Hidden Complexity of Lichen Symbiosis

Insights into Functionality, Reproduction and Composition

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Cover: *Letharia vulpina,* the lichen behind several results presented in this thesis (photo: Veera Tuovinen, modified by David Nogerius)

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Hidden complexity of lichen symbiosis - Insights into functionality, reproduction and composition

Abstract

Lichens are tremendously diverse physical outcomes of symbiotic relationships involving fungi, algae and bacteria. This thesis aims to give insight into the functionality, composition and reproduction of lichens from the fungal perspective. When previous results from a barcoding study were re-evaluated, no support for a free-living life phase of *Cladonia* mycobionts could be found. Genomic and transcriptomic data were used to identify the fungal partners in thalli, and a fluorescent *in situ* hybridization (FISH) method for the simultaneous visualization of the different fungi was developed. This approach led to the discovery of previously unknown basidiomycetes, *Cyphobasidium* spp., which are widespread components of the cortex of lichens in *Parmeliaceae*. In some cases, the abundance of *Cyphobasidium* correlates with previously unexplained phenotypic variation of the lichens. In the case example *Bryoria capillaris*, *Cyphobasidium* yeasts are the dominant cells in the cortex and hence the fungus that meets the eye when looking at the lichen. With the help of hologenomic data and FISH, it could also be shown that *Tremella lethariae*, a lichenicolous heterobasidiomycete known to induce galls on *Letharia*, is dimorphic and frequently occupies the cortex of asymptomatic *Letharia* thalli in its anamorphic state. Finally, genomic and transcriptomic data was used to investigate the mating system of the genus *Letharia*, which includes species with different reproductive strategies. All studied *Letharia* species have a heterothallic mating system, meaning they need to find a compatible mate in order to sexually reproduce. Thus, the variation of reproduction strategies within the genus cannot be explained only by the mating system of the *Letharia* mycobiont. Our data on the mating-type ratios indicates that no potential for sexual reproduction exists for the red-listed *L. vulpina* in Sweden, as only one mating type is found in the populations. The roles the secondary fungi have in the symbiosis is not yet fully understood. However, the results indicate that we should not automatically assume that some of the organisms are negligible for the function of the holobiont, or that all functions assigned to fungal origin are conducted by the primary mycobiont. Altogether, the findings presented in this thesis support the view of lichens as a community of bionts with complex interactions and calls for a more holistic approach for the study of the symbiosis.

Keywords: mycobiont, lichenicolous, mutualistic, parasitic, FISH, *Tremella, Cyphobasidium*, mating system, holobiont

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Dedication

To my dearest mom, dad and Emma-Maria

And yet here they were, a fungus and an alga, hanging out in lichen guise on a sun-bathed pine branch on one of the driest, least forgiving slopes in the Clearwater Valley.

Trevor Goward

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Tuovinen, V., Svensson, M., Kubartová, A., Ottosson, E., Stenlid, J., Thor, G., Dahlberg, A. (2015). No support for occurrence of free-living *Cladonia* mycobionts in dead wood. *Fungal Ecology* 14, 130-132.

II Tuovinen, V., Ament-Velásquez, S.L., Bergström, L., Spribille, T., Vanderpool, D., Nascimbene, J., Yamamoto, Y., Thor, G., Johannesson, H. Mating system in *Letharia*: insights from genomic and population analyses of the mating-type loci (manuscript).

III 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* 253, 488-492.

IV Tuovinen, V., Ekman, S., Spribille, T., Thor, G., Johannesson, H. Yeast-forming tremelloid fungi in the cortex of *Letharia* lichens (manuscript).

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The contribution of Veera Tuovinen to the papers included in this thesis was as follows:

I Main author. Carried out the laboratory work and analyses. Developed study design and wrote the paper with assistance from co-authors. Responsible for correspondence with the journal.

II Main author. Developed the hypothesis and study design together with supervisors and conducted parts of the fieldwork in Sweden, most of the laboratory work and sequence analyses. Took part in assembling the draft genomes and identifying the MAT loci. Wrote the paper with HJ and LA with assistance from GT.

III Second author. Developed the hypothesis about the location of *Cyphobasidium* with TS and had main responsibility for FISH method development and related laboratory work and confocal microscopy imaging. Conducted most of the laboratory work for *Letharia*. Took part in writing with other co-authors.

IV Main author. Developed the hypothesis and study design with co-authors. Designed new FISH probes. Carried out the microscopy work and laboratory work except for the cloning. Did the sequence analyses. Wrote the manuscript with assistance from co-authors.

Abbreviations

DNA	Deoxyribonucleic acid
LSCM	Laser scanning confocal microscopy
FISH	Fluorescent *in situ* hybridization
MAT	The mating-type locus
NGS	Next generation sequencing
ORF	Open reading frame, *i.e.*, the protein coding part of a gene
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

1 Introduction

Symbiosis is a widespread and successful life strategy found in all kingdoms of life. This fascinating life style is ancient and has led to many evolutionary innovations, from the origin of eukaryotic cell (see Archibald, 2011) to complex networks between insects cultivating and feeding on fungi (*e.g.*, Caldera et al., 2009; Hussa and Goodrich-Blair, 2013). Throughout this thesis I am going to use the term symbiosis as "unlike organisms living together", a definition proposed by de Bary already in 1879. This definition does not account for the type of interactions observed between the partners (bionts), nor if the symbiotic relationships are obligate (*i.e.,* the organisms cannot survive without each other) or facultative (*i.e*., the organisms are not existentially dependent on each other). Traditionally, symbiosis has been divided into three categories: mutualistic, parasitic, and commensalistic. In the first scenario, all symbionts benefit from the symbiosis compared to their free-living states. Parasites lower the fitness of their host, whereas commensalists can be considered as free-passengers, not harming nor benefiting the symbiosis. However, several authors stress the unnatural division of organismal relationships to these categories, as symbiosis in nature is often observed as a dynamic parasitic-mutualistic continuum varying in time, depending on external factors and the life cycle phase of the organism (*e.g.,* Sapp, 2004; Zook, 2015; Vasiliuskas et al., 2007; Martos et al., 2009).

Lichens, the unique symbiotic interactions between fungi and plants, play a leading role in this thesis. The lichenized life style has evolved independently several times both in Ascomycota and Basidiomycota (Fig. 1) and 415 my old fossils suggest the existence of lichens already in early Devonian (Honegger et al., 2013). Reflecting the success of this life strategy, 19 000 species of lichenized fungi are currently accepted, accounting for 17 % of all known fungal species (Lücking et al., 2016). In addition, epiphytic lichens offer a neat study system of the symbiotic interactions, as the symbiotic structures (thalli) are visible and delimited in space. In this thesis I aim to widen the perception

of lichens in the families Parmeliaceae and Cladoniaceae (Ascomycota) with symbiotic associations with basidiomycetous fungi (Fig. 1).

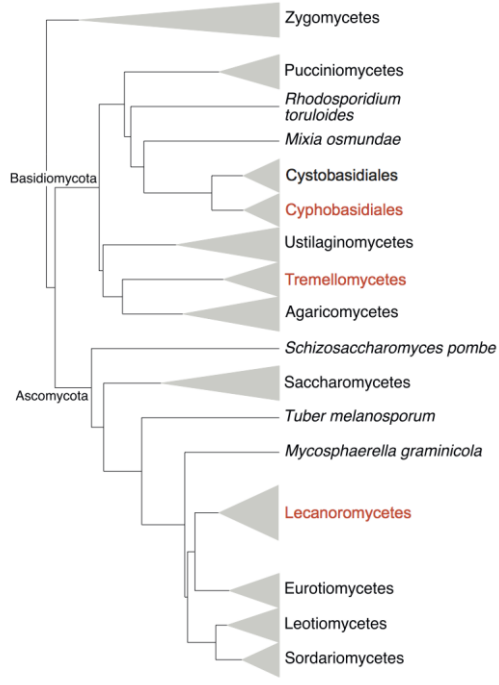

Figure 1. Phylogenetic placement of the groups studied in this thesis (marked in red). Cladoniaceae and Parmeliaceae belong to the largest class of lichens, Lecanoromycetes. Tree modified from the Paper II.

1.1 Lichen symbiosis: inhabitants of the thalli

Lichens are symbiotic, self-replicating composite organisms composed of fungi, algae, and bacteria. Definitions of lichens, including the predicted relationships between the organisms involved, are many, and despite more than a century of research no consensus exists (*e.g.*, Bates et al., 2012; Lücking et al., 2016). A definition introduced by Hawksworth (1982) considers lichens as stable self-supporting associations of a fungus (mycobiont) and a photosynthesizing partner (photobiont) in which the mycobiont is the exhabitant, *i.e.*, forming the external structures that enclose the photobionts. The composite structures that the involved organisms (bionts) together form, the lichen body, is called thallus. The exhabitant mycobiont is often considered

primary, existing in an ecologically obligate symbiosis (Honegger, 1991), as no free-living states of most mycobionts have been found in nature (but see Wedin et al., 2004). In contrast, at least some photosynthesizing partners can live without a mycobiont in nature and are considered facultative symbionts (Honegger, 1991). The life cycle and growth of the photobionts are somewhat suppressed and controlled by the mycobiont (Honegger, 1991; Molina et al., 1998), but as these symbionts together can tolerate extreme habitats where none could survive alone (De la Torre et al., 2010), the nature of the symbiosis is often considered mutualistic.

Despite the various definitions that stress the dual nature of lichens, the occurrence of additional microbes in thalli has been known for decades (Hawksworth, 1982). In recent years, culture-based and molecular studies have shed light on the organismal diversity of these communities, including bacteria, lichenicolous fungi and endolichenic fungi (*e.g.*, Cardinale et al., 2006; Bates et al., 2012; Park et al., 2014; Girlanda et al., 1997; Arnold et al., 2009; U'ren et al., 2010). Lichenicolous fungi can be defined as lichen-associated fungi that range in their relationship to their host lichen from parasitic to commensalistic (Hawksworth, 1983; Lawrey and Diederich, 2003). Most of them have been found exclusively in lichens and are regarded as obligately lichen-associated, but some show transitions in their nutritional state and can be found in saprotrophic or parasitic states outside of lichens (Hawksworth, 1982; Adams, 1996). Endolichenic fungi have been defined as commensalistic lichen inhabiting fungi, which are ecologically and taxonomically different from lichenicolous fungi (Arnold et al., 2009). Already Farrar (1976) proposed that lichens should be viewed as miniature ecosystems, but in spite of the growing amount of evidence on the diversity of lichen-associated microbes, these "secondary organisms" are still not regularly included in the study of lichens.

1.1.1 How to call lichens?

> Lichens have no names! There, I've said it. Lord knows somebody had to. Lichens have been going around nameless for nearly half a century now. It's indecent. Surely it's time we showed them a little more respect. (Goward, 2008).

A peculiarity in lichenology, stemming from the composite nature of lichens, is that thallus, the actual phenotype of the symbiosis, does not have a name. According to the International Code of Botanical Nomenclature (2011), "for nomenclatural purposes names given to lichens shall apply to their fungal components". Thus, the species names given for lichens refer only to the primary mycobiont and are also used as such in the following text. Throughout this thesis, I refer to the lichen thallus as including all associated organisms and

call it a holobiont, a term introduced by Margulis and Fester (1991). Furthermore, I use the term primary mycobiont for the named lichenized fungus that is the exhabitant of the thallus and call all other fungi "secondary" for clarity. Though, I want to note, that the term "secondary" does not here refer to the nature of the fungus, its function or importance for the symbiotic holobiont.

1.2 Thallus structure and the roles of the bionts

An outstanding feature of lichen symbiosis is that individual bionts, when cultured separately, do not resemble the unique phenotypes found in nature (Ahmadjian 1973). In addition, the resynthesis of fully complex thalli from primary mycobionts and photobionts in sterile conditions *in vitro* has not been successful (Ahmadjian, 1973; Honegger, 1993; Büdel and Scheidegger, 2008). The structure of the lichen thalli in nature varies from minute crustose to leafy and shrubby, with tremendous diversity in morphology and color. In this thesis, the focus is on stratified macrolichens, *i.e*., lichens with large and complex thalli, from the families Parmeliaceae and Cladoniaceae, which have green algae as their photobionts (Fig. 1). The thallus of stratified macrolichens is differentiated to a compact cortex consisting of fungal cells and rich in polysaccharides, the medulla with fungal hyphae, and a layer of photobionts (Büdel and Scheidegger, 2008).

The primary mycobiont is traditionally assigned the role of providing structure and shelter against the surrounding environment, while the photobiont provides energy for the mycobiont by photosynthesis. The assumption that primary mycobionts obtain energy only from the photobiont has seldom been questioned. Primary mycobionts that can develop to either saprotrophs or form symbiosis with algae, a phenomenon called "optional lichenization" (Wedin et al., 2004), are known only from crustose lichens (Wedin et al., 2006; Muggia et al., 2011). However, many lichens have the capacity to produce extracellular enzymes with potential for degradation of organic matter (reviewed in Becket et al., 2013). It has been suggested that some may obtain supplementary carbon by saprotrophic activities when the algal photosynthesis is low (Beckett et al., 2015). A barcoding study on wood-decaying fungal communities discovered several lichen-forming fungal taxa, including both crustose and macrolichens, several centimeters deep inside decaying wood of Norway spruce (Kubartová et al., 2012). These findings suggest that these fungi might have saprotrophic phase in their life cycle, which would widen the perception on ecological plasticity of primary mycobionts and give new insights into their life cycles. These results are studied and discussed further in Paper I.

The energy acquisition of lichenicolous fungi has seldom been studied in detail but in the case of parasitic interactions, haustoria attacking both the primary mycobiont (Grube and de Los Ríos, 2001) and the photobiont (Feige et al., 1993) have been observed. Hawksworth (1988) suggested that commensalistic lichenicolous fungi would be nutritionally similar to primary mycobionts and endolichenic fungi are found to be associated mainly with the photobiont (Arnold et al., 2009). In many other eukaryotic symbioses it has been widely accepted that associated microbiota may improve host fitness (Hussa and Goodrich-Blair, 2013). During recent years, this view has started to get a foothold also among lichenologists. Several researchers have suggested that lichen-associated microbes may add a mutualistic contribution to the symbiosis (*e.g.*, Grube et al., 2015; Kellog and Raja, 2016). However, the roles and effects of the secondary mycobionts for the holobiont are largely unknown. These issues are discussed further in the Paper IV.

1.3 The pretty colors

> No feature is more valuable to their fitness than their production of a laboratory full of secondary compounds, commonly labeled as lichen acids. (Zook, 2015).

A characteristic feature of lichens is the color diversity of thalli, created by secondary metabolites that are produced in lichens, but not always in the pure cultures of the individual bionts (Huneck, 1999). It is often assumed that the primary mycobiont is the producer of the secondary metabolites (Elix and Stocker-Wörgötter, 2008) but also the endolichenic community stands for a different set of substances (revised in Kellog and Raja, 2016). Secondary metabolites have been suggested to function *inter alia* as self-defence against pathogens and herbivores and as sunscreens (Solhaug and Gauslaa, 2012), although their antimicrobial effects vary (Lauterwein et al., 1995, Merinero et al., 2015; Kosanić and Ranković, 2015), and the fungal tissue *per se* was found to be a more important UV blocker than the secondary metabolites in *Lobaria pulmonaria* in a recent study by Gauslaa et al. (2017).

Lichen secondary metabolites have a long history in anthropogenic use, one well known but toxic example being vulpinic acid, extracted among others from the genus *Letharia* (Parmeliaceae). *Letharia* lichens are recognised by even many non-lichenologists - the bright yellow color draws attention to the shrubby, branched epiphytic thalli even from a distance. These lichens have traditionally been used in dying but also as a poison to kill foxes and wolves in western North America and Fennoscandia, which is mirrored in the names of

two of the species *Letharia vulpina* and *L. lupina* (Santesson, 1939; Altermann et al., 2016). Secondary metabolites have been used as a taxonomic character in many groups (Hawksworth, 1976), but it has been shown that chemotypes do not always reflect the phylogeny of the primary mycobionts (*e.g.*, Boluda et al., 2015). One such puzzling species pair has been *Bryoria fremontii – Bryoria tortuosa* (Parmeliaceae), where the *tortuosa* phenotype produces the toxic vulpinic acid, whereas *B. fremontii* is edible and has been widely used as food by indigenous peoples of North America (Turner, 1979). These two epiphytic hairy lichens have genetically identical primary mycobionts and photobionts (Velmala et al., 2009) and the cause behind the phenotype variation has not been known. The secondary metabolite in question, vulpinic acid, led us to the detailed study of the *Bryoria* and *Letharia* holobionts in Paper III.

1.4 Reproduction

From an evolutionary perspective, reproduction can be argued to be one of the most important traits organisms possess, affecting the species ability to adapt and survive in long term. Understanding of species reproductive system is also of crucial importance for applied research in biodiversity and conservation. Many lichens have mixed reproductive strategies including vegetative dispersal with symbiotic propagules and sexual reproduction via spores of the primary mycobiont. A special feature of the primary mycobionts is that the symbiotic state is needed for the sexual reproduction, fruiting bodies are often perennial and both vegetative and sexual structures persist in parallel over long periods of time. Endolichenic fungi are not known to reproduce sexually in lichen thalli, but are horizontally transmitted (U'ren et al., 2010). They have also been suggested to be transmitted in vegetative propagules (Peršoh and Rambold, 2012), whereas lichenicolous fungi are mostly known from their teleomorphic, sexual states (Hawksworth, 1982; Diederich, 1996).

The mating systems of lichen mycobionts are expected to be similar to mating systems in non-lichenized fungi. However, the mating loci have been studied in detail only in ascomycetous primary mycobionts (Scherrer et al., 2005; Seymour et al., 2005; Singh et al., 2012). Homothallism implies that an individual is self-fertile and not dependent on finding a mate, whereas a heterothallic individual needs a mate with opposing mating type in order to reproduce sexually. In heterothallic Ascomycota, the mating system is bipolar and each species has only two different mating types (Butler, 2007). The mating types are determined by alternative sequences, called idiomorphs, at the MAT loci (Glass et al. 1988). Both homothallic and heterothallic mating systems are found even in closely related primary mycobionts (Honegger et al.,

2004; Scherrer et al., 2005). It has been suggested that homothallism is a successful reproductive strategy for lichens in climatically extreme but undisturbed habitats, where a stable, highly adapted lineages would be most advantageous (Murtagh et al., 2000).

For the heterothallic species, the even distribution of mating types may be considered as an indication for outcrossing in a natural population. However, it has been pointed out that in lichens with dominant vegetative reproduction, the ratio can be skewed despite an outcrossing population structure (Singh et al., 2015). *Letharia* is an example of a molecularly fairly well studied lichen genus that includes species with mixed reproductive strategies. It has been suggested to consist of at least six taxa, although the taxonomic ranks of these are not completely resolved (Kroken and Taylor, 2001a; Altermann et al., 2016). Two of the species reproduce mainly by asexual means (soredia, isidioid soredia), while other species are always abundant in apothecia (Kroken and Taylor, 2001a; McCune and Altermann, 2009; Altermann et al., 2014; Altermann et al., 2016). However, the sorediate species also produce occasional apothecia and have been suggested to have outcrossing populations (Kroken and Taylor, 2001b; Högberg et al., 2002) and some of the apotheciate species also produce isidia (Kroken and Taylor, 2001a; Altermann et al., 2014). In addition, one of the species, *Letharia vulpina*, is red-listed in Sweden and Norway and hence of conservation interest (ArtDatabanken, 2015; Henriksen and Hilmo, 2015). These features make *Letharia* attractive for the study of mating systems, which was investigated in Paper II.

1.5 How to study the parts and the whole

1.5.1 Next-generation sequencing for understanding the holobiont

Barcoding with next generation sequencing (NGS) techniques, of both thalli and environmental samples, offers a means to investigate the organismal diversity, and to some degree the metabolically active members, of the communities. However, there are some limitations regarding the markers commonly used, that are at least partly based on the prevailing knowledge of sequence diversity of the organisms, discussed in Paper I.

As primary mycobionts grow slowly in axenic cultures, experimental studies on their mating systems or factors affecting phenotypic variation of thalli have been challenging. However, constantly cheaper NGS methods offer a possibility to study all the associated organisms in a holobiont, their natural environment, at once (hologenomics, holotranscriptomics, Papers II, III and IV). Genomic data can then be used to study the capabilities of different bionts, for example by investigating candidate genes for alternative carbon metabolism

of the primary mycobionts. With holotranscriptomics, functional questions about the role of the different organisms can be addressed, and comparative transcriptomics make the study of the factors affecting phenotypic variation possible even without cultures (Paper III). Despite the slow growth rates of the mycobionts, genome sequencing of the axenic cultures provides valuable reference data that can be used for separating the different fungal sequences derived from the hologenomes (Paper II).

1.5.2 Potential of fluorescent *in situ* hybridization and confocal lase scanning microscopy in visualizing organismal diversity

A powerful tool for the study of non-culturable organisms in their natural environment is rRNA targeted fluorescent *in situ* hybridization (FISH) (DeLong et al., 1989). Detecting different organisms in a community is based on differences in their rRNA sequence. Several differently labeled probes can be used simultaneously to target specific organism groups, *e.g.*, fungi in general or a specific group of fungi (Yin et al., 2015). In addition to the phylogenetic study of a microbial community, the spatial distribution of the different organisms can be studied (Amann and Ludwig, 2000). The samples stained with fluorescent probes can then be visualized using confocal laser scanning microscopy (CLSM). The obvious advantage with CLSM compared to traditional epifluorescence microscopy is that the out-of-focus light is excluded resulting in crisp, high-resolution images. In addition, series of images can be taken through the z-axis of the sample without destructive sampling. These images can be stacked and used for three-dimensional reconstructions of the sample.

FISH is commonly used in medicinal studies, *e.g.*, to detect foreign organisms in human tissue and fluids, and has also been increasingly used in ecological studies of microbial communities (Baschien et al., 2008; Wagner and Haider, 2012) and mycorrhizal symbioses (Desirò et al., 2014; Vági et al., 2014). In lichens, it has been used to visualize the associated bacterial communities (Cardinale et al., 2008; Cardinale et al., 2012; Grube et al., 2015). However, fungal rRNA targeted FISH has been applied only rarely to lichen mycobionts (Grube and de Los Ríos, 2001). I developed FISH methods for specific staining of fungal bionts and it was used for simultaneous visualizing of multiple mycobionts in lichen thalli in Papers III and IV.

2 Thesis aims

The overall aim of this thesis is to gain deeper understanding on lichen symbiosis. In the included Papers, the functionality and reproduction of the individual bionts as well as the composition of lichen thalli are addressed. These issues are investigated from the fungal perspective using genomic and transcriptomic tools and confocal laser scanning microscopy. The specific aims of this project were:

I To identify fungal components in lichen thalli and understand their prevalence in natural populations using hologenomic and holotranscriptomic data (Papers III and IV).

II To develop FISH methods for specific staining of different fungal bionts in lichen thalli (Paper III).

III To localize and simultaneously visualize different fungal bionts in lichen thalli by FISH and CLSM (Papers III and IV).

IV To investigate the potential of lichen-forming fungi to utilize wood as an alternative energy source and to re-evaluate previous findings from a barcoding study (Paper I).

V To increase knowledge on the reproduction of *Letharia* lichens by investigating the mating system of the primary mycobiont, characterizing the mating loci, and studying the distribution of different mating types in natural populations (Paper II).

3 Results and discussion

3.1 Hologenomic data shed light on the fungal building blocks of lichens (papers III and IV)

Hologenomic and holotranscriptomic data allowed us to identify and study the additional components in lichen thalli, those usually regarded as non-essential for the symbiosis. The holotranscriptomic data of *Bryoria fremontii, B. tortuosa, Letharia columbiana, L. lupina, L. 'rugosa'*, and *L. vulpina*, revealed a constant presence of previously unknown basidiomyceteous fungi, *Cyphobasidium,* in lichen thalli (Paper III). In addition, the data led us to the finding of a previously unknown anamorphic state of a lichenicolous heterbasidiomycete, *Tremella lethariae*, in *Letharia* lichens (Paper IV). Common for all the thalli used for genome and transcriptome sequencing was that none displayed clear symptoms of fungal infections.

3.1.1 Discovery of *Cyphobasidium* in macrolichens (Paper III)

The comparative analyses of holotranscriptomic data of two phenotypically different *Bryoria* species, vulpinic acid rich *B. tortuosa* and vulpinic acid deficient *B. fremontii*, revealed that the abundance of a previously unknown basidiomycete was the only factor explaining the separation into the two phenotypes. Phylogenomic analyses based on 349 single copy loci placed these fungi as a sister group to *Cystobasidium minutum* (Cystobasidiomycetes, Pucciniomycotina). According to a relaxed molecular clock, the split between *Cyphobasidium* and *Cystobasidium minutum* occurred around the same time as three major groups of macrolichens leading to *Xanthoria, Cladonia* and *Bryoria* originated. Based on fossil calibrations the split between *Cyphobasidium* and *Cystobasidium minutum* is 200 million years old.

When the sampling was expanded to other lichens in the class Lecanoromycetes, the largest class of lichenized fungi, several closely related

Cyphobasidium lineages were found, among others from the vulpinic acid rich *Letharia* lichens. A majority of these lineages form a well-supported monophyletic clade that was described as a new order, *Cyphobasidiales. Cyphobasidium* lineages are found to be present in thalli of 52 lichenized genera on six investigated continents, suggesting that these are widespread components of lichens. Notably, the *Cyphobasidium* lineages seem to be constantly present and lichen-specific, which together with the simultaneous emergence suggests a long shared evolutionary history.

3.1.2 Tremelloid fungi in *Letharia* lichens Paper (IV)

In addition to the discovery of *Cyphobasidiales*, holotranscriptomic and hologenomic data from four different *Letharia* taxa led us to find another common basidiomycetous component in *Letharia* thalli, *Tremella lethariae* (which is, to be noted, not closely related with *Cyphobasidiales*, see Fig. 1). *Tremella lethariae* was previously described as a rare, gall-inducing parasite, and only reported from *Letharia vulpina* from a few localities in western North America. We sequenced *T. lethariae* galls and confirmed that the same fungus was found in the hologenomes of all sequenced *Letharia* species. We found *T. lethariae* induced galls only from eight out of more than 600 studied *Letharia* thalli and only from North America. In contrast, we found tremelloid fungi to be frequent in asymptomatic specimens of *Letharia* lichens, when we screened for their presence with *Tremella*-specific PCR primers. *Tremella lethariae* was prevalent in specimens collected in the United States, whereas another species, *Tremella* sp. B. (Lindgren et al. 2015) was more common in *Letharia* collected in Europe. The same ITS haplotypes for both species are found both in Europe and North America and *T. lethariae* was found in all *Letharia* taxa studied (*L. 'barbata'*, *L. columbiana, L. gracilis, L. lupina, L. 'rugosa'* and *L. vulpina*). Phylogenetic analysis with nuclear ribosomal markers placed *T. lethariae* as a sister to *T. hypogymniae*, a lichenicolous fungus known from the lichen *Hypogymnia physodes* (Parmeliaceae). *Tremella* sp. B. has been previously sequenced from different *Bryoria* species (Lindgren et al. 2015), but has not been formally described as no teleomorphic states or other typical cells for it has yet been found. The results suggest that tremelloid fungi are common inhabitants of *Letharia* lichens.

3.2 Development of fluorescent *in situ* hybridization for simultaneous visualization of mycobionts

In order to locate the different fungal bionts and investigate their spatial distribution in lichen thalli, including the primary mycobionts and the newly detected *Cyphobasidium* and *Tremella lethariae*, we developed FISH protocols for use in lichens. All experiments were performed on fixed lichen thalli or cultured cells. The protocols need separate optimization for each lichen species and each probe used.

3.2.1 Permeabilization

Fungal cell walls are chitinazed and require an extra permeabilization treatment for probes to enter the cells. In addition, many macrolichens have a thick cortical layer, the exact content of which is not known in most species, making the specific enzymatic treatment challenging. Inspired by the study of Anglesea (1983), who used a commercially available laundry detergent to gradually wash away the cortical layer of *Usnea subfloridana*, we tried different concentrations of a laundry detergent "Ariel" (Procter and Gamble) on lichen thalli. We also tested different kinds of enzyme cocktails including chitinase, cellulase and proteinase K in combination with the abovementioned laundry detergent. The laundry detergent turned out to be very effective in washing away the polysaccharides and revealing the medulla, but also resulted in loss of large amount of cortical cells that had consequently become loosened during the washing steps. The best working solution might vary between different lichen species, but according to our experience a mixture of chitinase, sodium dodecyl sulphate SDS (a surfactant that lowers surface tension) and buffer works for many lichens (Papers III and IV). The same method has been effectively used in the study of fresh-water fungi by Baschien et al. (2001; 2008). In order to test that the permeabilization treatment was sufficient and the overall hybridization steps well optimized, we used a previously published probe designed to target a wide range of Eumycota (MY1574) (Baschien et al., 2008). However, we noticed that this probe is not compatible with all lichen-forming fungi and its usefulness needs to be tested for each target species separately.

3.2.2 Probe design

We used fluorescently monolabeled oligonucleotide probes designed for ribosomal RNA in all of our experiments. As metabolically active cells are expected to have plenty of ribosomes, several probes are expected to anneal to their complementary rRNA in each cell and hence give a multiplied signal. The design of discriminative probes between unwanted taxa is possible as rRNA is

well sequenced for many fungi and easily assessable via databases like NCBI. For Papers II and IV, we designed several probes covering the ribosomal 18S and 28S regions.

The signal intensity is dependent on the ribosomal content of the cells but also the accessibility of the ribosomal site targeted by the probe (Behrens et al., 2003), which can be structurally hindered or involved in molecular interactions with proteins (Amann et al., 1995). Ribosomal regions can be divided into different accessibility classes based on observed probe signal intensity from a particular site. We noticed that the accessibility classes and signal intensities from the same target sites vary a lot between different fungal taxa. For example, regions that have been reported to give strong signal in *Saccharomyces cerevisiae* (Behrens et al., 2003; Inácio et al., 2003) gave no or only faint signals in the species from Lecanoromycetes in our studies. In addition, the most conserved regions between different fungi are often the regions most accessible to the probes (Behrens et al., 2003; Inácio et al., 2003). Hence, bringing about a trade-off between signal intensity and probe specificity. Whenever possible, homologous probes for the different target organisms should be used in order to enhance the specificity. According to our experience, probe accessibility and related signal strength is the most critical factor affecting the success of FISH in lichens. The accessibility of the probes can be enhanced with specific unlabeled helper probes, complementary to the flanking regions of the probe target site, which help exposing the site to the probe (Fuchs et al., 2000).

3.2.3 Seeing is not always believing – pros and cons of autofluorescence

Some biological structures, *e.g.*, chloroplasts, naturally emit light, a phenomenon called autofluorescence. Different cell types in lichens have strong autofluorescence characteristics, which are something to be aware of in order to avoid interpretation of false positive signal. In addition, some lichen secondary metabolites are heavily autofluorescing, making the use of fluorophores with similar emission wavelengths impractical. However, the effect they have on the imaging is to some extent time dependent, as some of the metabolites are soluble in the FISH protocol liquids or the mounting media. As different secondary metabolites have different autofluorescence characteristics, species-specific adjustments are necessary. Thus, we strongly recommend a scan of each different lichen species and cell type prior to choosing fluorophores. However, autofluorescence characteristics can be useful as well. For example, the autofluorescence of chloroplasts in the algal cells helps separating the structures in CLSM images when there is no need for staining the algae with specific probes. In addition, cell walls of

Cyphobasidium and conidiate cells of *Tremella*, for example, have characteristic autofluorescence in certain wavelengths, which help the identification of cells even when the probe signal is weak.

We needed to test the newly developed probes to either closely related fungal cultures or directly on lichen thalli, as many of the primary mycobionts grow slowly in a culture and despite several attempts we did not manage to culture *Cyphobasidium*. The specificity of the probes can be verified by simultaneous application of different probes targeting the same organism but labeled with different fluorophores. Also, probes for different organisms should be tried out simultaneously in order to verify signal unambiguity. The stringency of the hybridization reaction can be controlled with both the chemical composition of the hybridization buffer and temperature and needs to be optimized for each probe. The time used is critical in minimizing unspecific binding and optimizing the signal-to-noise ratio.

3.3 The lichen cortex as a community (Papers III and IV)

With the newly designed taxon specific FISH probes and protocols and confocal laser scanning microscopy, we could investigate where in lichen thalli the different fungi are located. We could show that both *Cyphobasidium* and *Tremella lethariae* are located in the lichen cortex in a unicellular, anamorphic life state, embedded into the layer of polysaccharides and secondary metabolites. Also, cells of both fungi were found budding, indicating that they really live inside the cortex and are not random intruders or contaminants landed on the surface of thalli. In *Letharia*, catenate conidia of *T. lethariae* were observed. In addition, some yet unidentified cells are present. According to our preliminary testing, some of these cells are dead but some might be inactive cells (or active but beyond the detection limit) of the known bionts. Alternatively, they could belong to yet unidentified organisms, a hypothesis for testing in future.

In *Bryoria capillaris*, *Cyphobasidium* seems to be the only fungal cells in the cortex and hence actually the fungus seen when looking at a *B. capillaris* thallus from the outside. In other species, like *Letharia*, the amount of *Cyphobasidium* cells varies in the cortex. We hypothesize that in *Letharia*, at least some of the cells are lost during the several washing steps in the protocol, as the cortical layer flakes and gets dissolved in a way different from *Bryoria.* However, clearly not all macrolichens have as large amounts of *Cyphobasidium* cells in the cortex as *Bryoria*. The factors affecting the variation of *Cyphobasidium* cells in different lichens should be a task for future studies.

The only previously known members of *Cyphobasidium* were suggested to be rare gall-inducing parasites in *Parmeliaceae*, one example being *Cyphobasidium hypogymniicola* (Millanes et al., 2015). In our FISH experiments, we found both anamorphic and teleomorphic life states of *Cyphobasidium hypogymniicola* and *Tremella lethariae* from lichen thalli, and we suggest that these species can complete their life cycles inside the lichens. However, as the anamorph yeast state does not seem to harm the lichen thallus in any visible way, we suggest that these species should not be considered parasitic throughout their entire life cycle.

3.4 Functionality of mycobionts

3.4.1 *Cyphobasidium* related to the vulpinic acid production (Paper III)

The symbiotic role of *Cyphobasidium* remains unclear, but in the case of *Bryoria fremontii* and *B. tortuosa* the abundance of the *Cyphobasidium* cells in the cortex correlates with the variation in phenotype caused by secondary metabolite production. Holotranscriptome analyses revealed that vulpinic acid rich *B. tortuosa* harbors more *Cyphobasidium* cells than vulpinic acid deficient *B. fremontii*. The vulpinic acid production by *Bryoria* could be induced as an immune response or triggered by the presence of large amount of *Cyphobasidium* cells, or *Cyphobasidium* could produce the secondary metabolites alone or together with *Bryoria*. The complete biosynthetic pathway leading to vulpinic acid needs to be resolved before the transcriptomic data can be used to address its origin. In *B. capillaris,* the cortical polysaccharide layer is filled with *Cyphobasidium* cells. The lichen cortex is important for structure and rigidity of lichen thalli. We hypothesize that the incorporation of *Cyphobasidium* into the symbiosis is related to the formation of a robust cortex and led to evolution of structurally more complex lichens. Whether *Cyphobasidium* consumes the secondary metabolites or polysaccharides as energy or actually produces some of them is not known.

3.4.2 Tremelloid fungi and their virulence in *Letharia* - hypotheses for the future studies

In the Paper IV, we showed that *Tremella lethariae* is dimorphic, and we could identify yeast cells, conidia, hyphae, and different teleomorphic states inside lichen thalli. Anamorph *T. lethariae* was frequently observed in asymptomatic thalli and galls were induced only rarely. Excluding malformations, *i.e.*, the galls, induced by the teleomorphic state in the host cortex, we did not detect any visible harm to *Letharia* cells, like haustorial intrusions, in galls, cortex or medulla. Instead, we found hyphae of *Tremella* in close affinity to algal cells

and we suggest that *T. lethariae* may be nutritionally similar to *Letharia* and acquires its energy from the photobiont. It has been suggested that lichenicolous fungi with high host specificity are likely to be less parasitic (Hawksworth, 1982; Lawrey and Diederich, 2003), a hypothesis that our findings in *T. lethariae* seem to support. *Tremella* sp. B. is not specific to *Letharia,* but further studies on its morphology and cellular interactions with its hosts are needed to clarify the nature of the relationship. One option is, that the sexual reproduction of *T. lethariae* might be triggered by the infection of *Letharia* thalli by pathogenic lichenicolous fungi, and used as an escape mechanism. As the *Letharia* cortex seems to include a variety of dead cells, some fungi (also the primary mycobionts) in the cortex might obtain their energy saprotrophically from dying cells. I note that these scenarios, however, are still hypothetical and need to be tested in future studies.

3.4.3 Alternative energy sources for primary mycobionts – evaluating results from a previous barcoding study (Paper I)

The ability of primary mycobionts to utilize alternative energy sources to the algal photosynthates has seldom been suggested. However, Kubartová et al. (2012) discovered several primary mycobiont taxa more than six cm deep inside decaying Norway spruce logs. This was the first molecular trace of lichen primary mycobionts from inside wood and could indicate partly saprotrophic capacity or deep penetrating hyphae. The first step towards understanding whether some of these reported mycobionts could utilize alternative energy sources in nature would be to find them without their algal symbionts. The most abundant, reliably identified mycobiont from the barcode data of Kubartová et al. (2012) belonged to the genus *Cladonia*. When we re-examined the same samples used in that study we could not find any samples without known lichen photobionts. Decaying wood is a changing substrate and environment that gets cracked and fissured in the course of time. Lichen-derived DNA recorded in Paper I and in the study by Kubartová et al., (2012) could originate from several sources: symbiotic propagules or thallus fragments dispersed into the wood by animals, wind or rain, deep-penetration hyphae of the mycobiont or both free-living photo- and mycobionts. Alternatively, it could be indicative of contamination during the field sampling.

Barcoding studies can serve as a great complement to traditional inventories and in describing the composition of microbial communities. However, they cannot give any direct evidence of the functional roles of the organisms found. Major drawback of the barcoding methods is that the level of taxonomic information obtainable relies on good-quality databases. The number of sequenced lichen species is continuously growing, but the label on the

sequences is only as good as the taxonomic expertise of the identifier. In addition, molecular studies on primary mycobionts have revealed a large amount of yet undetected cryptic diversity in lichens (Leavitt et al., 2016).

The nuclear ribosomal internal transcribed spacer (ITS) is the most widely sequenced gene region used for species identification in fungi (*e.g.*, Nilsson et al., 2009) and was proposed as a universal barcoding marker for fungi by Schoch et al. (2012). However, ITS does not allow separation of lichen primary mycobionts on species level in all, especially recently diverged, groups (Leavitt et al., 2016). In *Cladonia*, for example, there is no barcoding gap to separate the operational taxonomic units (OTUs) at the species level (Kelly et al., 2011). In order to set a label "lichenized" to an OTU derived from a barcoding study, the sequence similarity needs to be high, as lichenization as a life style has been derived and lost several independent times in the evolution of fungi and a lot of fungal diversity is estimated to be still undiscovered (Lücking et al., 2016). Also, the primers used for barcoding studies are based on existing knowledge on sequence variation in target organisms, and some organism groups might go undiscovered due to mismatches in priming sites. For example, the commonly used ITS1 primers do not bind to *Cyphobasidium* species. In addition, use of a marker with length variation within target organisms can skew the picture of community composition due to amplification and sequencing bias. Whether some lichen-forming fungi have saprotrophic properties and utilize them in nature, remains the subject for future studies and can be approached *e.g.*, using genomic and transcriptomic tools.

3.5 Mating system in *Letharia* (Paper II)

In order to gain more understanding on the reproduction in *Letharia* lichens, we studied the mating system of *Letharia* fungi. We used a pure culture of *L. lupina* to generate a reference MAT locus for *Letharia*, free of contamination from secondary mycobionts present in thalli. In addition, we used the hologenomes of *L. columbiana*, *L. lupina*, *L. vulpina* and *L. 'rugosa'* and PCR screening to investigate the mating systems in the genus. We were able to characterize the mating locus in each of these taxa (Fig. 2). All individuals studied carried only one idiomorph indicative of a heterothallic mating system. PCR screens confirmed that the mating system of *L. 'barbata'* and *L. gracilis* is also heterothallic, as only one of the idimorphs was present in each thallus. Similar to many other Pezizomycotina (Debuchy and Turgeon, 2006), the MAT locus in *Letharia* is flanked by the conserved regions including genes *APN2* and *SLA2*. In addition, both idiomorphs include apparently *Letharia*

specific open reading frames (ORFs). The MAT1-2 idiomorph includes a truncated piece of the *MAT1-1-1* gene, which together with a region of intermediate sequence similarity towards the *SLA2* between the idiomorphs, could be indicative of a recombination event ancestral to species divergence in *Letharia*. For the MAT1-1 idiomorph, natural antisense transcripts were present in all studied species, a little studied phenomenon suggested to be related to developmental regulation (Muraguchi et al., 2015).

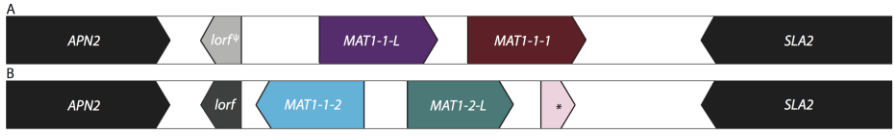

Figure 2. Schematic representation of the MAT locus and its flanking genes in the genus *Letharia* showing the typical gene content of the MAT1-1 idiomorph (A) and MAT1-2 (B). The *lorf* is pseudogenized in this figure for MAT1-1, as its functionality remains unclear. An asterisk (*) shows the location of a highly divergent locus orthologous to the last exon of the *MAT1-1-1* gene.

Since all studied *Letharia* species are heterothallic, the mating system of the primary mycobiont does not alone explain the variation in reproductive strategies in the lichens formed by this genus. Possibly, the switches in the reproductive strategies are triggered by symbiotic relationships in lichen thalli. Screening of natural populations for the mating types in different *Letharia* taxa revealed the presence of both mating types, suggesting sexual outcrossing. However, in Europe the mating-type ratio in *Letharia vulpina* populations is significantly skewed and in Swedish populations of *L. vulpina* we found only one mating type. Combined with the lack of apothecia, it is indicative of that sexual reproduction does not currently occur in Sweden. We suggest that the diminishing population sizes of *L. vulpina* in Sweden are due to a combination of fragmentation of suitable habitats and lack of compatible mates. Transplantation of the missing mating type to the existing populations has been suggested as a conservation method for other red-listed lichen species (Singh et al., 2012; Singh et al., 2015). However, as the enclosed microbial community of lichen thalli is still poorly known, *e.g.*, for the fungal pathogens, I find these kinds of conservation actions risky.

4 Conclusions – towards seeing the lichen for the bionts

Similar to not seeing the forest for the trees, the biology of a lichen, the holobiont, cannot be fully understood from the perspective of only one of the organisms at a time. However, understanding the functions and capabilities of the individual bionts is an important first step towards comprehending the holobiont. In this thesis, I have scrutinized lichens from the fungal perspective.

We could not find any support for the capacity of *Cladonia* to utilize alternative energy sources (*i.e.,* facultative saprotrophy). In order to gain knowledge on the complete life cycle of primary mycobionts in nature, candidate gene approach and transcriptomics, combined with environmental barcoding studies, could be used to investigate their ecological plasticity. It seems that the symbiotic nature of the mycobionts, both primary and secondary, moves on a parasitic – mutualistic continuum depending on the life cycle phase of the bionts, a characteristic similar to other symbiotic systems. *Tremella lethariae* serves as an example of a lichenicolous fungus previously considered as a rare parasite. Our results show that it frequently occurs in asymptomatic *Letharia* thalli in an anamorph life state. Based on its close affinity to photobiont cells, it seems nutritionally similar to primary mycobionts. Slightly parasitic interactions towards one of the symbionts might still benefit the holobiont, *e.g.,* by producing functional secondary metabolites that protect the thallus, but obtaining energy from, and controlling the growth, of the photobiont – a scenario that is usually accepted as mutualistic when scrutinizing the algal - primary mycobiont interactions. After all, symbiosis always includes trade-offs. The investigation of mating system in *Letharia* species revealed that despite different reproductive strategies within the genus, the mating system of the primary mycobiont is similar for all the species. What triggers the evolution of one reproductive strategy (sexual via fungal spores versus vegetative joint dispersal) over another in closely related species, can

thus not to be explained only by the mating system of the primary mycobiont. I hypothesize that the prevailing reproductive strategy in thalli is influenced also by the symbiotic interactions and not only by the genetics of the primary mycobiont, an interesting object for the future studies.

Many of the results presented in this thesis boil down to a question about which of all lichen-associated organisms are needed for a functional lichen to exist. The discovery of previously undetected, lichen-specific *Cyphobasidium* bionts in the largest radiation of macrolichens, clearly affecting the holobiont, has forced us to rethink some of the basic assumptions in lichen biology. The functions assigned to fungal origin should not automatically be assigned to the primary mycobiont. In some *Bryoria* lichens, *Cyphobasidium* seems to be related to the phenotypic variation by somehow affecting the secondary metabolite production. Furthermore, the structure of *B. capillaris* thalli is clearly affected by the abundant *Cyphobasidium*: the outermost cell layer in cortex is not formed by *B. capillaris*, but consists of *Cyphobasidium* cells, making the latter the exhabitant enclosing the primary mycobiont inside the thallus. Accordingly, the possible role of the secondary mycobionts for the holobiont should not be ignored. The case example *B. capillaris* points out the feasibility in naming also the lichen phenotypes (*i.e.,* the holobiont), as calling the lichen after the primary mycobiont (in this case *B. capillaris*), that is not visible for the observer in nature, feels arbitrary.

The studies presented in this thesis yield more questions than answers and numerous exciting hypotheses to be tested on our way to seeing the lichen for the bionts. A colleague asked me after I gave a talk on *Cyphobasidium* in lichens, if I “still think lichens are special”. The answer should stand clear: When I look at a lichen, I see a thallus, whose existence is dependent on the collective contribution from the diversity of inhabitants with a more or less shared ancient evolutionary history. I see a life strategy that results in a community of complex interactions leading to a self-replicating holobiont nothing alike, with higher tolerance for external circumstances than the inhabitants would have on their own. Needless to say - how could I not?

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