM1uf+G, K80+I and HKY+I for ITS1, 5.8S and ITS2, respectively.

Verrucaria ditmarsica is related to *Turgidosculum ulvae*, forming a long branch within the family based on analysis of four makers (Pérez-Ortega et al. 2018). There are only three *V. ditmarsica* sequences available in GenBank and they differ significantly from each other. For example, two specimens from Wales (vouchers Orange 16340 (NMW - C.2005.001.318) and Orange 16447 (NMW - C.2005.001.424), GenBank accessions FJ664845 and FJ664846, respectively) have only 90% identity in the nuITS and part of LSU rDNA sequence. The dataset included all the available *V. ditmarsica* and *T. ulvae* sequences and *W. mucosa* as the outgroup, consisted of 15 unique sequences and 432 positions (218 V, 132 Pi). The selected substitution models were HKY+G, K80+I and TrN+I for ITS1, 5.8S and ITS2, respectively.

Verrucaria ceuthocarpa is related to *V. degelii* and *W. mucosa* according to Heidmarsson et al. (2017). It was aligned with all the described taxa and representatives of undescribed lineages known to belong to the group (Guedain et al. 2009, Pérez-Ortega et al. 2010, Heidmarsson et al. 2017, Černajová et al. 2022a) and *H. maura* as the outgroup. The alignment contained 12 unique sequences and 532 positions (284 V, 197 Pi). The selected substitution models were HKY+G, TPM2+I and HKY+I for ITS1, 5.8S and ITS2, respectively.

The photobiont sequences belonged either to the family Kornmanniaceae (Ulvales) or matched the genus *Urospora* (Ulotrichales). The Kornmanniaceae datasets included all the currently recognized species with available DNA sequence data (Darienko and Proschold 2017, Škaloud et al. 2018, Liu et al. 2019, Černajová et al. 2022a). The SSU alignment contained 28 unique sequences, including *Bolbocoleon piliferum* as the outgroup, and consisted of 1587 positions (204 V, 138 Pi). The ITS alignment contained 29 unique sequences and 530 positions (230 V, 204 Pi). The selected substitution models were SYM+I+G, K80+G, K80+I and K80+G for SSU, ITS1, 5.8S and ITS2, respectively.

The Ulotrichales dataset was based on Škaloud et al. (2018) with additional *Urospora* species as in Černajová et al. (2022a). The concatenated alignment consisted of 36 unique sequences, including *Desmochloris molenhaueri* (Chlorocystidales), *Halochlorococcum moorei* (Oltmannsiellopsidales) and *Pseudendochloris marina* (Ulvales) as the outgroup. It contained 2251 positions (462 V, 321 Pi). The selected substitution models were K80+I+G, SYM+I+G, K80+I and SYM+G for SSU, ITS1, 5.8S and ITS2, respectively.

The phylogenetic trees were inferred by maximum likelihood analyses (ML) in RAxML v. 8.2.12 (Stamatakis 2014) and Bayesian Inference (BI) in MrBayes v. 3.2.6 (Ronquist et al. 2012) using partitioned datasets. The ML bootstrap support values were calculated based on 1000 replications. In BI, two parallel Monte Carlo Markov Chain (MCMC) runs, with one cold and three heated chains, were carried out. The convergent diagnostic of the potential scale reduction factor approached 1 in all cases. The average standard deviation of split frequencies (SDSF) was 0.0024 for *Hydropunctaria* (15 million generations), 0.0017 for the *V. ditmarsica* dataset (5 million generations), 0.0007 for the

Wahlenbergiella group (5 million generations), 0.0021 and 0.0043 for Kornmanniaceae SSU and ITS, respectively (5 million generations both) and 0.0046 for Ulotrichales (10 million generations). All the analyses were run on the CIPRES Science Gateway v. 3.3 web portal (Miller et al. 2010).

2.3 Photobiont culturing

Isolation of photobionts from lichen thalli was performed as in Černajová et al. (2022a) and identity of the obtained strains was verified by sequencing ITS rDNA as above.

2.4 Symbiotic interactions

Interaction network and maps were built in the free software R v. 4.1.0 (R Core Team 2021) using the packages bipartite (Dormann et al. 2009), GISTools (Brundson and Chen 2014), raster (Hijmans 2015), rgdal (Bivand et al. 2015) and scales (Wickham and Siedel 2022).

2.5 Metabarcoding

DNA of the free-living algal communities was isolated using Fast DNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturer´s instructions. ITS2 amplicons from Illumina MiSeq sequencing were produced by nested PCR with the newly designed primer 1378j02 (5'-TTG CCT TGT CAG GTT GAT TCC-3') and the primer ITS4 (White et al. 1990) in the first step and barcoded 5.8F-Chlorophyta (Vančurová et al. 2020) and ITS4 primers in the second step. The PCRs were performed useing the Q5 High-Fidelity DNA polymerase (BioLabs Inc.), they were run in 22 and 24 cycles in the first and second step, respectively and the conditions were: initial denaturation at 98 °C for 30 s, 98 °C denaturation for 10 s, 52° C amplification for 45 s and 72 °C elongation for 1 min, with a final 72 °C extension for 2 min. Each sample was run in three replicates and three PCR negative controls (PNC) were included. The PCR products were purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter), pooled equimolarly and sent for library preparation and sequencing to Fasteris (Plan-les-Ouates, Switzerland). Sequencing was performed on the Illumina MiSeq platform with paired end mode (2 × 300 bp). Quality control of the Illumina MiSeq paired-end reads was carried out using FastQC v. 0.11.8 (Andrews 2010). Raw reads were processed according to Báilint et al. (2014), including quality filtering, paired-end assembly, removing primer artifacts, extracting reads by barcodes, reorienting reads to 5'-3', demultiplexing, dereplicating, OTU clustering (this step carried out using Swarm v. 2 (Mahé et al. 2015), with denoising set to d = 3) and chimera filtering. Each sample was sequenced in triplicate, and both negative controls (distilled water as template) and multiplexing controls (unused combinations of left and right barcodes) were used in library preparation. Only OTUs that were found in at least 100 reads and at least two replicates, while not being found in more than 0.5% of negative controls, were considered.

The OTUs were identified by BLAST searches in SEED2 (Větrovský et al. 2018), and only Chlorophyceaen sequences were further processed. To identify the algae present in the free-living communities, the obtained sequences were aligned with the closest BLAST matches and a ML tree was constructed as above. Additional trees of the two dominant photobiont genera were constructed to compare the diversity of the free-living communities with the lichen photobiont diversity.

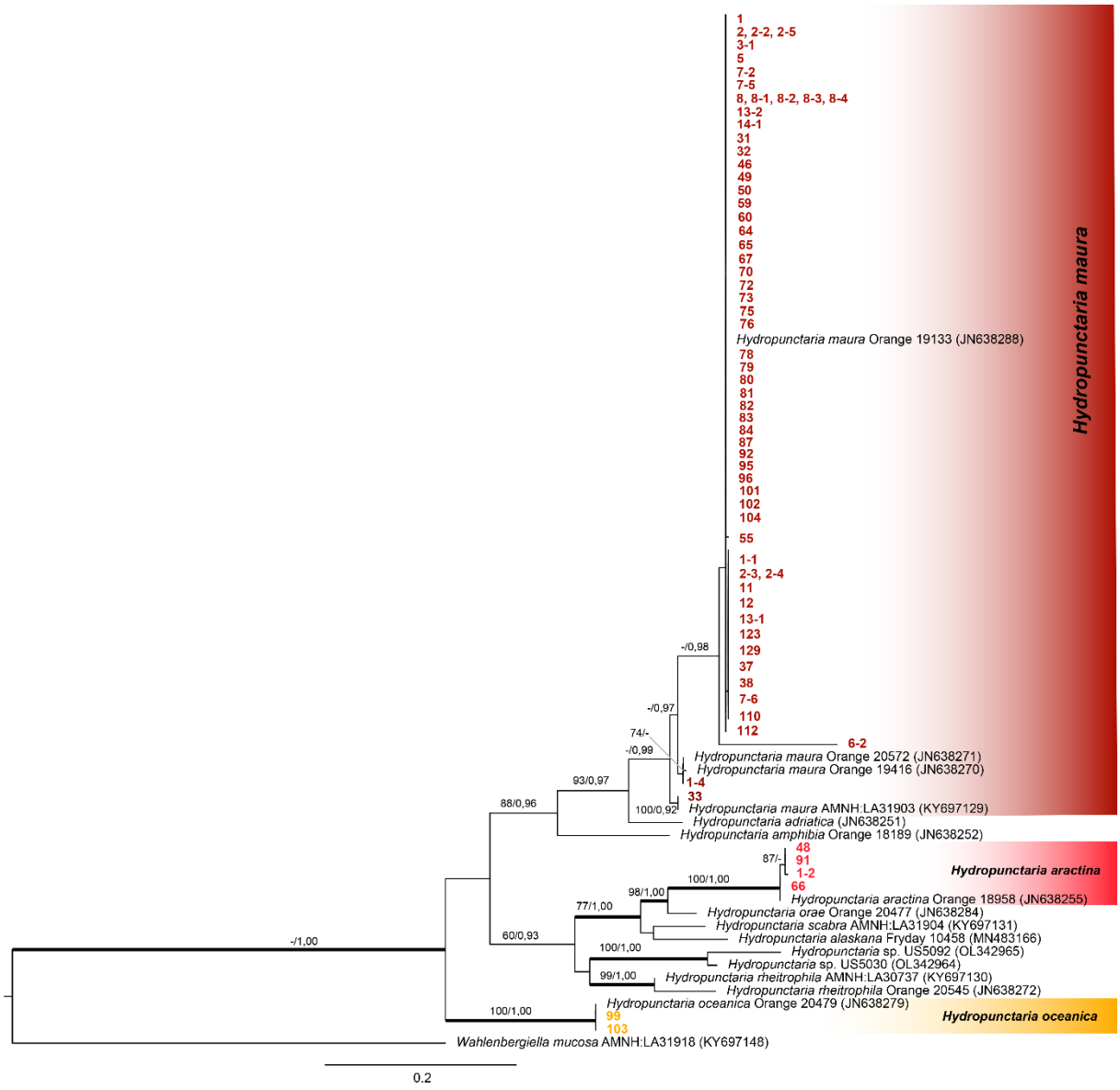

Figure 2 Phylogeny of the genus *Hydropunctaria* group based on maximum likelihood (ML) of ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown. Scale bar represents the expected number of substitutions per site.

3 Results

Both mycobiont and photobiont sequences were successfully obtained from 91 samples, photobiont sequence was not obtained from a single specimen of *H. oceanica* (sample 93).

3.1 Mycobiont diversity

Altogether, nine lichen mycobiont species or species-level lineages were found, their distribution is shown in Fig. 1A. The highest diversity was at the shore of the North Sea although it was the least sampled.

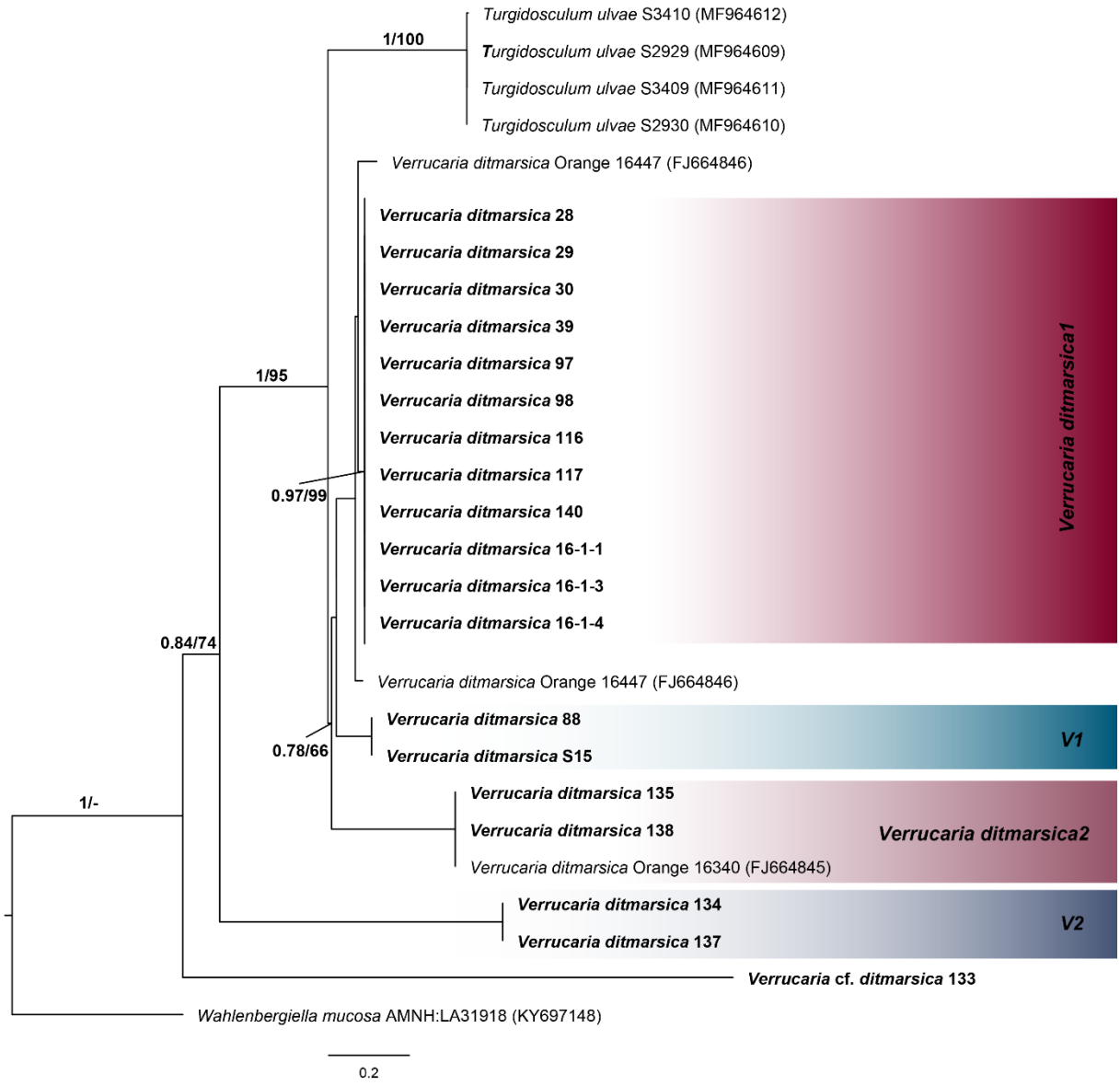

Figure 3 Phylogeny of the *Verrucaria ditmarsica*/*Turgidusculum ulvae* lineage based on maximum likelihood (ML) of ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown. Scale bar represents the expected number of substitutions per site.

The phylogeny of *Hydropunctaria* (Fig. 2) revealed, that in addition to the dominant *H. maura*, also *H. aractina* (4 specimens) and *H. oceanica* (2 specimens) were present. *H. aractina* had previously been only known from northern Norway and differs from *H. maura* in dull green cortical pigments and thallus thickness, however, with great overlaps between the two species (Orange 2012). As stated in the *H. aractina* description (Orange 2012), the distinction between the species was obvious when found together (Fig. S1A) but isolated thalli were virtually impossible to be distinguished (Fig. S1B, S1C). This was partly due to the fact that the thalli were often overgrown by filamentous cyanobacteria, which completely disguised the cortical pigments (Fig. S1D). Additionally, only few spores were found. In our dataset it was only found in the Baltic Sea. *H. oceanica* (Fig. S1E) had

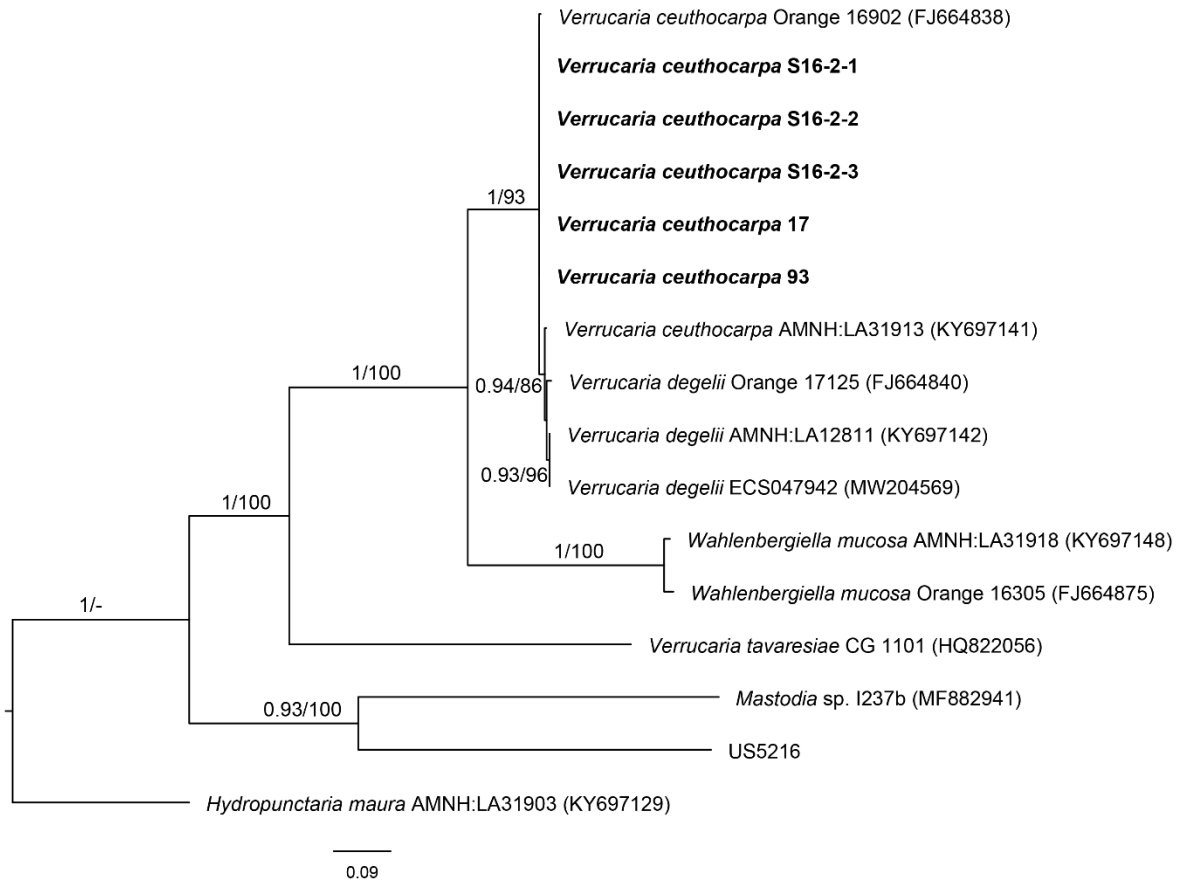

Figure 4 Phylogeny of the *Wahlenbergiella* group based on maximum likelihood (ML) of ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown. Scale bar represents the expected number of substitutions per site.

previously been only known from the British Isles, here we found it in Kattegat only. It should differ from *H. maura* mainly in the conspicuously protruding perithecia (Orange 2012), however, this characteristic was not seen in our collections. The phylogeny of the *V. ditmarsica/T. ulvae* lineage (Fig. 3) showed that the morphospecies *V. ditmarsica* is polyphyletic and probably contains various cryptic species. Our specimens were placed in four separate lineages (labelled *V.ditmarsica1*, *V. ditmarsica2*, V1 and V2 here). However, the support values for some of the lineages were too low (Fig. 3) to draw conclusions and the group will require further studies.

The phylogenetic analysis of ITS rDNA (Fig. 4) of the *Wahlenbergiella* group positioned *V. ceuthocarpa* (morphologically easily-recognizable based on the combination of black sides of areoles and vertically oriented hyphae, Fig. S2) as an inner lineage of the genus *Wahlenbergiella*, intermixed with *V. degelii*, with high posterior probability/bootstrap values (1/93).

3.2 Photobiont diversity

Altogether, nine lichen photobiont species or species-level lineages were found, their distribution is shown in Fig. 1B. Again, the highest diversity was found in the North Sea despite the smallest sampling size. Multiple photobionts were detected in seven samples (Table 1).

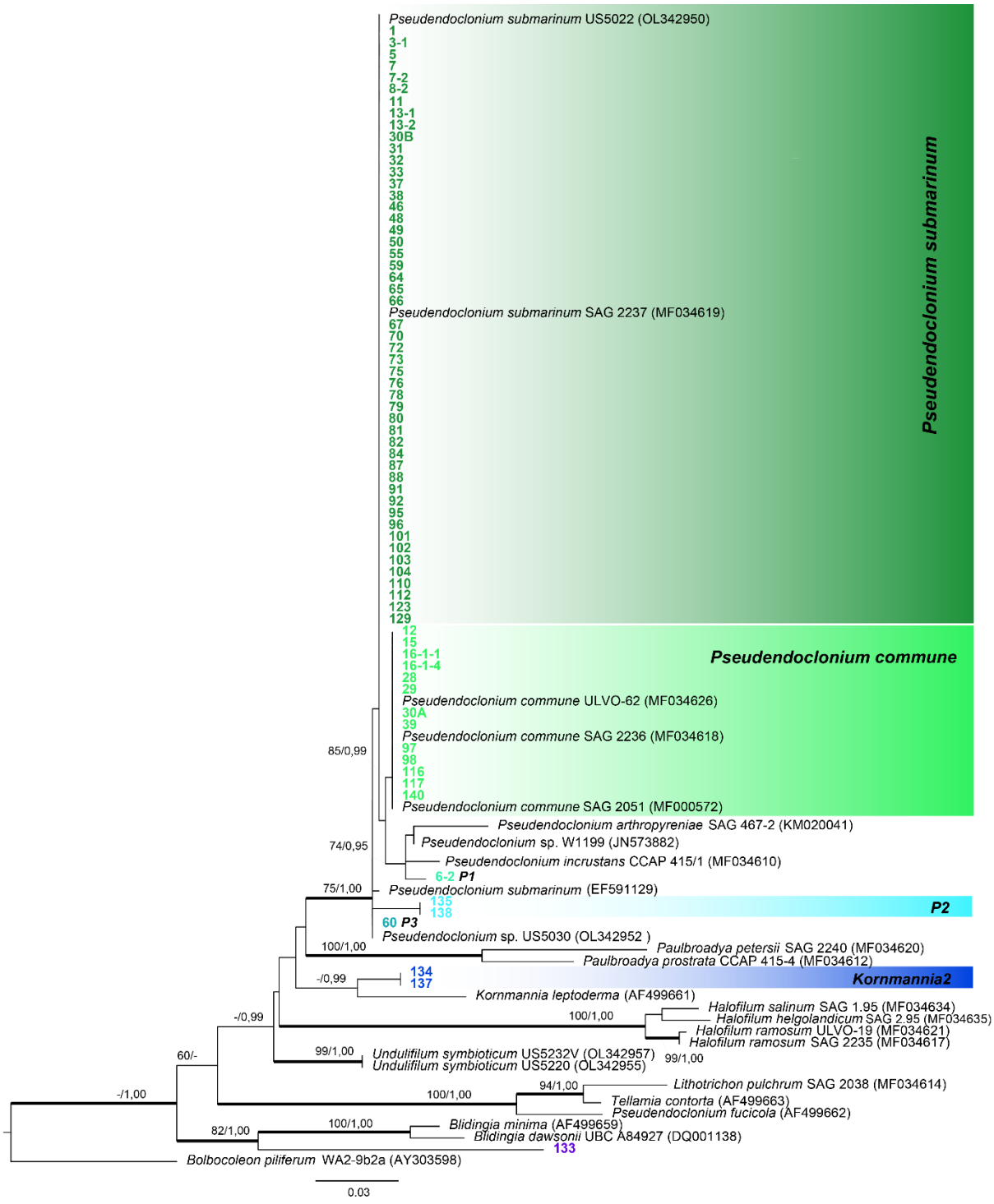

Figure 5 Phylogeny of the family Kornmanniaceae based on maximum likelihood (ML) of nuSSU rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown. Scale bar represents the expected number of substitutions per site.

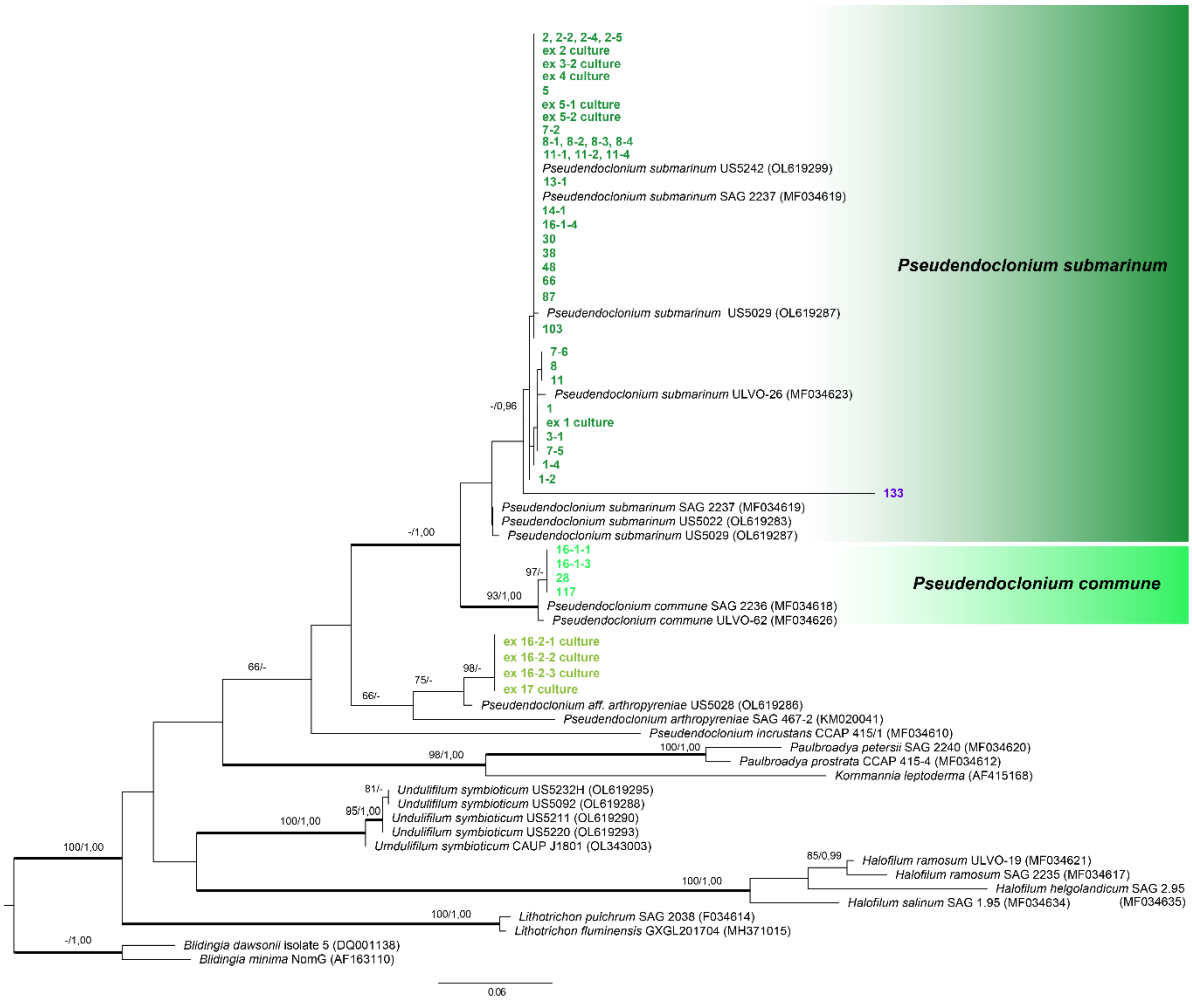

Figure 6 Phylogeny of the family Kornmanniaceae based on maximum likelihood (ML) of ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown.

Out of the 98 photobionts obtained, 93 belonged to the family Kornmanniaceae (Fig. 5). *Pseudendoclonium submarinum* was the most common (68 samples), followed by *P. commune* (14 samples). The two species are well distinguished based on the ITS rDNA sequence while they only differ in two nucleotides in the SSU rDNA sequence; specifically, T (thymine) vs. C (cytosine) at position 610 of the alignment and C vs. A (adenine) at position 1565 in *P. submarinum* and *P. commune*, respectively. This two-nucleotide difference turned out to be consistent in the present dataset and was supported by the phylogeny based on ITS (Fig. 6). Thus, we believe that the species distinction is reliable even based on SSU, if clear chromatographs are obtained. The only discrepancy occurred in sample 16-1-4, which we consider a case of algal plurality. Four other lineages within the genus were found (labelled P1, P2, P3 and *P.* aff. *arthropyreniae*, Fig. 5). They did not match any of the known species and their position was not statistically supported. *P.* aff. *arthropyreniae* was not obtained by direct sequencing of the thalli. Instead, it was isolated in culture from the photobiont layer of four of the *Urospora*-containing specimens.

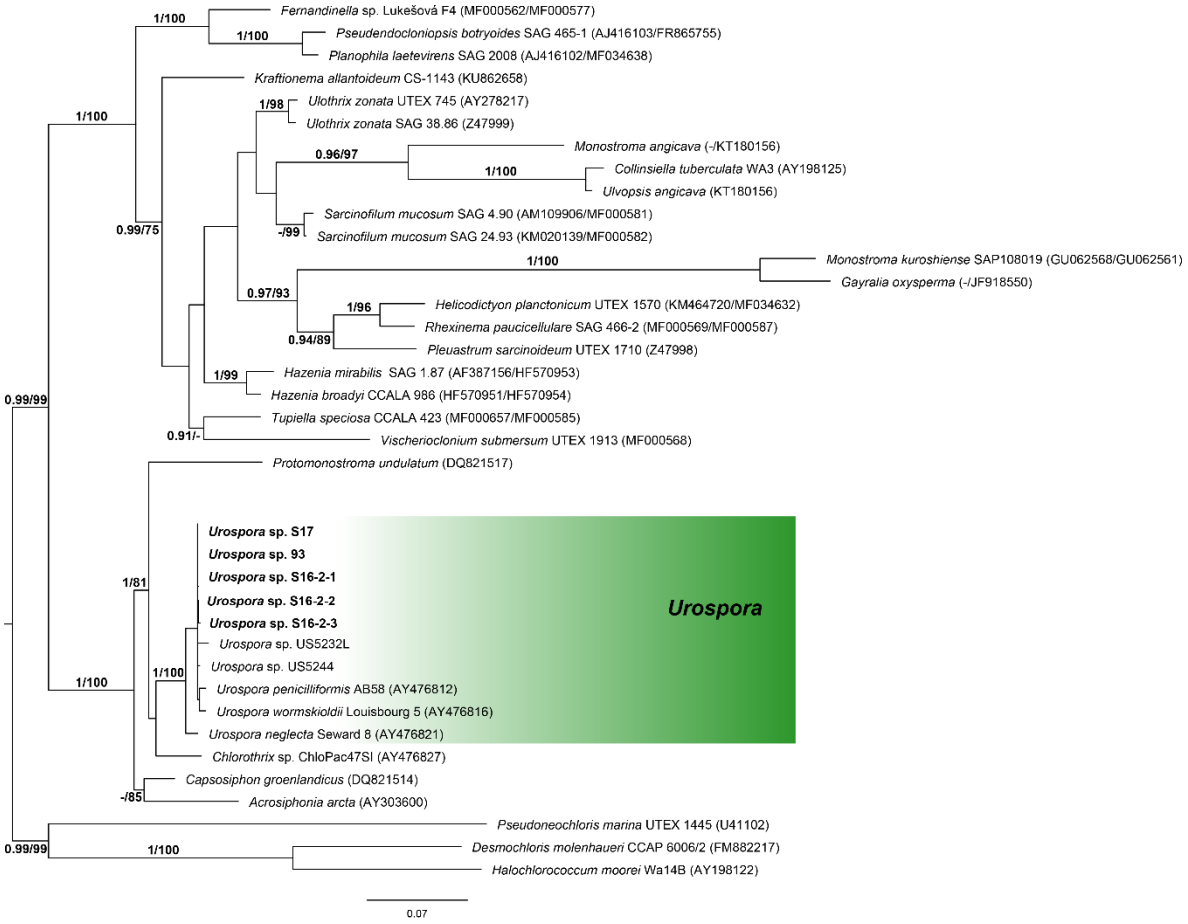

Figure 7 Phylogeny of the order Ulotrichales based on maximum likelihood (ML) of concatenated nuSSU and ITS rDNA. Values at nodes show statistical support calculated by MrBayes posterior-node probability (PP)/ML bootstrap. Only statistical supports with PP > 0.7 are shown.

Two specimens (134 and 137) contained photobionts belonging to a previously unknown lineage related to *Kornmannia leptoderma*, however the lineage placement was not statistically supported by ML analysis. Additionally, the photobiont of sample 133 was placed in an unknown lineage related to *Blidingia* (Fig. 6).

The phylogeny of Ulotrichales revealed *Urospora* (Fig. 7) as a photobiont in five lichen samples. The affiliation to the genus is highly supported (1/100). However, as the relationships within the genus are unresolved on molecular level, its specific identity remains unknown.

Urospora photobionts are reported for only the second time as lichen photobionts here. They were found in *V. ceuthocarpa* from the shore of Kattegat (Barsebäckshamn, Sweden). The site was first visited in September 2019 and revisited in November 2021. The recollection confirmed the presence of *Urospora*, so we consider this finding reliable. Unfortunately, culturing attempts were not successful.

Importantly, morphological distinction of *Urospora* from the more common Kornmanniaceae photobionts is possible directly within the lichen thallus. When stained with iodine (Lugol's solution),

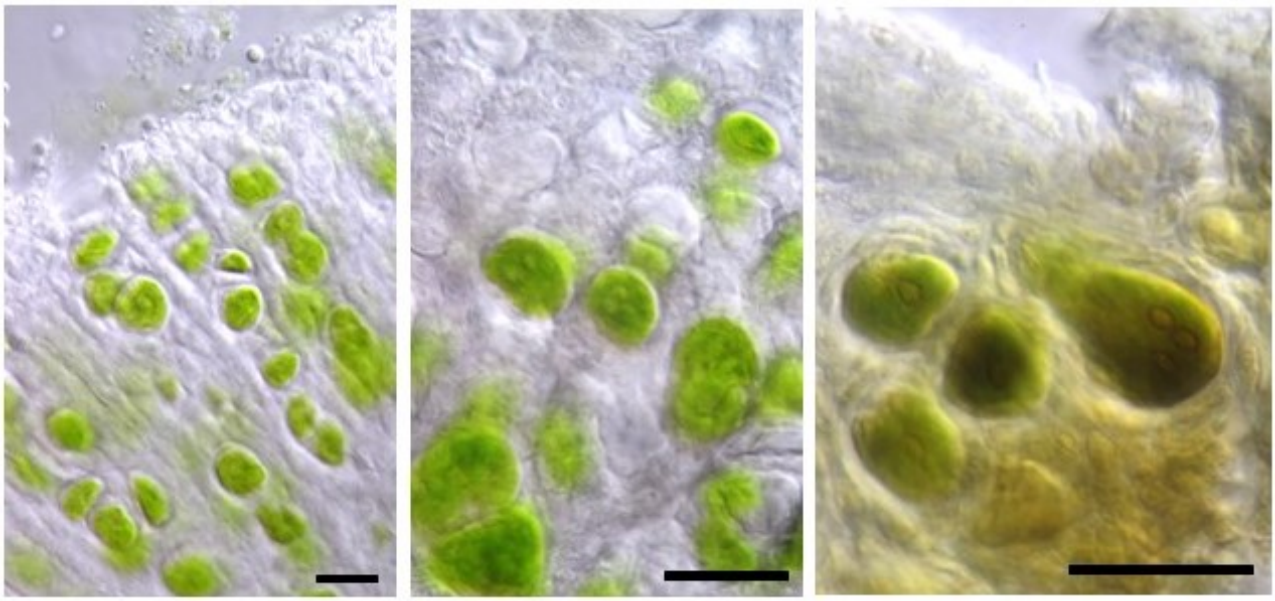

Figure 8 Morphology of *Urospora* within lichen thallus (**A-B**), pyrenoids visible after staining with Lugol´s solution (**C**). Scale bars represent 20 µm.

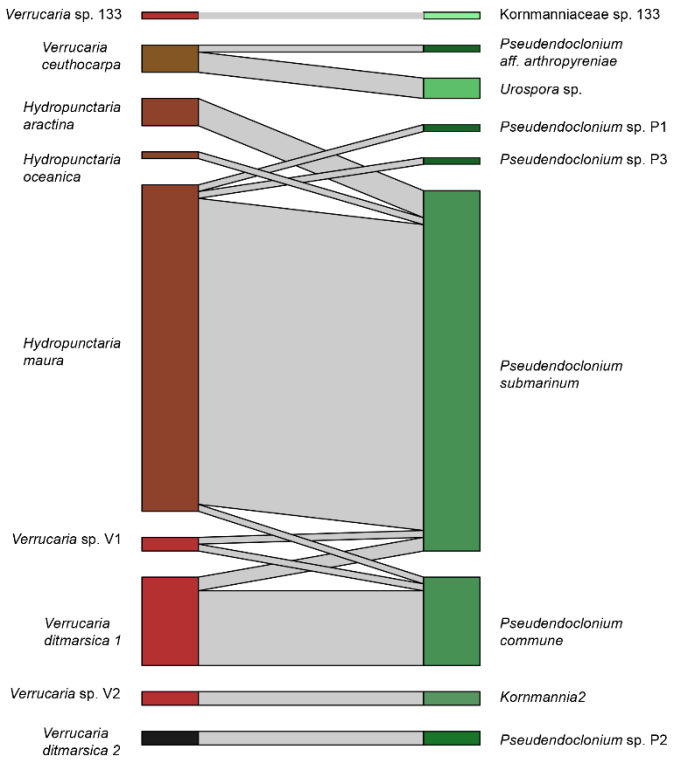

Figure 9 Interaction network between the mycobionts and the photobionts. Link widths are proportional to the number of samples in the association.

several pyrenoids within the cell were clearly visible (Fig. 8), a feature characteristic of the genus (Leliaert et al. 2009).

3.3 Symbiont interactions

Majority of the mycobiont species showed high specificity (Fig. 9). The infrequently sampled mycobiont species associated with one photobiont only; *H. aractina* (4 samples) and *H. oceanica* (1 sample) associated only with *P. submarinum*, *V. ditmarsica*2 (2 samples) with *Pseudendoclonium* sp. P2, *Verrucaria* sp. V2 (2 samples) with *Kornmannia*2 and *Verrucaria* sp. 133 with Kornmanniaceae sp. 133. The specificity in these cases might be a result of low number of samples. *V. ceuthocarpa* (5 samples) and *V. ditmarsica*1 (11 samples) always associated with *Urospora* sp. and *P. commune*, respectively.

Table 2 Total number of reads of corresponding to individual algal genera, obtained by Illumina metabarcoding of free-living algal communities.

Locality:	1	2	5	7	11	14	16
Ulvophyceae							
Pseudendoclonium	96641	67603	210019	277287	137666	103666	152289
Urospora	140464	0	117940	19510	194746	18557	105189
Halofilum	0	11450	36326	156	0	2965	276
Chlorothrix	0	17	38739	0	0	340	869
Ulva	0	2385	3114	0	0	74	10514
Pseudendoclonium-like	0	13382	0	0	0	0	0
Paulbroadya	0	102	5103	94	0	84	2286
Capsosiphon	0	0	2384	0	0	0	0
Lithotrichon	0	2316	0	0	0	0	0
Blidingia	0	0	341	0	0	0	0
Hazenia	0	0	0	92	0	0	0
Trebouxiophyceae							
Trebouxia	21861	365	6767	0	23770	3485	38272
Desmococcus	8608	3816	0	0	0	0	48
Prasiola	0	0	0	0	0	11333	0
Diplosphaera	0	0	2121	423	0	1638	20
Chlorella	0	0	0	245	0	0	0
Symbiochloris	0	0	158	0	0	0	0
Chloroidium	0	0	0	70	0	0	0
Apatococcus	0	0	0	0	61	0	0
Chlorophyceae							
Chlamydomonas	0	0	0	0	134	0	0

However, in four specimens of *V. ceuthocarpa* and two specimens of *V. ditmarsica*1, an additional photobiont was detected (*P.* aff. *arthropyreniae* and *P. submarinum*, respectively).

Lower specificity was observed for V1 which associated with *P. submarinum* (1 sample) and *P. commune* (1 sample). *H. maura* exhibited the lowest specificity, as it associated with four species of *Pseudendoclonium* – *P. submarinum* (70 samles), *P. commune* (2 samples), P1 (1 sample) and P3 (1 sample).

At the same time, *H. maura* exhibited high selectivity; despite its obvious ability to switch photobionts, it strongly preferred *P. submarinum* and was consistent in this preference across different salinity zones (Figs. 1 and 9). The high selectivity is also supported by the metabarcoding data (see below).

3.4 Available photobiont pool

The Chlorophycean free-living rock-inhabiting algal communities were dominated by Ulvophyceae and Trebouxiophyceae (Fig S3). List of the detected genera together with their read numbers at each site are given in Table 2. All the photobionts found in lichen thalli were also detected in the free-living communities (Figs. S4 and S4). Additional unknown lineages closely related to *Urospora* and

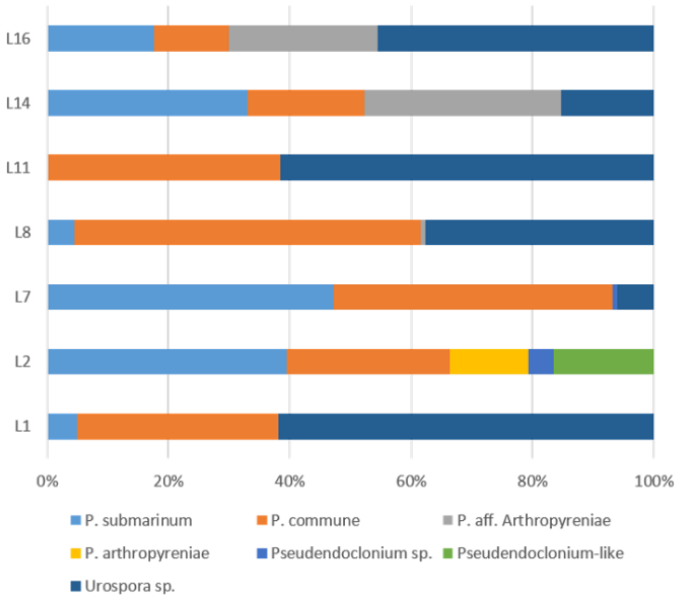

Figure 10 Proportions of photobiont taxa in the free-living algal pool in the proximity of sampled lichens. *H. maura* associated with *P. submarinum* at each of these sites.

within the genus *Pseudendoclonium* were also found (Figs. S4 and S4). Ratios of the photobiont species available at each site are illustrated in Fig. 10.

The composition of the available free-living photobiont pool highlights the high selectivity of *H. maura*. It was sampled at all the seven metabarcoding sites and at each of them it associated with *P. submarinum* although the choice of its compatible partners (algae it associated with at other sites) was wider (Fig. 10). On the other hand, *Urospora* was available at six of the seven sites but was only selected by *V. ceuthocarpa* which occurred at one site only. This suggests incompatibility of *Urospora* with the other mycobiont species studied on one hand, and high specificity of *V. ceuthocarpa* towards *Urospora* on the other, as various *Pseudendoclonium* species were also available at the site.

4 Discussion

One of the major innovations of the lichen symbiosis is the resilience to physiological stress induced by fluctuating water content (Spribille et al. 2022), which allows them to inhabit various hostile habitats. In fact, the alternation of drying and re-wetting is necessary for the formation of the symbiotic thalli (Armstrong 1976, Jahns 1993). In seashore habitats, the stress is even augmented by the periodic water immersion and salinity-induced water loss. Coping mechanisms, mainly on cell and biochemical level, were reviewed by Delmail et al. (2013). Gasulla et al. (2019) highlighted the role of photobiont choice in the lichen ecological performance in these habitats.

The photobiont diversity recovered in this study is comparable to the diversity obtained in our previous study of the same habitat in Patagonia (Černajová et al. 2022a). In both studies, vast majority of the photobionts belonged to the family Kornmanniaceae, Ulvales and a few to the genus *Urospora*, Acrosiphoniaceae, Ulotrichales. In both cases, *Urospora* was associated with mycobionts belonging to the *Wahlenbergiella* group, which is itself understudied and includes a number of deep undescribed lineages (Pérez-Ortega et al. 2010). Possibly, *Urospora* will turn out to be not an uncommon lichen photobiont when the lichen group will have been more closely studied. Additionally, it has also been recorded as an accessory photobiont of the intertidal cyanolichen *Lichina pygmaea* (Chrismas et al. 2021). Although we did not succeed in obtaining *Urospora*

photobiont culture and could not proceed with studying its identity, we provide a convenient microscopical diagnostic characteristic to distinguish it from the more common Kornmanniace photobionts within the lichen thallus – staining with Lugol's solution reveals three pyrenoids in *Urospora* cells (Fig. 8).

Among Kornmanniaceae, *Pseudendoclonium submarinum* was, by far, the most common. In addition to two yet-undescribed species-level lineages already reported from Patagonia (*P.* aff. *arthropyreniae* and *Pseudendoclonium* sp. P3), four novel lineages were found within the family (Fig. 5), suggesting that the Kornmanniaceae diversity is still far from being well-explored. The members of the family have evolved a range of mechanisms to deal with fluctuating salinity and thus facilitate the ability of the holobiont to thrive in the intertidal zone conditions (see the discussion in Gasulla et al. 2019 and Černajová et al. 2022a).

Another suggested mechanism for coping with fluctuating environments is the maintenance of multiple algae with distinct physiological properties within the lichen thalli (Castano et al. 2010). This has also been suggested for the intertidal *L. pygmaea*, a cyanolichen that was shown to host a variety of accessory photobionts, both cyanobacterial and Ulvophycean (Chrismas et al. 2021). Although we did not focus on photobiont plurality, it was detected in three cases; both *P. submarinum* and *P. commune* were found in a thallus of *Verrucaria ditmarsica1* (sample 30) and *Urospora* sp. was found in a *V. ceuthocarpa* together with *P.* aff. *arthropyreniae* (samples 16 and 17, collected at a single site). Abundances of multiple photobiont species within the thalli and differences in their ecophysiological performance should be subject to further studies in order to understand their effect on the lichen fitness.

The ecological niche of certain lichen species widens with the range of photobionts the mycobiont is capable of associating with (Rolshausen et al. 2018, 2020, Oszycka et al. 2020, Vančurová et al. 2020). In other species, the mycobiont is only capable of switching among photobiont species with similar ecological preferences (Peksa et al. 2022, Škvorová et al. 2022). *Hydropunctaria maura* is a cosmopolitan species with wide ecological amplitude in terms of salinity or substrate; it tolerates salt concentrations from 1 to 35 PSU (Schieffelbein 2009) and grows on various substrates from siliceous rocks to limestone (Smith et al. 2009). In our study, it was collected along the whole salinity gradient where it exhibited low specificity, associating with four *Pseudendoclonium* species/species-level lineages (Fig. 9). At the same time, it exhibited high selectivity towards *P. submarinum.* Not only was it the most frequently associated photobiont (65 out of 69 samples), it was selected even at sites where it was in minority or even virtually absent from the free-living algal community (Fig. 10). Data on the occurrence and ecological requirements of *P. submarinum* is limited, but it can apparently be considered a generalist species, at least in terms of salinity range. Here, it was found in the Baltic Sea, Kattegat and the North Sea, it was also isolated from the West Coast of Chile (Černajová et al. 2022a) and the authentic strain comes from West Scotland (SAG 2237). Therefore, photobiont switch is not the mechanism behind the wide distribution of *H. maura* in the habitat. Contrarily, it seems that the success of the holobiont is given by more or less stable association of two generalists (both the mycobiont and the photobiont).

Recruitment of photobionts de novo at the beginning of thallus formation is one of the fundamental questions in lichen biology. The lingering doubts about the occurrence of free-living lichen

photobionts are a heritage of earlier authors opinions (Ahmadjian, e.g., 1967, 1970, 1987) that were based mainly on the scarcity (both in frequency and abundance) of *Trebouxia* cells in the environment and the belief that the degree of coevolution of lichen symbionts does not allow for non-symbiotic life (Ahmadjian 1988). However, free-living photobiont populations and contact between them and mycobiont hyphae have been documented various times (Sanders and Masumoto 2021 and references therein). Interestingly, these doubts have never been raised for filamentous Ulvophycean photobionts (Tschermak-Woess 1989) although the co-specificity of the free-living and lichenized strains in the pre-molecular era can be reasonably questioned today. While the potential has not been largely exploited yet, the recent accessibility of NGS methods brings a powerful tool into the debate.

Metabarcoding studies of algal diversity in the environment are few (e.g., Frey et al. 2013, Lutz et al. 2015, Rippin et al. 2018) and a single one has focused on diversity of photobionts so far (Vančurová et al. 2020). It confirmed the presence of lichen photobionts in soil and also showed high selectivity of *Stereocaulon* lichens, as algae commonly found in soil were rare in lichen thalli and vice versa (Vančurová et al. 2020). Here, we report a similar pattern. The genera of photobiont species comprise a significant portion of the whole Chlorophycean community (Table 2). But, in half of the cases, the choice of photobionts by lichen thalli at a site was rather a result of strong mycobiont selectivity than algal availability (Fig. 10). A comparable picture can be drawn from the data of Chrismas et at. (2021) who showed high selectivity even for accessory photobionts in the case of the intertidal *L. pygmaea.* Comparing the endothallic and epithallic communities of algae and cyanobacteria they found that the lineages that were the most abundant within the lichen thalli were rare on their surface and vice versa (Chrismas et at. 2021).

In conclusion, the present paper suggests a high selectivity of intertidal lichens which is consistent along a salinity gradient. It is evidenced by both the frequency of the associations and composition of the available photobiont pool in free-living algal communities. Photobiont switch as the main mechanism for lichen niche widening is thus contradicted here.

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References

Abrego D., Ulstrup K. E., Willis B. L. & Van Oppen M. J. H. (2008) Species-specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. Proceedings of the Royal Society B: Biological Sciences 275: 2273-2282.

Andrews S. (2010) FastQC: a quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Ahmadjian V. (1967) A guide to the algae occurring as lichen symbionts: Isolation, culture, cultural physiology, and identification. Phycologia 6: 127-160.

Ahmadjian V. (1970) The lichen symbiosis: Its origin and evolution. In: Dobzhansky T., Hecht M. K. & Steere W. C. (Eds.) Evolutionary Biology 4, Appleton-Century-Crofts, New York, pp. 163-184.

Ahmadjian V. (1987) Coevolution in lichens. In: Lee J. J. & Fredrick J. F. (Eds.) Proceedings of endocytobiology, III. Symposium, New York, pp. 301-315.

Ahmadjian V. (1988) The lichen alga *Trebouxia*: does it occur free-living? Plant Systematics and Evolution 158: 243-247.

Armstrong R. A. (1976) The influence of the frequency of wetting and drying on the radial growth of three saxicolous lichens in the field. New Phytologist 77: 719-724.

Asplund J. & Wardle D. A. (2016) How lichens impact on terrestrial community and ecosystem properties.B iological Reviews 92: 1720-1738.

Baur A., Baur B. & Froberg L. (1994) Herbivory on calcicolous lichens: different food preferences and growth rates in two coexisting land snails. Oecologia 98: 313-319.

Bivand R., Keitt T. & Rowlingson B. (2015) rgdal: bindings for the geospatial data abstraction library. R package version 1.1-3. https://CRAN.Rproject.org/package=rgda.

Brunsdon C. &Chen H. (2014) GISTools: some further GIS capabilities for R. R package version 0.7-4. https://CRAN.R-project.org/package=GISTools.

Castano L. M., del Campo E. M., García-Breijo F. J., Reig-Armiñana J., Gasulla F., del Hoyo A., Guéra A. & Barreno E. (2010) Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus competition? Environmental Microbiology 13: 806-818.

Castresana J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540-552.

Cubero O. F., Crespo A., Fatehi J. & Bridge P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243-249.

Černajová I., Schiefelbein U. & Škaloud P. (2022) Lichens from the littoral zone host diverse Ulvophycean photobionts. Journal of Phycology 58: 267-280.

Černajová I., Steinová J, Škvorová Z. & Škaloud P. (2022) The curious case of *Cladonia luteoalba*: no support for its distinction. Lichenologist 54: 345-354.

Chrismas N. A. M., Allen R., Hollingsworth A. L., Taylor J. D. & Cunliffe M. (2021) Complex photobiont diversity in the marine lichen *Lichina pygmaea*. Journal of the Marine Biological Association of the United Kingdom 101: 667-674.

Darienko T. & Pröschold T. (2017) Toward a monograph of non-marine Ulvophyceae using an integrative approach (Molecular phylogeny and systematics of terrestrial Ulvophyceae II.). Phytotaxa. 324: 1-41.

Darriba D., Taboada G. L., Doallo R. & Posada D. (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Delmail D., Grube M., Parrot D., Cook-Moreau J., Boustie J., Labrousse P. & Tomasi S. (2013) Halotolerance in Lichens: Symbiotic Coalition Against Salt Stress. In: Ahmad P., Azooz M. M. & Prasad M. N. N. (Eds.) Ecophysiology and Responses of Plants under Salt Stress, Springer, New York, pp. 115-148.

Dobson F. S. (2014) A Field Key to Coastal and Seashore Lichens. Frank S. Dobson, New Malden, UK, 100 pp.

Dormann C. F., Frueund J., Bluethgen N. & Gruber B. (2009) Indices, graphs and null models: analyzing bipartite ecological networks. Open Ecology Journal 2: 7-24.

Frey B., Bühler L., Schmutz S., Zumsteg A. & Furrer G. (2013) Molecular characterization of phototrophic microorganisms in the forefield of a receding glacier in the Swiss Alps. Environmental Research Letters 8: 015033.

Gardes M. & Bruns T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Gasulla F., Guéra A., De Los Riós A. & Pérez-Ortega S. (2019) Differential responses to salt concentrations of lichen photobiont strains isolated from lichens occurring in different littoral zones. Plant and Fungal Systematics 64: 149-162.

Geburzi J. C., Heuer N., Homberger L., Kabus J., Moesges Z, Ovenbeck K. Brandis D. & Ewers-Saucedo C. (2021) A marginal habitat, but not a sink: Ecological genetics reveal a diversification hotspot for marine invertebrates in the brackish Baltic Sea. bioRxiv. 2021.06.06.447071

Gueidan C., Savić S., Thüs H., Roux C., Keller C, Tibell, L., Prieto, M., Heidmarsson S., Breuss O., Orange A., Fröberg L., Wynns A. A., Navarro-Rosinés P., Krzewicka B., Pykälä J, Grube M. & Luztoni F. (2009) Generic classification of the Verrucariaceae (Ascomycota) based on molecular and morphological evidence: Recent progress and remaining challenges. Taxon 58: 184-208.

Heiđmarsson S., Gueidan C., Miądlikowska J. & Lutzoni F. (2017) Multilocus phylogeny supports the placement of *Endocarpon pulvinatum* within *Staurothele* s. str. (lichenised ascomycetes, Eurotiomycetes, Verrucariaceae). Phytotaxa 306: 37-48.

Helms G. (2003) Taxonomy and symbiosis in associations of Physciaceae and *Trebouxia*. University of Göttingen (Doctoral thesis), 156 pp.

Hijmans R. J. (2015) Raster: Geographic data analysis and modeling. R Package Version 2.4-15. http://CRAN.R-project.org/package=raster

Jahns H. M. (1993) Culture experiments with lichens. Plant Systematics and Evolution 187: 145-174.

Jones D. (1988) Lichens and pedogenesis. In: Galun M. (Ed.) CRC Handbook of lichenology, Vol. 3, CRC Press, Boca Raton, pp. 109-124.

Katoh K., Rozewicki J. & Yamada K. D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20: 1160-1166.

Lalley J. S., Viles H. A., Henschel J. R. & Lalley V. (2006) Lichen-dominated soil crusts as arthropod habitat in warm deserts. Journal of Arid Environments 67: 579-593.

Leliaert F., Rueness J., Boedeker C., Maggs C. A., Cocquyt E., Verbruggen H. & De Clerck O. (2009) Systematics of the marine microfilamentous green algae *Uronema curvatum* and *Urospora microscopica* (Chlorophyta). European Journal of Phycology 44: 487-496.

Liu B., Wang Q., Li S., Fang J., Liu G. & Hu Z. (2019) Taxonomic transfer of *Gongrosira fluminensis* Fritsch (Chaetophorales, Chlorophyceae) to *Lithotrichon* Darienko et Pröschold (Ulvales, Ulvophyceae) based on morphological observation and phylogenetic analyses. Fottea 19: 25-32.

Lücking R., Hodkinson B. P. & Leavitt S. (2017) The 2016 classification of lichenized fungi in the Ascomycota and Basidiomycota – Approaching one thousand genera. Bryologist 119: 361-416.

Lutz S., Anesio A. M., Edwards A. & Benning L. G. (2015) Microbial diversity on Icelandic glaciers and ice caps. Frontiers in Microbiology 6: 307.

Mahé F., Rognes T., Quince C., de Vargas C. & Dunthorn M. (2015) Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ 3: e1420.

Miller M. A., Pfeiffer W. & Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop, 14 Nov. 2010: 1-8.

Nash III. T. H. (2008) Lichens as self-contained miniature ecosystems. In: Nash III. T. H. (Ed.) Lichen Biology. Cambridge University Press, Cambridge, UK, pp. 7-8.

Von Nordheim H. & Boedeker D. (1998) Introduction and Scientific Concept for the Red List of Marine and Coastal Biotopes and Biotope Complexes of the Baltic Sea, Belt Sea and Kattegat. Baltic Sea Environment Proceedings 75: 1-14.

Olofsson M., Hagan J. G., Karlson B. & Gamfeldt L. (2020) Large seasonal and spatial variation in nano- and microphytoplankton diversity along a Baltic Sea – North Sea salinity gradient. Scientific Reports 10: 1-12.

Orange A. (2012) Semi-cryptic marine species of *Hydropunctaria* (Verrucariaceae, lichenized Ascomycota) from north-west Europe. Lichenologist 44: 299-320.

Orange A. & Chhetri S. (2022) Verrucariaceae from Nepal. Lichenologist 54: 139-174.

Osyczka P., Lenart-Boroń A., Boroń P. & Rola K. (2021) Lichen-forming fungi in postindustrial habitats involve alternative photobionts. Mycologia 113: 43-55.

Paavola M., Olenin S. & Leppäkoski E. (2005) Are invasive species most successful in habitats of low native species richness across European brackish water seas? Estuarine, Coastal and Shelf Science 64: 738-750.

Peksa O. & Škaloud P. (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). Molecular Ecology 20: 3936-3948.

Peksa O., Gebouská T., Škvorová Z., Vančurová L. & Škaloud P. (2022) The guilds in green algal lichens – an insight into the life of terrestrial symbiotic communities. FEMS Microbiology Ecology 98: 1-17.

Pérez-Ortega S., Miller K. A. & De Los Ríos A. (2018) Challenging the lichen concept: *Turgidosculum ulvae* (Verrucariaceae) represents an independent photobiont shift to a multicellular blade-like alga. Lichenologist 50: 341-356.

Pérez-Ortega S., De Los Ríos A., Crespo A. & Sancho L. G. (2010) Symbiotic lifestyle and phylogenetic relationships of the bionts of *Mastodia tessellata* (Ascomycota, incertae sedis). American Journal of Botany 97: 738-752.

R Core Team (2021) R: A Language and Environment for Statistical Computing. http://www.r-project.org/.

Rikkinen J. (2003) Ecological and evolutionary role of photobiont-mediated guilds in lichens. Symbiosis 34: 99-11.

Rippin M., Borchhardt N., Williams L., Colesie C., Jung P., Büdel B., Karsten U. & Becker B. (2018) Genus richness of microalgae and Cyanobacteria in biological soil crusts from Svalbard and Livingston Island: morphological versus molecular approaches. Polar Biology 41: 909-923.

Rolshausen G., Dal Grande F., Sadowska-Deś A. D., Otte J. & Schmitt I. (2018) Quantifying the climatic niche of symbiont partners in a lichen symbiosis indicates mutualist-mediated niche expansions. Ecography 41: 1380-1392.

Rolshausen G., Hallman U., Dal Grande F., Otte J., Knudsen K. & Schmitt I. (2020) Expanding the mutualistic niche: parallel symbiont turnover along climatic gradients. Proceedings of the Royal Society B 287: 20192311.

Ronquis F., Teslenko M., van der Mark P., Ayres D., Darling A., Hohna S., Larget B., Liu L., Suchard M. A. & Huelsenbeck J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539-542.

Rosentreter R., Eldridge D. J., Westberg M., Williams L. & Grube M. 2016. Structure, composition, and function of biocrust lichen communities. In: Weber B., Büdel B. & Belnap J. (Eds.) Biological soil crusts: an organizing principle in drylands. Springer, Berlin, pp. 121-138.

Sanders W. B. & Masumoto H. (2021) Lichen algae: the photosynthetic partners in lichen symbioses. Lichenologist 53: 347-393.

Savić S., Tibell L., Gueidan C. & Lutzoni F. (2008) Molecular phylogeny and systematics of *Polyblastia* (Verrucariaceae, Eurotiomycetes) and allied genera. Mycological Research 112: 1307-1318.

Schieffelbein U. (2009) The marine and maritime lichens of the Baltic Sea – an overview. Bibliotheca Lichenologica 100: 463-494.

Škaloud P. & Rindi F. (2013) Ecological differentiation of cryptic species within an asexual protist morphospecies: A case study of filamentous green alga *Klebsormidium* (Streptophyta). Journal of Eukaryotic Microbiology 60: 350-362.

Škaloud P., Rindi F., Boedeker C. & Leliaert F. (2018) Freshwater Flora of Central Europe, Vol 13: Chlorophyta: Ulvophyceae. Springer Spektrum, Berlin, 289 pp.

Škvorová Z., Černajová I., Steinová J., Peksa O., Moya P. & Škaloud P. (2022) Promiscuity in lichens follows clear rules: partner switching in *Cladonia* is regulated by climatic factors and soil chemistry. Frontiers in Microbiology 12: 781585.

Smith C. W., Aptroot A., Coppins B. J., Fletcher A., Gilbert O. L., James P. W & Wolseley P. A. (2009) The Lichens of Great Britain and Ireland. British Lichen Society, London, 1046 pp.

Spribille T., Fryday A., Pérez-Ortega S., Svensson M., Tønsberg T., Ekman S., Holien H., Resl P., Schneider K., Stabentheiner E., Thüs H., Vondrák J. & Sharman L. (2020) Lichens and associated fungi from Glacier Bay National Park, Alaska. Lichenologist 52: 61-181.

Spribille T., Resl P., Stanton D. E. & Tagirdzhanova G. (2022) Evolutionary biology of lichen symbioses. New Phytologist 234: 1566-1582.

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

Strandmark A., Bring A., Cousins S. A. O., Destouni G., Kautsky H., Kolb G., de la Torre-Castro M. & Hambäck P. A. (2015) Climate change effects on the Baltic Sea borderland between land and sea. AMBIO 44: S28-S38.

Thüs H., Muggia L., Pérez-Ortega S., Favero-Longo S. E., Joneson S., O'Brien H., Nelsen M. P., Duque-Thüs R., Grube M., Friedl T., Brodie J., Andrew C. J. Lücking R., Lutzoni F. & Gueidan C. (2011) Revisiting photobiont diversity in the lichen family Verrucariaceae (Ascomycota). European Journal of Phycology 46: 399-415.

Tschermak-Woess E. (1976) Algal taxonomy and the taxonomy of lichens: the phycobiont of *Verrucaria adriatica*. In: Brosn D. H., Hawksworth D. L. & Bailey R. H. (Eds.) Lichenology: Progress and Problems. Academic Press, New York, pp. 79-88.

Tschermak-Woess E. (1989) The algal partner. In: Galun (Ed.) CRC Handbook of Lichenology, Volume I, CRC Press, Boca Raton, Florida, pp. 39-92.

Vančurová L., Kalníková V., Peksa O., Škvorová Z., Malíček J., Moya P. Chytrý K., Černajová I. & Škaloud P. (2020) Symbiosis between river and dry lands: Phycobiont dynamics on river gravel bars. Algal Research 51: 102062.

Větrovský T., Baldrian P. & Morais D. (2018) SEED 2: a user-friendly platform for amplicon high-throughput sequencing data analyses. Bioinformatics 34: 2292-2294.

Watanabe S., Nakano T. & Deguchi H. (1997) Photobionts isolated from maritime lichens. Journal of Marine Biotechnology 5: 103-112.

White T. J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (Eds.) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp. 315-322.

Wickham H. &Seidel D. (2022) scales: Scale Functions for Visualization. https://scales.r-lib.org, https://github.com/r-lib/scales.

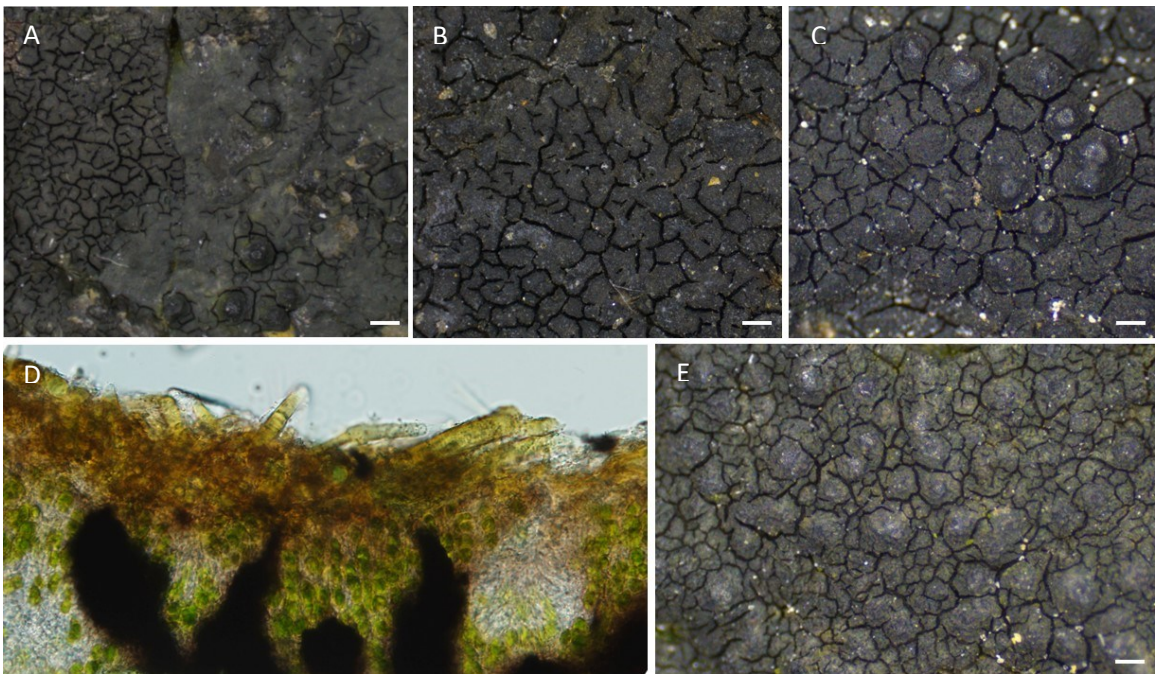


Figure S1 *Hydropunctaria* species. A *H. maura* (left) and *H. aractina* (right) when growing together. **B** *H. maura* alone. **C** *H. aractina* alone. **D** Cyanobacteria on the thallus surface disguising cortical pigments. **E** *H. oceanica*. Scale bars represent 200 µm.

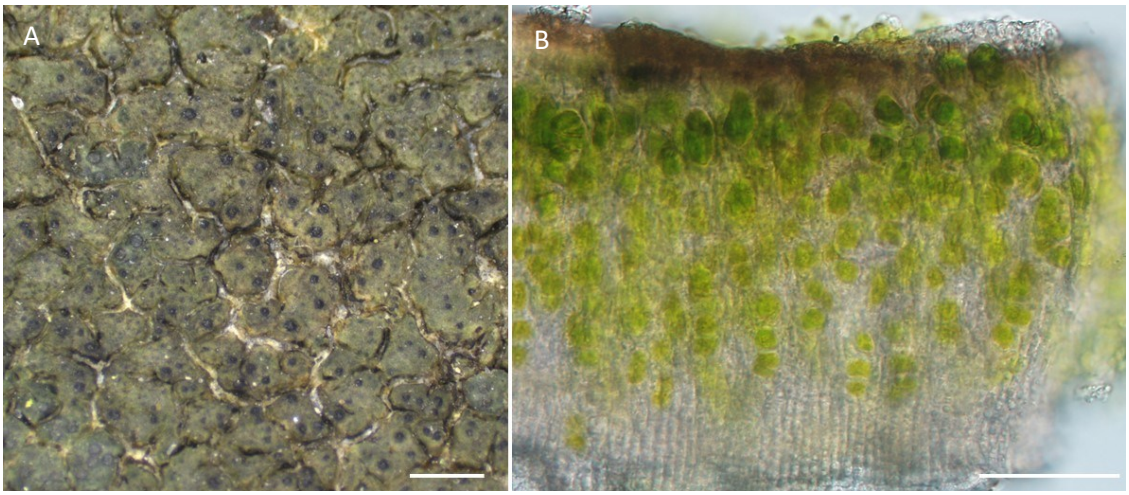
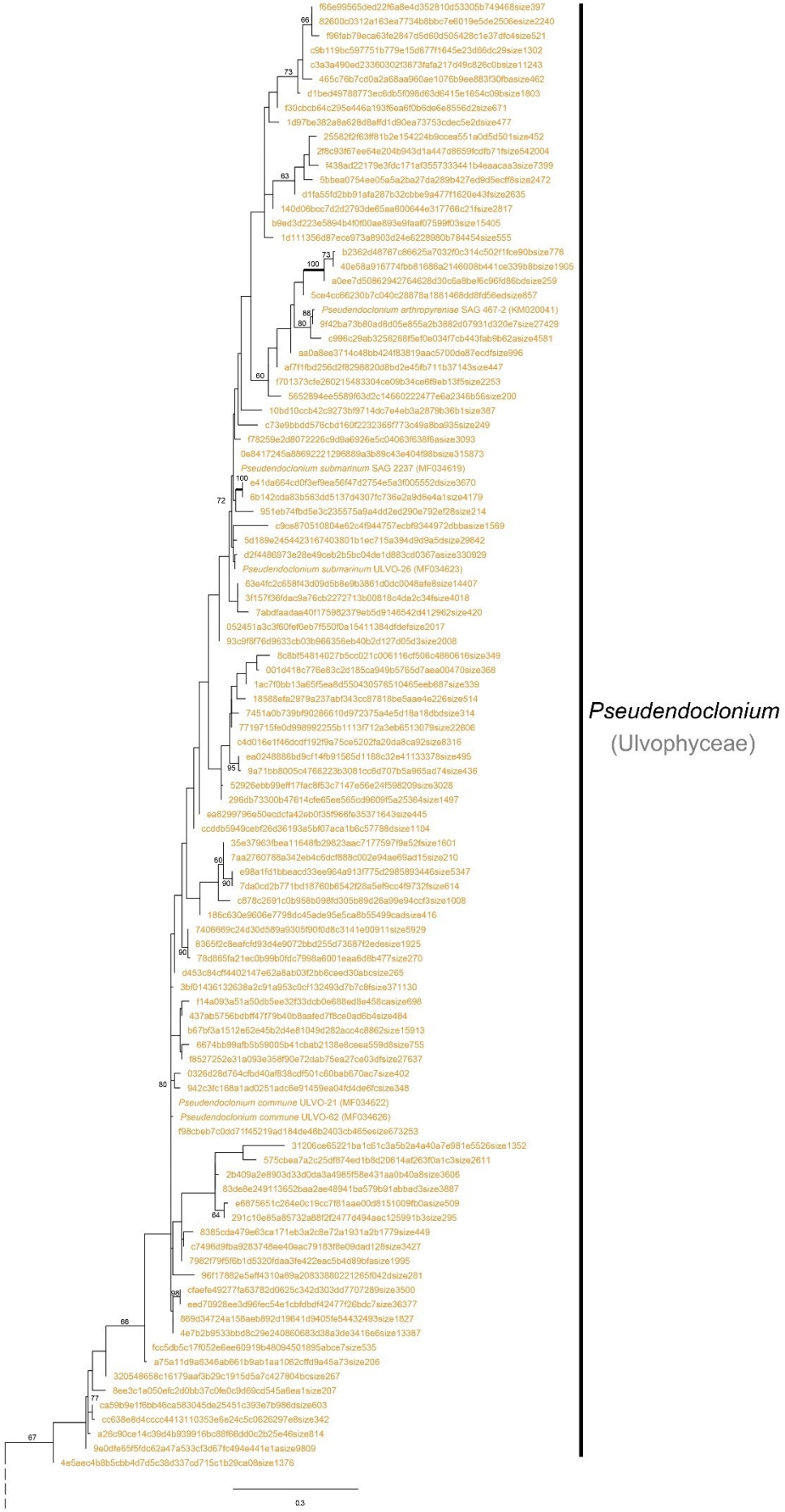


Figure S2 *Verrucaria ceuthocarpa*. **A** morphology **B** cross-section. Scale bars represent 1 mm (**A**) and 50 µm (**B**).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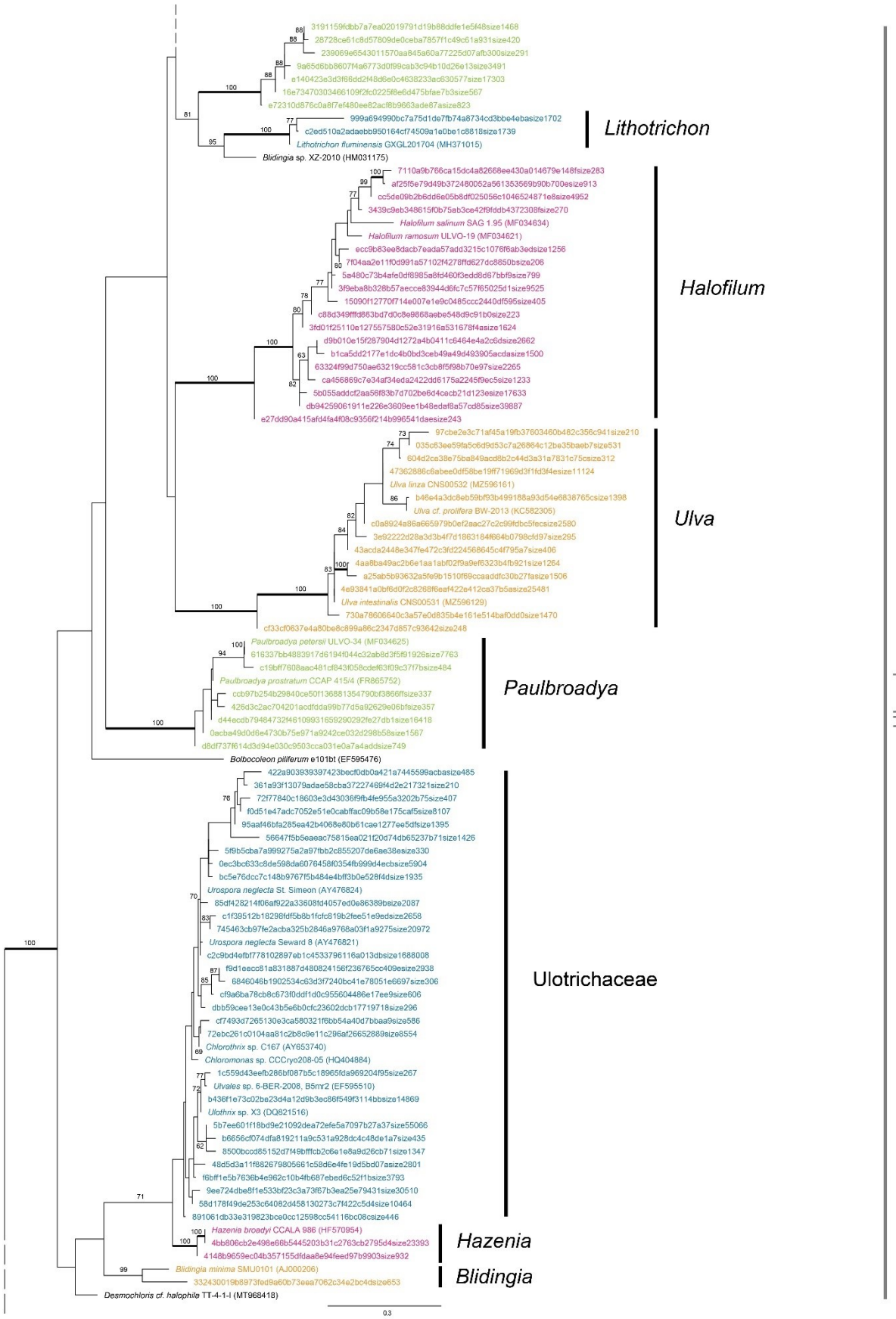
Pseudendoclonium
(Ulvophyceae)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Ulva
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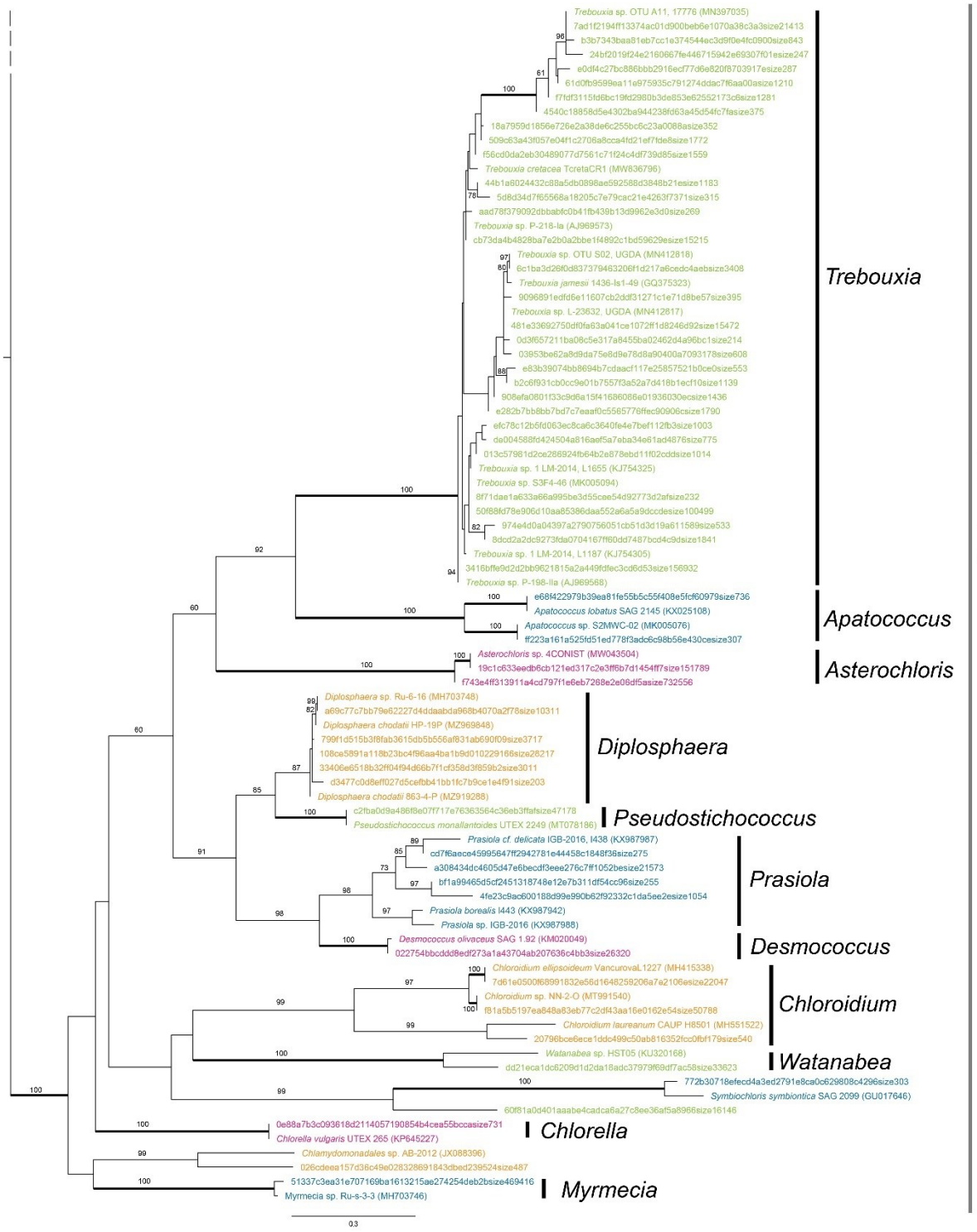

Figure S3 Phylogenetic tree of Ulvophyceae and Trebouxiophyceae based on Maximum Likelihood (ML) of ITS2 rDNA obtained by Illumina metabarcoding of the free-living algal communities. Only ML bootstrap values > 60 are shown. Branches with full bootstrap values are thickened. Scale bar represents the expected number of substitutions per site.

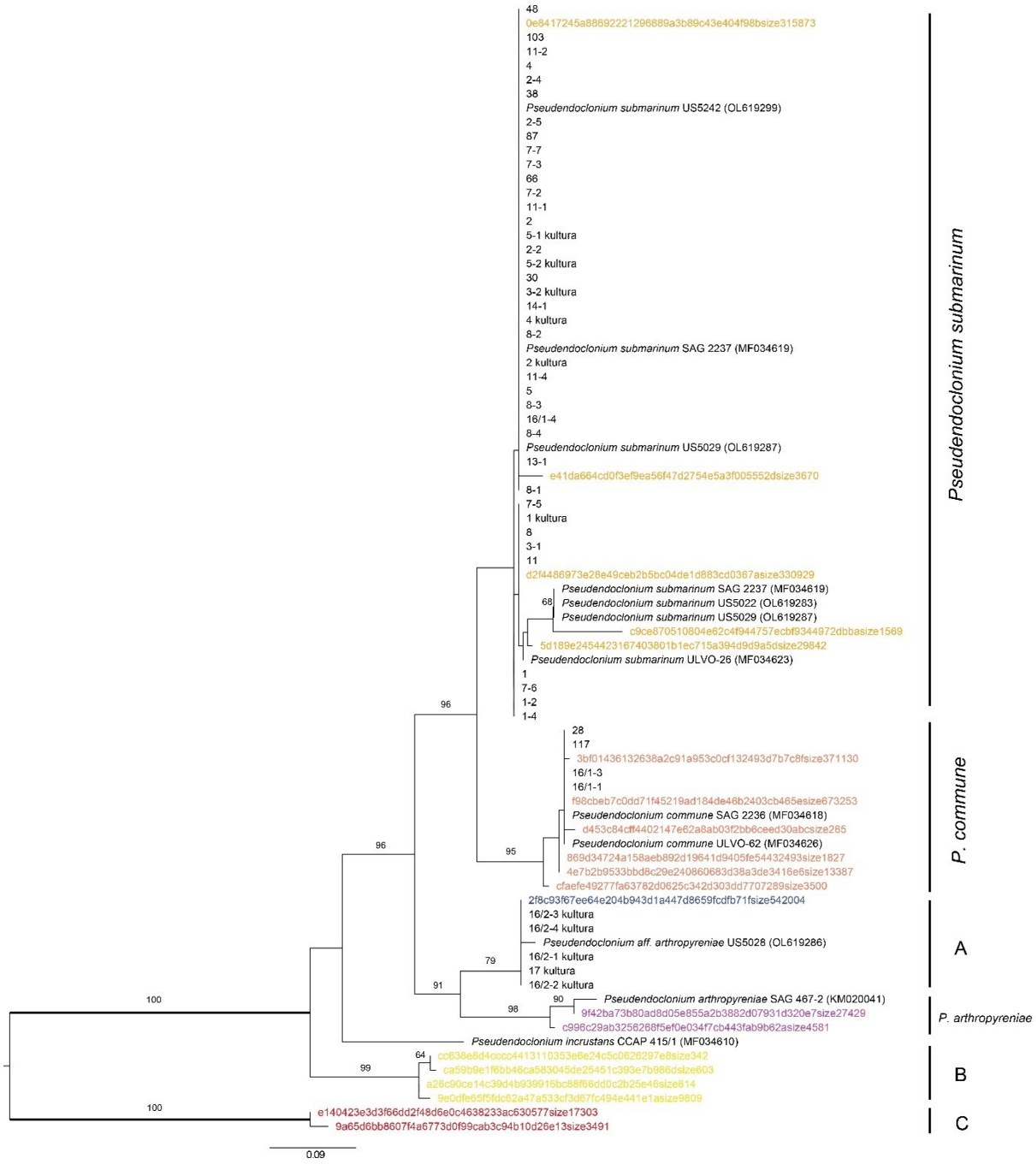

Figure S4 Phylogeny of the genus *Pseudendoclonium* based on Maximum Likelihood (ML) of ITS2 rDNA. Sequences obtained by Sanger sequencing of lichen thalli and by Illumina metabarcoding of the free-living algal communities (in colour) are included together with reference sequences from GenBank. Only ML bootstrap values > 60 are shown. Scale bar represents the expected number of substitutions per site. A = *P. aff. arthropyreniae*; B = *Pseudendoclonium* sp. a C = *Pseudendoclonium*-like.

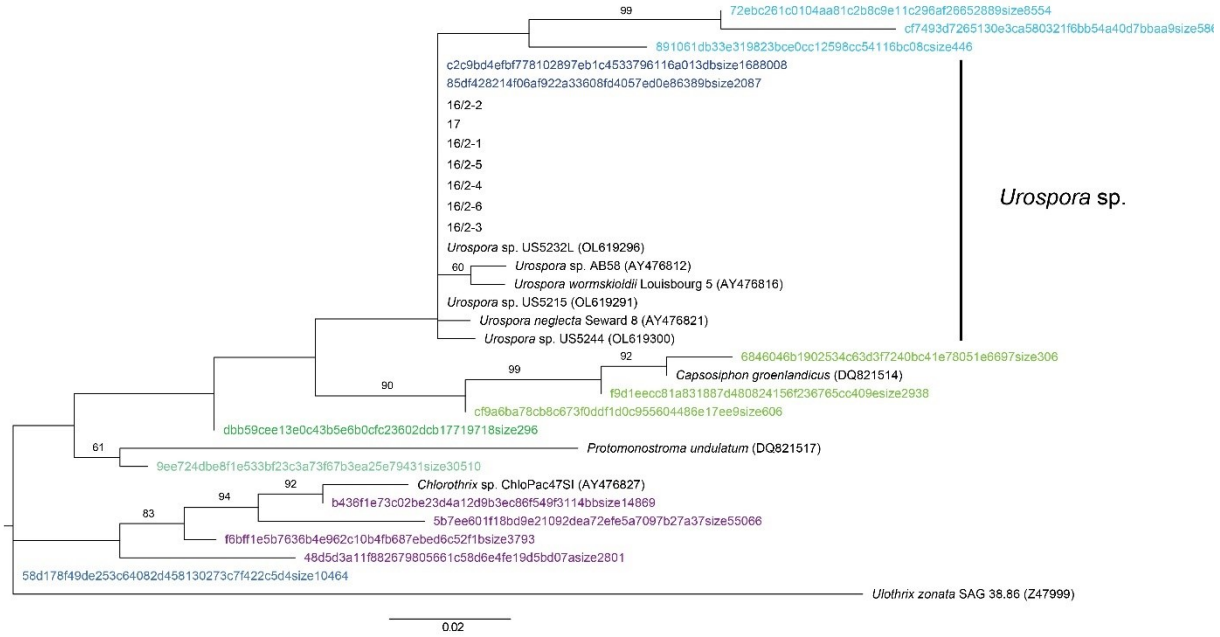

Figure S5 Phylogeny of Ulotrichales based on Maximum Likelihood (ML) of ITS2 rDNA. Sequences obtained by Sanger sequencing of lichen thalli and by Illumina metabarcoding of the free-living algal communities (in colour) are included together with reference sequences from GenBank. Only ML bootstrap values > 60 are shown. Scale bar represents the expected number of substitutions per site.